ACTIONS OF BALSALAZIDE AND SULPHASALAZINE ON MAST CELLS

THESIS PRESENTED FOR THE DEGREE

OF

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BY

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TO MY PARENTS
ABSTRACT

The mode of action of sulphasalazine (SASP) and balsalazide (BSZ), therapeutic agents of inflammatory bowel disease (IBD), is not clear. This thesis describes studies of the pharmacological actions of SASP, BSZ, and cyclosporin A (CsA, an immunosuppressant), with particular reference to histamine release (HR) from mast cells.

In acute rat colitis, topical pretreatment with BSZ and SASP showed marked prophylactic effect by reducing myeloperoxidase activity, tissue oedema, lactate dehydrogenase release, and HR. Oral treatment of chronic and active rat colitis showed some improvement with BSZ, but not with SASP.

Secretagogues for HR showed differential effects on dispersed guinea pig rectocolonic mucosal cells (RCMC) and isolated rat peritoneal mast cells (RPMC). Deoxycholic acid evoked dose-dependent HR (partly cytotoxic). Peroxidase/H₂O₂/halide system (free radical generating system) also induced HR from both cell types. This was inhibited by metabolites of BSZ and SASP, while parent molecules were less effective. CsA only inhibited the induced HR from RPMC, but not that from RCMC. Some studies of the signal transduction pathways involved in HR from RPMC showed that SASP and BSZ might have a mode of action different to that of CsA. The modulatory effect of both SASP and BSZ on HR by selected secretagogues studied showed tachyphylaxis.

BSZ, SASP, and in some cases their metabolites were shown to stabilize erythrocyte membrane against various membrane labilizers. CsA had the opposite effect.

This study demonstrated that BSZ and SASP share similar actions and efficacy, the parent molecules may not merely be pro-drugs, and their carrier moieties may contribute to the beneficial effect of both compounds. CsA was found to have different actions from both BSZ and SASP. The demonstration that IBD therapeutic agents show different pharmacological actions further indicates the involvement of multiple mechanisms in IBD pathogenesis. The study yields useful information which may lead to new, more effective, and less toxic approaches to treat IBD.
CHAPTER 1  INTRODUCTION  1-63

1.1. HISTORICAL BACKGROUND OF INFLAMMATORY BOWEL DISEASE  1

1.1.1. Ulcerative colitis  1
1.1.2. Crohn’s disease  1

1.2. FEATURES OF INFLAMMATORY BOWEL DISEASE  2

1.3. AETIOLOGY OF INFLAMMATORY BOWEL DISEASE  2

1.3.1. Genetic factors  3
1.3.2. Infectious agents  4
1.3.3. Immunological factors  5
1.3.3.1. Systemic immunity  5
1.3.3.2. Intestinal immunity  7
1.3.4. Increased mucosal permeability  9
1.3.5. Dietary factors  9
1.3.6. Psychological factors  10
1.3.7. Mucin defect 10
1.3.8. Ischaemia 10
1.3.9. Summary 11

1.4. TREATMENT OF INFLAMMATORY BOWEL DISEASE 11
1.4.1. Sulphasalazine (SASP) 11
1.4.2. Balsalazide (BSZ) 13
1.4.3. Cyclosporin A (CsA) 14
1.4.4. Focus of the present study 15

1.5. ANIMAL MODELS OF INFLAMMATORY BOWEL DISEASE 15

1.6. SOME ASPECTS OF PATHOPHYSIOLOGY IN INFLAMMATORY BOWEL DISEASE 17
1.6.1. The mast cell 17
1.6.1.1. Structure and distribution of mast cells 17
1.6.1.2. Role of mast cells 17
1.6.1.3. Mast cells and inflammatory bowel disease 18
1.6.1.4. Mast cell heterogeneity 21
1.6.1.5. Origin of mast cells 24
1.6.1.6. Mast cell mediators 25
1.6.1.6.1. Histamine 25
1.6.1.6.2. 5-Hydroxytryptamine 27
1.6.1.6.3. Proteoglycans 27
1.6.1.6.4. Neutral protease 28
1.6.1.6.5. Preformed chemotactic mediators 28
1.6.1.6.6. Other preformed mediators 29
1.6.1.6.7. Newly synthesized mediators 29
1.6.1.7. Mast cell activation 30
1.6.1.8. Activation mechanism of mast cells 31
1.6.1.8.1. Serine esterase activation 32
1.6.1.8.2. Guanosine triphosphate (GTP)-binding proteins 32
1.6.1.8.3. Role of calcium 34
    Calcium pools involved in histamine release 35
    Role of calcium binding protein calmodulin 37
1.6.1.8.4. Role of phosphoinositide metabolism
Inositol triphosphate and calcium signalling
1,2-diacylglycerol and the activation of protein kinase C

1.6.1.8.5. Role of cyclic AMP

1.6.1.8.6. Role of tyrosine kinase

1.6.1.8.7. Phospholipase activation and arachidonic acid liberation

1.6.2. Bile acids

1.6.2.1. Bile acids in the intestine

1.6.2.2. Bile acids and colonic function and integrity

1.6.2.2.1. Transport and permeability

1.6.2.2.2. Degeneration, proliferation, and dysplasia

1.6.2.3. Mechanisms underlying interaction of bile acid with colonic mucosa

1.6.2.4. Bile acid and mast cells in present study

1.6.3. Reactive oxygen metabolites

1.6.3.1. Production of reactive oxygen metabolites

1.6.3.1.1. Endogenous sources

1.6.3.1.2. Oxidized metabolites of nitrogen

1.6.3.1.3. Exogenous sources

1.6.3.2. Cell and tissue injury by reactive oxygen metabolites

1.6.3.3. Antioxidant defense mechanisms

1.6.3.3.1. Endogenous-enzymatic antioxidants

1.6.3.3.2. Endogenous-nonenzymatic antioxidants

1.6.3.3.3. Exogenous antioxidants

1.6.3.4. Focus of study

1.6.4. Glutathione

1.6.4.1. Role of glutathione against free radical toxicity

1.6.4.2. Free radical production during thiol group oxidation

1.6.4.3. Interest of study

1.6.5. Cell Membrane Stabilization

1.6.5.1. Erythrocyte membrane stabilization

1.6.5.2. Erythrocyte membrane stabilization and inflammation

1.6.5.3. Focus of study
<table>
<thead>
<tr>
<th>1.7.</th>
<th>AIMS OF THE PRESENT STUDY</th>
<th>56</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CHAPTER 2</strong></td>
<td>MATERIALS AND METHODS</td>
<td>64-86</td>
</tr>
<tr>
<td>2.1.</td>
<td>MATERIALS AND SOURCES</td>
<td>64</td>
</tr>
<tr>
<td>2.2.</td>
<td>ANIMALS</td>
<td>66</td>
</tr>
<tr>
<td>2.3.</td>
<td>BUFFERS</td>
<td>66</td>
</tr>
<tr>
<td>2.3.1.</td>
<td>HEPES-buffered Tyrode solution (HBTS)</td>
<td>66</td>
</tr>
<tr>
<td>2.3.2.</td>
<td>Hank's balanced salt solution (HBSS)</td>
<td>67</td>
</tr>
<tr>
<td>2.3.3.</td>
<td>Krebs solution</td>
<td>68</td>
</tr>
<tr>
<td>2.3.4.</td>
<td>TRIS-buffered electrolyte solution (TBES)</td>
<td>68</td>
</tr>
<tr>
<td>2.3.5.</td>
<td>Phosphate buffer</td>
<td>68</td>
</tr>
<tr>
<td>2.3.6.</td>
<td>Bicarbonate buffer</td>
<td>68</td>
</tr>
<tr>
<td>2.3.7.</td>
<td>Isotonic saline</td>
<td>68</td>
</tr>
<tr>
<td>2.3.8.</td>
<td>Heparinized saline</td>
<td>68</td>
</tr>
<tr>
<td>2.4.</td>
<td>PREPARATION OF CHEMICAL AND BIOCHEMICAL SOLUTIONS</td>
<td>69</td>
</tr>
<tr>
<td>2.5.</td>
<td>ISOLATION OF MAST CELLS</td>
<td>70</td>
</tr>
<tr>
<td>2.5.1.</td>
<td>Rat peritoneal mast cells (RPMC)</td>
<td>70</td>
</tr>
<tr>
<td>2.5.2.</td>
<td>Guinea pig rectocolonic mucosal mast cells (RCMC) prepared by scraping (non-enzymic dispersion)</td>
<td>70</td>
</tr>
<tr>
<td>2.5.3.</td>
<td>Guinea pig RCMC prepared by enzymic dispersion</td>
<td>71</td>
</tr>
<tr>
<td>2.6.</td>
<td>HISTAMINE RELEASE FROM ISOLATED MAST CELLS</td>
<td>71</td>
</tr>
<tr>
<td>2.6.1.</td>
<td>Effect of histamine liberators</td>
<td>71</td>
</tr>
<tr>
<td>2.6.1.1.</td>
<td>Time-course of histamine release</td>
<td>72</td>
</tr>
<tr>
<td>2.6.1.2.</td>
<td>Liberator concentration-dependence of histamine release</td>
<td>72</td>
</tr>
<tr>
<td>2.6.1.3.</td>
<td>Temperature-dependence of histamine release</td>
<td>73</td>
</tr>
<tr>
<td>2.6.1.4.</td>
<td>Calcium-dependence of histamine release</td>
<td>73</td>
</tr>
<tr>
<td>Section</td>
<td>Page</td>
<td></td>
</tr>
<tr>
<td>------------------------------------------------------------------------</td>
<td>------</td>
<td></td>
</tr>
<tr>
<td>2.6.1.5. pH-dependence of histamine release</td>
<td>73</td>
<td></td>
</tr>
<tr>
<td>2.6.1.6. Effect of metabolic inhibitors on histamine release</td>
<td>73</td>
<td></td>
</tr>
<tr>
<td>2.6.2. Effect of modulators on induced histamine release</td>
<td>73</td>
<td></td>
</tr>
<tr>
<td>2.7. HISTAMINE ASSAY</td>
<td>74</td>
<td></td>
</tr>
<tr>
<td>2.7.1. Histamine radioimmunoassay</td>
<td>74</td>
<td></td>
</tr>
<tr>
<td>2.7.2. Spectrofluorometric assay of histamine</td>
<td>74</td>
<td></td>
</tr>
<tr>
<td>2.7.3. Interference test of fluorometric assay of histamine</td>
<td>75</td>
<td></td>
</tr>
<tr>
<td>2.8. ANIMAL MODELS WITH ETHANOL-INDUCED RECTOCOLONIC INFLAMMATION IN RATS</td>
<td>76</td>
<td></td>
</tr>
<tr>
<td>2.8.1. In vivo model</td>
<td>76</td>
<td></td>
</tr>
<tr>
<td>2.8.1.1. Induction of inflammation</td>
<td>76</td>
<td></td>
</tr>
<tr>
<td>2.8.1.2. Effect of drugs on the induced inflammation</td>
<td>77</td>
<td></td>
</tr>
<tr>
<td>2.8.1.3. Damage score</td>
<td>77</td>
<td></td>
</tr>
<tr>
<td>2.8.2. In situ ethanol (EtOH)-perfused rat rectocolon model</td>
<td>78</td>
<td></td>
</tr>
<tr>
<td>2.8.2.1. Preparation of animal</td>
<td>78</td>
<td></td>
</tr>
<tr>
<td>2.8.2.2. Perfusion procedure</td>
<td>79</td>
<td></td>
</tr>
<tr>
<td>2.9. DETERMINATION OF MYELOPEROXIDASE (MPO) ACTIVITY</td>
<td>79</td>
<td></td>
</tr>
<tr>
<td>2.9.1. Preparation of tissue homogenate</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>2.9.2. MPO activity assay</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>2.10. DETERMINATION OF GLUTATHIONE (GSH)</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>2.10.1. Preparation of tissue homogenate</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>2.10.2. Preparation of perfusate</td>
<td>81</td>
<td></td>
</tr>
<tr>
<td>2.10.3. Glutathione assay</td>
<td>81</td>
<td></td>
</tr>
<tr>
<td>2.11. DETERMINATION OF LACTATE DEHYDROGENASE (LDH)</td>
<td>81</td>
<td></td>
</tr>
<tr>
<td>2.12. ERYTHROCYTE MEMBRANE STABILIZATION</td>
<td>82</td>
<td></td>
</tr>
</tbody>
</table>
2.12.1. Preparation of erythrocytes for haemolysis experiments 82
2.12.2. Procedure of heat-induced haemolysis 83
2.12.2.1. Tests with unmodified erythrocytes 83
2.12.2.2. Tests with modified erythrocytes 83
2.12.2.2.1. Modification by trinitrobenzene sulphonic acid (TNBS) 84
2.12.2.2.2. Modification by phospholipase C (PLC) 84
2.12.2.2.3. Modification by deoxycholic acid (DA) 84
2.12.3. Haemolysis induced by other membrane labilizers 84
2.12.4. Assay of haemoglobin content and expression of haemolysis 85
2.12.5. Procedure of heat-induced protein coagulation (denaturation) 85

2.13. TREATMENT OF RESULTS 86

CHAPTER 3 ANIMAL MODELS OF ETHANOL- AND HAPten-induced RECTOCOLONIC INFLAMMATION 87-103

3.1. INTRODUCTION 87

3.2. MATERIALS AND METHODS 88

3.3. RESULTS 88
3.3.1. Determination of GSH content and MPO activity in rat rectocolon (ex vivo) following EtOH-induced damage 88
3.3.1.1. GSH content 89
3.3.1.2. MPO activity 89
3.3.1.3. Damage score 90
3.3.2. Effect of BSZ and SASP on chronic rectocolonic damage induced by EtOH+TNBS 90
3.3.2.1. Damage score 90
3.3.2.2. GSH content
3.3.2.3. MPO activity
3.3.3. Effects of BSZ and SASP on In Situ EtOH-perfused rat rectocolon
3.3.3.1. LDH release
3.3.3.2. Histamine release
3.3.3.3. MPO activity
3.3.3.4. GSH content
3.3.3.5. Tissue weight

3.4. DISCUSSION

CHAPTER 4 THE EFFECT OF DEOXYCHOLIC ACID ON MAST CELLS ON GUINEA PIG RECTOCOLON-IC MUCOSA AND RAT PERITONEAL CAVITY

4.1. INTRODUCTION

4.2. MATERIALS AND METHODS

4.3. RESULTS

4.3.1. Effect of DA on histamine release from guinea pig RCMC
4.3.1.1. Effect of varying concentration of DA
4.3.1.2. Effect of incubation time
4.3.1.3. Effect of extracellular calcium
4.3.1.4. Effect of temperature
4.3.1.5. Effect of pH
4.3.1.6. Effect of metabolic inhibitors
4.3.2. Effect of DA on histamine release from RPMC
4.3.2.1. Effect of varying concentration of DA
4.3.2.2. Effect of incubation time
4.3.2.3. Effect of extracellular calcium
4.3.2.4. Effect of pH
4.3.2.5. Effect of temperature 107
4.3.2.6. Effect of metabolic inhibitors 107
4.3.3. Authentication of histamine released from cell preparation from guinea pig rectocolonic mucosal scraping 107

4.4. DISCUSSION 107

CHAPTER 5 FREE RADICAL (PEROXIDASE/H₂O₂/HALIDE-INDUCED HISTAMINE RELEASE FROM MAST CELLS OF GUINEA PIG RECTOCOLONIC MUCOSA AND RAT PERITONEAL CAVITY AND MODULATORY EFFECT OF SASP, BSZ, AND CsA 120-138

5.1. INTRODUCTION 120

5.2. MATERIALS AND METHODS 121

5.3. RESULTS 121

5.3.1. Effect of H₂O₂/Nal/HRP system on histamine release from guinea pig RCMC and RPMC 121
5.3.1.1. Effect on guinea pig rectocolonic mucosal cells (RCMC) 121
5.3.1.2. Effect on rat peritoneal mast cells (RPMC) 122
5.3.2. Effect of BSZ, SASP, and metabolites on H₂O₂/Nal/-HRP system-induced histamine release from guinea pig RCMC and RPMC 123
5.3.2.1. Effect on histamine release from guinea pig RCMC 123
5.3.2.2. Effect on histamine release from RPMC 123
5.3.3. Effect of CsA on H₂O₂/Nal/HRP system-induced histamine release from guinea pig RCMC and RPMC 124
5.3.3.1. Effect on histamine release from guinea pig RCMC 124
5.3.3.2.  Effect on histamine release from RPMC 124
5.3.4.  Effect of GSH on H₂O₂/Nal/HRP system-induced histamine release from guinea pig RCMC and RPMC 124
5.3.4.1.  Effect on histamine release from guinea pig RCMC 124
5.3.4.2.  Effect on histamine release from RPMC 124

5.4.  DISCUSSION 125

CHAPTER 6  MODULATORY EFFECT OF SASP, BSZ, AND CsA ON HISTAMINE RELEASE INDUCED BY SELECTED SECRETAGOGUES FROM GUINEA PIG RCMC AND RPMC 139-172

6.1.  INTRODUCTION 139

6.2.  MATERIALS AND METHODS 140

6.3.  RESULTS 141
6.3.1.  Effect of selected secretagogues on histamine release from RPMC and guinea pig RCMC 141
6.3.1.1.  Response of RPMC to selected secretagogues 141
6.3.1.2.  Response of guinea pig RCMC to selected secretagogues 141
6.3.2.  Effect of BSZ, SASP, and their metabolites on histamine release induced by selected secretagogues from RPMC and guinea pig RCMC 141
6.3.2.1.  Effect on histamine release from RPMC 141
6.3.2.1.1.  Platelet activating factor-induced histamine release 141
6.3.2.1.2.  Compound 48/80-induced histamine release 142
6.3.2.1.3.  Substance P-induced histamine release 142
6.3.2.1.4.  Sodium fluoride-induced histamine release 143
6.3.2.1.5.  Phospholipase C-induced histamine release 143
6.3.2.1.6.  Dic8-induced histamine release 143

xii
6.3.2.1.7. Calcium ionophore A23187-induced histamine release

6.3.2.2. **Effect on histamine release from guinea pig RCMC**

6.3.2.2.1. Phospholipase C-induced histamine release

6.3.2.2.2. Calcium ionophore A23187-induced histamine release

6.3.3. Effect of cyclosporin A on histamine release from RPMC and guinea pig RCMC induced by selected secretagogues

6.3.3.1. **Effect on histamine release from RPMC**

6.3.3.1.1. NaF-induced histamine release

6.3.3.1.2. Phospholipase C-induced histamine release

6.3.3.1.3. Dic8-induced histamine release

6.3.3.1.4. Calcium ionophore A23187-induced histamine release

6.3.3.2. **Effect on histamine release from guinea pig RCMC**

6.3.3.2.1. Phospholipase C-induced histamine release

6.3.3.2.2. Calcium ionophore A23187-induced histamine release

6.3.4. Effect of calmodulin antagonists on compound 48/80-induced histamine release from RPMC

6.4. DISCUSSION

---

**CHAPTER 7** EFFECT OF BALSALAZIDE AND SULPHASALAZINE ON MEMBRANE STABILIZATION

7.1. INTRODUCTION

7.2. MATERIALS AND METHODS

7.3. RESULTS
7.3.1. Heat-induced haemolysis
7.3.1.1 Effects of drugs on heat-induced lysis of unmodified rat erythrocytes
7.3.1.2 Heat-induced haemolysis of modified rat erythrocytes
7.3.1.2.1 Effect of modification procedure on erythrocytes
7.3.1.2.2 Effect of drugs on heat-induced haemolysis of modified rat erythrocytes
7.3.2. Haemolysis induced by other membrane labilizers
7.3.2.1 Pilot tests for determining experimental conditions using other labilizers
7.3.2.2 Effect of BSZ and SASP on melittin-induced human erythrocyte lysis
7.3.2.3 Effect of drugs on PLC-induced rat erythrocyte lysis
7.3.2.4 Effect of drugs on DA-induced rat erythrocyte lysis
7.3.3. Effect of SASP and BSZ on heat-induced albumin denaturation
7.4. DISCUSSION

CHAPTER 8 GENERAL DISCUSSION

REFERENCES
ACKNOWLEDGEMENTS

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PUBLICATIONS


ABBREVIATIONS FOR TEXT

AA: Arachidonic acid
4-ABA: 4-Aminobenzoyl-B-alanine
5-ASA: 5-Aminosalicylic acid
ATP: Adenosine triphosphate
BMCC: Bone marrow-derived mouse mast cell
BSZ: Balsalazide
C3a: Complement fragment 3a
C5a: Complement fragment 5a
cAMP: Cyclic AMP; adenosine 3',5'-cyclic monophosphate
CD: Crohn's disease
CD45: Leucocyte common antigen
CD45RA: CD45 isoform
CD45RO: CD45 isoform
CF: Calcium free
CMA: Calmodulin antagonist
CMF: Calcium-magnesium free
CsA: Cyclosporin A
CT: Cholera toxin
CTMC: Connective tissue mast cell
CuPIPS: Copper (II) 3,5-diisopropylsalicylic acid
DA: Deoxycholic acid, sodium salt
DAG: 1,2-diacylglycerol
DEM: Diethyl maleate
DFP: Diisopropyl fluorophosphate
Dic8: 1,2-Dioctanoyl-sn-glycerol
DMSO: Dimethyl sulfoxide
DNTB: 5,5'-Dithiobis-(2-nitrobenzoic acid)
DSCG: Disodium cromoglycate
EDTA: Ethylenediaminetetraacetic acid
EGTA: Ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid
ER: Endoplasmic reticulum
EtOH: Ethanol
FcεR1: High affinity IgE receptor
FCS: Foetal calf serum
FMLP: Formyl-methionyl-leucyl-phenylalanine
GDPβS: Guanosine-5′-O-(2-thiodiphosphate)
Gq: G protein distinct from Gp and involved in mediating the late event of exocytosis
GF: Glucose free
Gp: PLC-associated G protein
GSH: Glutathione, reduced form
GSSG: Glutathione, oxidized form
GTPβS: Guanosine-5′-O-(3-thiotriphosphate)
G-protein: Guanosine triphosphate (GTP)-binding protein
H₂O₂: Hydrogen peroxide
HBSS: Hank's balanced salt solution
HBTS: HEPES-buffered Tyrode solution
HEPES: N-2-hydroxyethyl piperazine-N'-2-ethane sulphonic acid
HETE: Hydroxyeicosatetraenoic acid
HLA: Histocompatibility locus antigens (human)
HOCI: Hypochlorous acid
HR: Histamine release
HRP: Horseradish peroxidase
5-HT: 5-Hydroxytryptamine
HTAB: Hexadecyltrimethylammonium bromide
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>IBD</td>
<td>Inflammatory bowel disease</td>
</tr>
<tr>
<td>IEL</td>
<td>Intraepithelial lymphocyte</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IP$_3$</td>
<td>Inositol 1,4,5-triphosphate</td>
</tr>
<tr>
<td>IP$_4$</td>
<td>Inositol 1,3,4,5-tetrakis-phosphate</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
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<tr>
<td>LPL</td>
<td>Lamina propria lymphocyte</td>
</tr>
<tr>
<td>LT</td>
<td>Leukotriene</td>
</tr>
<tr>
<td>MC$^1$</td>
<td>Human mast cells containing tryptase</td>
</tr>
<tr>
<td>MC$^2$</td>
<td>Human mast cells containing both tryptase and chymase</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MMC</td>
<td>Mucosal mast cell</td>
</tr>
<tr>
<td>MMCP</td>
<td>Mouse mast cell protease</td>
</tr>
<tr>
<td>MPO</td>
<td>Myeloperoxidase</td>
</tr>
<tr>
<td>NAD</td>
<td>Nicotinamide adenine dinucleotide, oxidized form</td>
</tr>
<tr>
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<td>Nicotinamide adenine dinucleotide, reduced form</td>
</tr>
<tr>
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</tr>
<tr>
<td>N-4ABSA</td>
<td>N-(4-aminobutyl)-5-chloro-1-naphthalenesulphonamide</td>
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<td>Phosphatidic acid</td>
</tr>
<tr>
<td>PAF</td>
<td>Platelet activating factor</td>
</tr>
<tr>
<td>PBZ</td>
<td>Phenylbutazone</td>
</tr>
<tr>
<td>PC</td>
<td>Phosphatidylcholine</td>
</tr>
<tr>
<td>PG</td>
<td>Prostaglandin</td>
</tr>
<tr>
<td>PG-PS</td>
<td>Peptidoglycan-polysaccharide</td>
</tr>
<tr>
<td>PI</td>
<td>Phosphatidylinositol</td>
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<tr>
<td>PIC</td>
<td>Picumast</td>
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<tr>
<td>PIP$_1$</td>
<td>Phosphatidylinositol 4-phosphate</td>
</tr>
<tr>
<td>PIP$_2$</td>
<td>Phosphatidylinositol 4,5-bis-phosphate</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PLA$_2$</td>
<td>Phospholipase A$_2$</td>
</tr>
<tr>
<td>PLC</td>
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</tr>
<tr>
<td>PLD</td>
<td>Phospholipase D</td>
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<tr>
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<td>Polymorphonuclear leucocyte</td>
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<tr>
<td>PT</td>
<td>Pertussis toxin</td>
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<tr>
<td>PTK</td>
<td>Protein tyrosine kinases</td>
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<tr>
<td>PTPase</td>
<td>Protein kinase phosphatases</td>
</tr>
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<td>Rat basophilic leukaemia cell line 2H3</td>
</tr>
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<td>RCMC</td>
<td>Rectocolonic mucosal mast cell</td>
</tr>
<tr>
<td>ROM</td>
<td>Reactive oxygen metabolite</td>
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<tr>
<td>RPMC</td>
<td>Rat peritoneal mast cell</td>
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<td>5-Sulphosalicylic acid</td>
</tr>
<tr>
<td>Sub P</td>
<td>Substance P</td>
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<tr>
<td>TBES</td>
<td>TRIS-buffered electrolyte solution</td>
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<tr>
<td>TFP</td>
<td>Trifluoperazine</td>
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<td>2,4,6-trinitrobenzene sulphonic acid</td>
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<td>12-O-tetradecanoylphorbol-13-acetate</td>
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<td>Tyrode solution</td>
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<td>TX</td>
<td>Thromboxane</td>
</tr>
<tr>
<td>UC</td>
<td>Ulcerative colitis</td>
</tr>
<tr>
<td>VIP</td>
<td>Vasoactive intestinal polypeptide</td>
</tr>
<tr>
<td>W7</td>
<td>N-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide</td>
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1.1. HISTORICAL BACKGROUND OF INFLAMMATORY BOWEL DISEASE

Inflammatory bowel disease (IBD), including ulcerative colitis (UC) and Crohn's disease (CD), until quite recently, were virtually unknown entities.

1.1.1. Ulcerative colitis

The origins of UC have been lost amid diagnostic confusion with the infectious and non-infectious diarrhoeas, but it is possible that the disease existed in Greek and Roman times. As cited in several references [1-3], the name UC was first used by Sir Samuel Wilks in a letter published in 1859 reporting his testimony at a court trial. In 1875, Wilks and Moxson published a now classic description of UC which they isolated from a specific form of dysentery, and modern considerations of the disease really date from that time. However, little attention was paid to this condition until a symposium on the aetiology of UC held in the Royal Society of Medicine in 1909 reviewed the collective experience with approximately 200 patients seen in several London hospitals.

1.1.2. Crohn's disease

The general recognition of granulomatous disease of the bowel (Crohn's disease) can be dated quite precisely to 1932 when Crohn, Ginzburg, and Oppenheimer published their description of regional ileitis seen in 14 patients at Mt. Sinai Hospital, New York. However, as cited in a historical review by Shapiro in 1939, the clinical awareness of a CD-liked process in the bowel, which was often confused with intestinal tuberculosis, may be traced back as early as 1806 [1].

It was first thought that CD was confined to the terminal ileum only; the other granulomas in the colon, such as what is now called the granulomatous colitis (and ileocolitis) described by Moschcowitz and Wilensky in 1923, were considered to be a different disease [1, 2]. However, in 1933, Harris, Bell, and Brunn described ileojejunitis [2]; and in 1934, Colp reported the first case of granulomatous colitis (ileocolitis) with disease in the ileum and caecum [1]. The same disease has been recognized to affect
Chapter 1

the colon or anus without involvement of the small bowel [1, 2, 4]. Involvement of
the upper digestive tract, especially the mouth, the distal portion of the stomach, and
the first portion of the intestine (duodenum) has become more apparent in recent years,
and Crohn’s-type lesion also has been reported in the skin, bone, muscles, lung, and
even the umbilicus [4]. It is now recognised that CD, while historically being assigned
to the terminal ileum can affect any part of the gastrointestinal tract — from the mouth
to the anus, while UC affects colon and rectum [5].

1.2. FEATURES OF INFLAMMATORY BOWEL DISEASE

IBD is still the major unsolved and complex clinical problem in gastroenterology
today. The disease is characterized by the presence of acute and chronic inflammation
of the gastrointestinal tract [5]. There are fundamental differences between UC and
CD to distinguish the two said diseases in the majority of patients, while in 15-20% of
the patients, the differentiation is difficult [3, 5]. The main points of differentiation are
summarized in Table 1.1.

1.3. AETIOLOGY OF INFLAMMATORY BOWEL DISEASE

The aetiology and aetiological agents of the diseases are unknown, the
pathophysiological mechanism of the attacks is unclear, and the chemical mediators of
the inflammation are largely undefined. This lack of knowledge as to the cause of the
diseases is reflected in many aetiological hypotheses. These hypotheses implicate
infectious, allergic, autoimmune, dietary, immunological, vascular, neuromotor, and
psychosomatic factors; genetic and constitution disorders; the destructive effect of
proteolytic enzymes; and deficiencies of various types - including lack of ‘protective
substances’ in the wall of the intestine [2, 6-12]. Preferences for one or another of
the above hypotheses have many times been determined, not by how well a theory has
been proven, but by how clearly it has been disproven by vigorous clinical,
epidemiologic, and laboratory evaluations. The only hypotheses that are best
scientifically supported and still under serious consideration are those of an infective
cause and immunological defects. The latter involves the concept of a genetically
determined alteration in the immunoregulation of gastrointestinal and host defences,
especially in the response to bacteria, viruses, and other injurious agents, and in the
imunological defences of the small and large intestine to various pathogenic agents [2, 13, 14].

1.3.1. Genetic factor

UC and CD do not associate with a definite pattern of inheritance (such as Mendelian pattern) [7, 15]. However, the genetic influences are important in the development of the diseases, since a high familial incidence of both UC and CD has been established [2, 8, 14, 16, 17]. The occurrence of UC or CD has been observed in the first-degree relative of 15-20% of patients, while an even higher familial incidence of 40% has also been documented [8, 14, 16]. Additional evidence of heredity as an aetiological factor has been shown by the high concordance rate in monozygotic twins of a large Swedish study based on an unselected twin register [18]. The same study also indicated unequivocally that the genetic contribution is stronger in CD than in UC. Furthermore the close association of IBD with other diseases which have an inherited basis and genetic factors playing an important predisposing role also contributes to the evidence [16]. A typical example of the association is the case of the disease ankylosing spondylitis which in turn is closely associated with histocompatibility locus antigen HLA-B27 [16]. However, the carriage rate of the genetic marker HLA-B27 is lower in IBD patients than in patients with anklylosing spondylitis alone, although in patients with UC and spondylitis as many as 80% had the HLA-B27 antigen [16]. Several studies of HLA class I (A, B, C) or class II (DR) have been performed. No consistent correlation has been found, and isolated reports of a significantly higher incidence of certain haplotypes probably reflect the genetic characteristics of a population rather than a true association [7, 8, 14, 16]. Due to the lack of reproducible association it is possible that the elevated familial incidence in IBD may not be based on genetic factors but on exposure to common environmental elements, or perhaps on a combination of both, though newer haplotypes have yet to be fully evaluated [14, 19]. It has been suggested that IBD is a heterogeneous disorder in that UC and CD are genetically and clinically heterogeneous diseases with overlapping morphological features and multifactorial origins, and what is now called CD may itself represent different disease entities (i.e. CD is heterogeneous) [2, 16].
1.3.2. Infectious agents

It has long been suspected that IBD has infectious aetiology. However, a specific microbial cause of IBD has been sought for unsuccessfully over the last 60 years despite intensive epidemiological, microbiological, immunological, and therapeutic investigations all of which have failed to yield an acceptable and consistent infectious microorganism that can be transferred to experimental animals [2, 6, 9, 11, 20-22]. The infectious agents studied include various intestinal pathogens, anaerobes, cell wall-deficient organisms, mycobacteria, and viruses.

In recent years, research has come full circle with a resurgence of interest in the concept of mycobacterial aetiology for IBD, particularly CD, after the failures in search of viruses (eg. cytomegalovirus) and cell-wall-defective bacteria (eg. *Pseudomonas maltophilia*) as potential aetiologial agents [2, 9, 11, 20, 21]. The presence of intestinal granulomas in what is now called Crohn’s disease caused speculation as early as the beginning of the century that the disease might be due to an infection similar to tuberculosis (mycobacterium related); however, no evidence of tuberculous infection was found then, and the disease was separated from intestinal tuberculosis [1, 2]. The current viable candidate as a pathogen in IBD is *Mycobacterium paratuberculosis*, whose isolation rate nevertheless remains low and whose role is yet to be proven [9, 11, 20-22]. Recently, several investigations have demonstrated that various mycobacterial proteins involved in immune recognition are stress proteins (heat shock proteins) which may be responsible for autoimmune reactions through the mechanism of molecular mimicry initiated by humoral and cellular immune system responses to conserved (common, cross-reacting) epitopes of stress protein of an infecting microorganism [11, 22]. Stress proteins are found in a wide range of organisms, including man, and show great sequence homology. However, the attempts in determining the involvement of mycobacterial stress proteins in IBD have yielded inconsistent and inconclusive results [11, 22].

Recent serological studies of other microorganisms have implicated the possible role of different strains of *Saccharomyces cerevisias* and *Chlamydia trachomatis* in the cause of IBD [22]. The findings await more critical analyses.

The whole enteric commensal flora, as opposed to an isolated organism, have been suggested to play a role in exacerbation and recurrence, if not pathogenesis, in IBD [9]. These commensal flora secrete or contain within them substances, including oligopeptides such as formyl-methionyl-leucyl-phenylalanine (FMLP), lipopolysaccharide (endotoxin), muramyl peptides, and peptidoglycan-polysaccharide (PG-PS), all of which
have potent inflammatory and immunoregulatory properties [9, 25, 26]. Based on the experimental animal models, these bacterial products have been implicated in IBD [9, 24-28]. However, information about their role in humans is lacking.

Evidence for an infectious agent as directly responsible for IBD is inconclusive, yet the hypothesis that microorganisms are responsible for the disease cannot be discarded, considering the immense number of potential pathogenic agents many of which have not been investigated in detail. Failure to isolate them is not proof that they are not implicated. It is possible that the microbial pathogens may act as inciting agents that initiate a pathological process that ultimately results in IBD, but play no part in the continuation or persistence of the disease as a result of being eliminated by local defense mechanisms, while the primary lesion could be perpetuated by a defective immune response [8].

1.3.3. Immunological factors

As the centre feature of IBD is the presence of both acute and chronic inflammation of the gastrointestinal tract, the nature of the immune mechanisms involved in this inflammation has been an important area for investigation into the aetiology of the disease [13, 14, 22, 29-33]. However, it remains difficult to determine whether the demonstrated abnormalities are primary or secondary phenomena. In addition, very often, investigation has been obscured by the variable and conflicting results. An abnormality in immunological homeostasis preceding the onset of either UC or CD has not been demonstrated. Although there is an inherited susceptibility to the disease, and although HLA associations are found, they are not as striking as classical autoimmune diseases, and no reproducible association can be established (see Section 1.3.1.). Chronic activation of the immune system could be secondary to a number of factors, such as: (a) unrecognised infection (see Section 1.3.2.); (b) increased mucosal permeability [34, 35] allowing entry of bacterial or dietary antigens which are normally excluded; (c) increased presentation of luminal antigens by epithelial cells to T cells [14, 33]; and (d) other subtle alterations in immunoregulation that could serve to perpetuate chronic immune reaction.

1.3.3.1. Systemic immunity

Studies of the numbers of T and B lymphocytes and their characteristics in IBD have yielded variable data. Though the data seem to vary with the disease (such as
increased number of activated cells), yet no cause-and-effect relationship has been established [13, 14, 29]. The presence of anticolon antibodies in patients with UC suggests that these antibodies could be involved in IBD, but they have also been found in other intestinal conditions and are not cytotoxic for the colonic epithelial cells [8, 14]. Furthermore, there has been lack of correlation between clinical activity and antibody titers. The hypothesis that colon-directed cytotoxicity which could be mediated by the cross-antigenicity of the enterobacterial common antigen of Kunin has suffered the same fate [8, 14]. Recently, an IgG immunoglobulin specially reactive with colon-derived antigen (40-kD protein) was isolated from the bowel of patients with active UC [36]. This 40-kD antigen is apparently present exclusively in colonic tissue, both in healthy patients and patients with IBD, and the IgG antibody can only be extracted from the large bowel of patients with UC, but not CD. Significantly increased levels of circulating antibodies against human colonic extract enriched with 40-kD protein have also been detected in the sera from UC patients compared to CD patients or normal controls [37]. These findings implicate the possible autoimmunity in at least one type of IBD. However, other investigators have not been able to confirm the findings [22].

The possibility that IBD is mediated by immune complexes has been explored, and the indirect evidence for the detection of material compatible with immune complexes in the circulation of patients with UC and CD has been conflicting [8, 14]. In some reports, a correlation between active disease and elevated titers of circulating immune complexes has been found, especially in patients with extraintestinal manifestation of IBD [8, 14]. Additional data supporting the presence of antigen-antibody reactivity in IBD has been provided by studies of some complement components. Recently, the parallel appearance of complement C3b and terminal complement complex C5b-9 in deposits in submucosal blood vessels in active IBD, and also the enhanced local production of complement components in unaffected jejunum of patients with CD have been reported [22]. However, the exact nature of immune complex material has never been elucidated and, more importantly, evidence that immune complexes are deposited at the site of tissue destruction has not been provided. Hence, the pathogenic role of immune complexes in IBD is still questionable [8, 14].

In the study of cell-mediated immunity in IBD, a wide range of T cell-mediated phenomena has been investigated in vivo, through tests of skin reactivity to recall antigens, and in vitro, through studies of lymphocyte proliferation to lectin mitogens, cytotoxic function, allogenic and autologous mixed lymphocyte reactivity, and helper and suppressor cell function [8, 13, 14, 32]. Unfortunately, varied results have been
observed, patient immune responses have ranged from decreased through normal to increased. These inconsistent abnormalities of cell-mediated immunity suggest that they are not the primary cause of the disease [14].

There is some evidence in UC and CD of decreased interleukin-2 production by T cells in peripheral blood where T cells were also less responsive to interleukin-2 [38]. Gamma-interferon production by peripheral blood monocytes is also lower in CD and untreated UC [38]. In contrast, CD monocytes produce increased interleukin-1 when stimulated in vitro regardless of the state of disease activity [38].

1.3.3.2. Intestinal immunity

More recently, the focus of investigation of immunological events in IBD has changed to intestinal immunity. With regard to the phenotypic studies, compared with normal mucosa, the total number of cells is higher in mucosa affected by IBD [39]. It is now clear that intraepithelial (IEL) and lamina propria lymphocytes (LPL) are distinctive populations. In human mucosa, IEL are predominantly suppressor/cytotoxic T cells [13, 40]. Despite its slight change in the helper/inducer to suppressor/cytotoxic T cells ratio in CD of the ileum, IEL appear to be largely unaffected by the disease process in total number and the ratio of helper/inducer to suppressor/cytotoxic T cells [22, 40]. In the LPL population, helper/inducer T cells are predominant over suppressor/cytotoxic T cells in a 2:1 ratio as do lymphocytes in the peripheral blood [13, 40]. The phenotypes and the relative proportions of LPL are essentially identical in IBD and control tissues [13, 40], but those in the diseased tissues express early activation antigens [22]. However, recently, in an immunohistochemical study of the expression of different leucocyte common antigen (CD45) isoforms, an increase in LPL T-helper cells and a reduction in LPL T-suppressor cells in CD patients were reported [22]. In the same study, it was also found in the inflamed lesions of UC and CD that there was a marked increase in the fraction of LPL T-cells which displayed the phenotype of antigen-primed cells (CD45RO+) — 85% vs 68% in IBD and normal subjects respectively. The findings indicate that the majority of these cells are activated in situ responding to unknown antigens. Correspondingly, the fraction of LPL with expression of isoform (CD45RA+) which displayed phenotype of naive cells was decreased in active CD and UC [22].

In IBD, the B cell system shows evidence of chronic immune stimulation with more IgG and IgM than IgA plasma cells, but for IgE plasma cells, variable results have been observed [8, 14]. The distribution of IgA and IgG subclass plasma cells and the
secretion of IgA and IgG subclasses are also different in healthy mucosa and mucosa of IBD [42-43]. A substantial increase in numbers of IgA plasma cells has been reported in the diseased mucosa [42] and the increase correlates with disease activity and degree of inflammation [44]. Furthermore, it has been observed that the proportion of IgG, plasma cells is significantly higher in UC than in CD, and conversely, the proportion of IgG2 plasma cells is significantly higher in CD than in UC [41]. Similar changes in IgA and IgG subclasses are seen with antibodies secreted by intestinal mononuclear cells isolated from IBD specimens [43, 45]. In normal mucosa, dimetric IgA is the predominant type of immunoglobulin secreted by cells, whereas in IBD, more monomeric IgA and IgA2, but less polymeric IgA and IgA2 are secreted [43]. With regard to IgG secretion, mononuclear cells isolated from UC intestinal specimens spontaneously secrete large amount of IgG1, with a slightly increased concomitant secretion of IgG2; whereas those from CD intestinal specimens exhibit moderate increases in the spontaneous secretion of all three IgG subclasses mentioned with the predominant increase being IgG2 [45]. These findings of abnormal immunoglobulin production are important, since the production of one type of immunoglobulin is not a random process, and different antigens induce preferential production of certain immunoglobulin classes or subclasses [45, 46]. In human, IgG, subclass reflects response to proteins and T-cell dependent antigens, while IgG2 subclass reflects response to carbohydrates and many bacterial antigens [45,46].

In cell-mediated immunity, no major differences are observed between control mucosal mononuclear cells and those in IBD with regard to proliferative responses, immunoregulatory capacity, and non-specific cytotoxic activity [13, 14]. However, it has been revealed that tissue involved in UC and CD but not of normal tissue, contains cells capable of specifically recognizing and destroying epithelial cell-associated components, a unique set of antigens only present in the gut epithelium [14]. Also it has been reported that the cytotoxicity to autologous colonic epithelial cells by intestinal lamina propria lymphocytes is augmented in IBD patients [47]. Nevertheless, natural killer cells are sparse in normal and not increased in IBD [40, 47]. It has been suggested that spontaneously cytotoxic or lymphokine activated killer cells are unlikely to play a role in the induction of colonic epithelial cell injury by direct cytotoxicity in IBD [48]. These results underscore the importance of studying well-defined gut related antigens to characterize the unique properties of the immune cells present in the IBD-affected intestine [14]. Recently, it has been demonstrated convincingly that gut epithelial cells can function as antigen-presenting cells by expressing major histocompatibility complex
Chapter 1

(MHC) class II HLA-DR, -DP, and -DQ antigens which are more profound in IBD [22]. In normal mucosa, epithelial cells present antigens with preferential activation of antigen unspecific suppressor T-cells, while epithelial cells derived from IBD patients selectively stimulate helper T-cells and fail to induce suppressor T-cells [22]. These findings suggest that an intrinsic defect of epithelial cells may contribute to the pathogenesis of IBD. Macrophage turnover is increased, and macrophages with characteristics of antigen-presenting cells are more abundant in inflamed colon of IBD [22].

Lymphokines, as immunoregulatory molecules, play critical roles in proliferation, clonal expansion, cytolytic activity, antigen presentation, chemotaxis, and macrophage activation [22, 31, 33]. From the studies on the production of lymphokines by mucosal cells from patients with IBD, there is evidence that significantly less interleukin-2 and gamma-interferon are produced by local T cells in both UC and CD than by cells from normal intestine [14]. Increased level of interleukin-1 has been shown to be present in intestinal supernatants of cultured intestinal tissue and intestinal mononuclear cells extracted from IBD patients when compared to normal controls [22, 49]. Production of interleukin-6 and interleukin-8 have also been shown in active IBD specimens [22, 49, 50].

1.3.4. Increased mucosal permeability

Increased intestinal permeability allows luminal antigenic compounds, which are normally excluded by the normal bowel, to penetrate through the intestinal mucosa to serve as an initiating factors in IBD [35]. In 1986, Hollander et al [34] found increased intestinal permeability in patients with CD and their clinically healthy relatives with no manifestation of the disease. This observed increase in permeability in healthy uninvolved relatives of the patients may reflect more than an injury secondary to inflammation. However, a recent parallel study has shown normal intestinal permeability in first degree relatives of patients with CD [51]

1.3.5. Dietary factors

As early as 1920s cow's milk was singled out as a potential cause of UC. However, subsequent investigations yielded variable results, and milk as a primary causative agent in UC has been practically dismissed [6, 8]. Investigations on other dietary factors such as refined sugar, cereal, fruit and fibre, total carbohydrate, total protein, and total caloric intake as one of the possible aetiological factors remain
Chapter 1

inconclusive [10, 52].

1.3.6. Psychological factors

The possibility of psychological factors being important in the aetiology of UC was predominant in the 1930s. It is generally accepted that emotional disturbances are common in patients with UC and CD, and are important in the course of the disease and in the response to therapy, but probably are not primary cause of the diseases. Hence, though not curative, psychotherapy may help and influence the clinical course of IBD [2, 8]. It has emerged recently that there is a "gut-brain" interaction in the form of bidirectional communication between the neuroendocrine system and the immune system (both systemic and mucosal) [53-55].

1.3.7. Mucin defect

Mucin glycoproteins are thought to have important roles in the host defense of mucosal integrity, including provision of lubricating properties, serving as an important interface with the microflora [31], and limiting oxidative insult by toxic oxidant [51]. Alterations in specific mucin species have been implicated in mucosal diseases in general and UC in particular [12]. Depletion of goblet cell mucus is a striking histological feature of active UC [56]. Alterations in the content of colonic mucins have been detected in patients with UC, not only in active disease state, but also in quiescent disease state, in uninvolved mucosa, and in clinical remission. In contrast, goblet cell mucus is relatively well preserved in CD despite the inflammation [12, 51]. Therefore a mucin defect may be more important in UC, but whether it is a primary cause of the disease remains to be clarified.

1.3.8. Ischaemia

In view of the clinical and morphological similarity existing at times between UC and ischaemic bowel disease, vascular impairment has been suggested to be of primary pathogenic relevance in UC [8]. It has been suggested that mucosal damage in the colon could be secondary to impairment of the microcirculation resulting from an increased frequency of pressure waves [8]. An ischaemia/reperfusion hypothesis with respect to the role of reactive oxygen metabolites for the pathogenesis of UC has been proposed [58].
Chapter 1

1.3.9. Summary

The multiplicity of abnormalities observed in IBD may implicate an array of aetiological factors acting independently or in concert, and resulting in pathological, immunological, and clinical manifestations, all of which may depend on the factor or factors inducing the disease in each individual patient.

1.4. TREATMENT OF INFLAMMATORY BOWEL DISEASE

The therapy of the disease remains empirical. There is no intervention that can be regarded as curative, other than perhaps surgical operation. Total colectomy and ileostomy is a curative operation for severe UC, but with postoperative complications, while recurrences of disease of the small bowel after intestinal resection and reanastomosis is a frequent feature of CD [59, 60]. Instead, medical therapy is directed to reducing acute inflammation and treating disease manifestations and complications.

1.4.1. Sulphasalazine

Since the introduction in the 1940s by Svartz originally for the treatment of rheumatoid arthritis, but with the unexpected symptomatic improvement in the treatment of UC [61], sulphasalazine has become the most commonly prescribed therapy for IBD. It is chemically salicylazosulphapyridine (SASP) and is a combination of 5-aminosalicylic acid (5-ASA) linked by an azo bond to sulphapyridine (SP) (Fig. 1.1.a).

SASP is efficacious in treating mild to moderate acute attacks of UC, with remission or significant improvement in up to 80% of patients at 3-4 g/day [62-64]. It is also established as a drug for maintenance treatment at 2 g/day to prevent flare-ups of UC in 75-88% of patients who have achieved a remission [62-64]. While in patients with mild to moderate Crohn’s colitis and ileocolitis, but not ileitis, SASP has also been shown to be effective, other studies have shown that it is also effective in patients with active small bowel Crohn’s (very small study), or only patients with Crohn’s colitis [38]. Thus, there is doubt about the use of SASP in small intestinal CD, and it does not seem to be effective as a maintenance treatment in CD in general [38].

After oral administration, only 10-20% of the ingested SASP is absorbed in the small intestine, while the majority of an ingested dose is delivered intact to the colon where it is split at the azo bond by the colonic bacteria with the liberation of 5-ASA and SP [64]. After absorption in the small intestine, only 2-10% (13% at maximum)
of the ingested SASP reaches the systemic circulation [65], while the rest of the absorbed SASP is excreted in the bile without modification [64, 65]. Virtually all the SP is absorbed in the colon and excreted in the urine after being metabolized in the liver by acetylation, hydroxylation, and glucuronidation, while 5-ASA remains largely unabsorbed and is excreted in the faeces either unchanged or in acetylated form [64, 65]. In 1977, 5-ASA was administered by enema and identified in patients with mildly active UC as the active moiety of SASP [66]. This finding has subsequently been confirmed by others [64]. In contrast SP seems to be the active agent in rheumatoid arthritis [67]. Studies of rectal instillation of acetylated form of 5-ASA have failed to show any clinical effect, although one study showed a significant effect in active UC [64].

The concentrations of SASP and its metabolites achieved in the inflamed colon are not known in man, but it is reasonable to expect them to be between serum and stool levels. The serum concentrations of SASP, 5-ASA, and SP in UC patients responded to an oral dose of 3-6 g of SASP per day are reported to be 0.045, 0.007, and 0.149 mM respectively; while stool concentrations for patients taking 3-12 g of SASP per day are reported to be up to about 2.0, 9.8, and 1.9 mM respectively [68]. Thus, interstitial concentrations of SASP, 5-ASA, and SP may be as high as 1.0, 5.0, and 0.9 mM respectively. Based upon perfusion studies in cats, mucosal interstitial concentrations of 0.65 mM 5-ASA in the ileum and 0.13 mM in the colon have been predicted [69].

Despite the extensive use of SASP in patients with chronic IBD, its mode of action has yet to be elucidated. However, the disposition of oral 5-ASA containing compounds and the response to enemas both indicate that the site of action is the colonic mucosa. As indicated above, the luminal concentrations of SASP and its metabolites, especially 5-ASA, in patients on treatment are high, thus, perhaps not surprisingly, a wide range of pharmacological effects have been observed and proposed as possible mechanisms of action [64, 70, 71, 72]. These include inhibition of synthesis of cyclooxygenase products, lipoxygenase products, or platelet activating factor; inhibition of prostaglandin metabolism; inhibition of neutrophil, macrophage, or mast cell functions; alteration of colonic permeability; free radical scavenging; inhibition of humoral immunity; and inhibition of production or action of cytokines. In addition to the effects just mentioned, there are other effects including binding to connective tissue, increased colonic fluid and electrolyte absorption, inhibition of oxidation of short-chain fatty acid, and inhibition of intestinal folate transport, folate metabolism, and folate action in lymphocytes [63, 73]. The binding to connective tissue has been considered as an important effect in relation
to the efficacy of the drug, in that connective tissue appears to be the origin of the
diseased process of UC, although the issue is not yet settled [61, 63]. In some
experimental systems the effects of SASP and its metabolites, 5-ASA and SP, differ. In
some instances, the parent compound SASP has activities which are not shared with
its component metabolites (Figure 1.2), hence giving rise to the doubt about whether
SASP is merely a pro-drug for delivering 5-ASA, or it may also contribute to the
therapeutic effect [74].

About one-third of the patients taking SASP develop dose-related side effects
which are predominantly attributed to high serum level of SP, and 10% of the patients
are completely precluded from the use of SASP [63, 64]. The main and common side
effects include nausea and vomiting, abdominal pain, anorexia, headache, macrocytosis,
reticulocytosis, cyanotic skin discoloration and impaired male fertility due to oligospermia
and reduced sperm motility [63, 64]. As the result of adverse effects, a new generation
of SASP-like drugs without the SP moiety is emerging.

1.4.2. Balsalazide

Balsalazide (BSZ) is designed as a SASP-like pro-drug with 5-ASA linked by an
azo bond to 4-aminobenzoyl-ß-alanine (4-ABA, an inert carrier) (Figure 1.1.b) and has
similar pharmacokinetics to SASP without detectable serum concentrations of the parent
compound or carrier moiety [75]. Following single oral doses of 2 g BSZ, serum
concentrations of 5-ASA, 4-ABA, and unchanged BSZ were below the limits of detection
(2 µg/ml); the amounts of unchanged BSZ, total 5-ASA, and total 4-ABA detected in the
faeces were 12, 46, and 72% of the ingested dose respectively, while those in the urine
were 0.5, 34, and 19% respectively. BSZ has been shown to be equipotent or superior
to SASP in animal models of colitis [76] and to be free from the side effect of male
infertility in man [77] and animals [78].

In a study of comparing BSZ (2 g/day) with SASP (2 g/day) as maintenance
treatment during 6-month period in patients with UC in remission, BSZ (remission rate:
51%) was found to be not significantly different from SASP (remission rate: 63%) in
maintaining remission but to have fewer side effects [79]. Two recent first phase
reports (12 month stage) of a longer-term study of BSZ (over 12 months) confirmed that
this new drug is highly effective in maintaining remission in UC (77% remission rate at
probable optimal dose of 3 g/day) and very well tolerated in a dosage of up to 6
g/day (equivalent to 5.5 g/day of SASP) at least over a prolonged period of 12 months
[80, 81].
1.4.3. Cyclosporin A

Cyclosporin A (CsA) is a lipophilic, cyclic fungal undecapeptide (MW 1202) which has been an effective immunosuppressant used widely in organ transplantation to prevent graft rejection with selective activity toward T-lymphocytes [82]. More recently, after the first case-report of the use of CsA in CD [83] followed by that in UC [84], it has received great attention in the treatment of IBD [85]. Oral doses of 5-15 mg/kg of body weight/day, continuous intravenous infusion of 4 mg/kg of body weight/day, and enema of one single dose of 350 mg/day have been used in the treatment. Several small preliminary uncontrolled studies and limited number of controlled trials have shown that CsA appears to be a potential agent in the management of IBD [85-87]. However, due to concerns about the toxicity and the not yet defined role of CsA in the treatment of IBD, it has been recommended that, other than in severe active UC which has failed steroid, the use of CsA in relation to IBD should be restricted to research protocol before the information of more extensive double-blind controlled trials using CsA in both UC and CD are available [85, 88]. Blood concentrations of CsA have been shown to be negligible while colonic tissue concentrations to be quite high after enema administration [64, 85]. The bioavailability of CsA after oral ingestion has been shown to vary greatly between and within individual patients because of variable absorption [82]. Hence, in view of its serious side effects, the blood level of CsA has to be monitored closely when it is used.

The exact mechanism of the action of CsA remains unclear. It has been suggested that its action is mediated by its capability of binding to the cytosolic calcium-binding protein calmodulin [89]. However, in some studies, CsA did not appear to bind with high affinity to calmodulin [90, 91], an aspect which is still debatable [92, 93]. The other cytosolic protein thought to be involved in the critical step of the CsA mechanism of action is cyclophilin [82, 90]. Based on several pieces of evidence, cyclophilin, which is abundant and broadly distributed in human tissues [94], is favoured as a CsA receptor [82]. In T-cells, it has been proposed that CsA manifests its immunosuppressive effect by inhibiting the activity of a calcium-, calmodulin-dependent protein phosphatase called calcineurin indirectly [95]. CsA first forms a complex with the abundant cytosolic cyclophilin, then the CsA-cyclophilin complex binds to and inhibits the phosphatase activity of calmodulin-calcineurin complex, and hence prevents the translocation of nuclear factor of activated T cell from the cytoplasm into the nucleus.
to form a competent transcriptional activator for lymphokine genes [95], such as that of interleukin 2.

There is increasing evidence suggesting that CsA may have multiple sites of action. In addition to lymphocytes, it may also alter the functional state of macrophages [96, 97], mast cells [98-102], basophils [103, 104], and neutrophils [105-106]. Thus in addition to its immunosuppressive effect, CsA may also have anti-allergic and anti-inflammatory effects. More recently, cyclophilin has been shown to be a secretory product of activated macrophages, and it has cytokine-like proinflammatory activity with chemotactic effects on neutrophils and eosinophils [107, 108]. The effects of CsA on this extracellular cyclophilin has been suggested to be the primary immunomodulatory mechanism of action of CsA, rather than the inhibition of cytokine production [109]. It has also been suggested that excessive production of lysophospholipids and the consequent inhibition of Na^+, K^+-ATPase during CsA treatment of mitogen- or antigen-activated lymphocytes is a possible biochemical mechanism of the immunosuppressive activity of CsA [110].

1.4.4. Focus of the present study

It is of interest to compare the pharmacological actions of a novel 5-ASA based drug (i.e. BSZ) with the established SASP and an immunosuppressant in some aspects of the inflammation, using animal models and isolated cell preparations.

1.5. ANIMAL MODELS OF INFLAMMATORY BOWEL DISEASE

There are various animal models of IBD [2, 111-115] and they can be broadly classified into two categories: (1) natural animal models with spontaneous bowel inflammation; (2) experimentally induced models. In the former category are diseases in which the animal is naturally exposed to an organism that causes disease or diseases in which the animal is genetically predisposed to manifest disease, possibly in response to material (causative agent) that is commonly found in the normal environment. Nevertheless, none of the natural animal models duplicates human IBD in at least one aspect, that is, in most (if not all) cases of natural models, an organism (usually an intracellular organism) was found; whereas in IBD, a causative organism has not been identified. One promising natural animal model is that described in cotton-top tamarins. The animals develop a disease with many features — such as chronic inflammation, high
incidence of colonic adenocarcinoma, selective alteration of colonic mucin composition, and response to sulphasalazine treatment — resembling those of human IBD [116-118]. Unfortunately, cotton-top tamarin is an endangered species and is not widely available.

In the experimentally induced models, induction is either by exposure to a disease-producing agent or by manipulation of otherwise normal physiological processes. The former includes bacterial toxin or products, patient diseased tissue, chemical inducers (such as carrageenin, dilute acetic acid, dilute ethanol, dilute ethanol+2,4,6-trinitrobenzenesulphonic acid); while the latter includes alteration of immune response (such as by sensitization to 2,4-dinitrochlorobenzene, ovalbumin, or the use of immune complexes) and alteration of the vascular system (ischaemia) [111, 113-115, 119-121].

A suitable model of IBD developed in laboratory animals is essential in serving as a screen for new drugs and studying their mode of action so as to discover more effective agents. It may also aid in understanding the pathogenic mechanisms for the disease. An ideal IBD model should consist of a disease in animals that is identical in every respect to human disease. In other words, the animal disease should be induced by the same primary factors (same cause) and maintained by the same secondary factors (same pathophysiology) as human disease. This will ensure that the animal disease has an equivalent spectrum of clinical and pathological manifestations such as spontaneously relapsing inflammation and a similar response to possible therapeutic agents. In addition, the ideal model must be a practical tool of study in that the animals used must be accessible, must lend themselves to experimental manipulation, must be hardy enough to allow easy management, easy maintenance, and rapid expansion, and, finally, must be treatable with therapeutic agents which can ultimately be tested in human. However, in practice, with unknown endogenous and exogenous aetiological factors involved in the disease and unknown nature of disease mechanisms present, as well as the absence of a sure disease-associated marker, it is not difficult to envisage the problem in developing animal model of IBD. The problem is that one cannot define a model of an entity which itself is poorly defined. Therefore, animal models observed to date are best classified as gastrointestinal inflammation rather than as models of IBD specifically. Nevertheless, as the central feature of IBD is in essence the inflammatory response, though not exclusive to IBD, studying these animal models under various conditions can still help in understanding human disease and can even lead to hypothesis concerning pathogenesis of IBD [113].
Chapter 1

1.6. SOME ASPECTS OF PATHOPHYSIOLOGY IN IBD

As mentioned above, not only the aetiology of IBD is unknown, the pathophysiological mechanism of the attack is in principle unclear. Nevertheless, there are a few potential factors which may be involved in the disease.

1.6.1. The mast cell

1.6.1.1. Structure and distribution of mast cells

In 1878, Ehrlich was first to identify mast cells in human connective tissue and their circulating counterpart basophils on the basis of the metachromatic staining properties of their prominent cytoplasmic granules [122]. Mammalian mast cells, each with a round or ovoid nucleus, are ovoid or irregularly elongate in situ with an average diameter of 10-30 μm and numerous elongated, thin folds on the cell surface, while the cytoplasm is filled with granules of average diameter ranging from 0.2 to 0.4 μm [123-126]. Rodent mast cell granular structure is more homogenously electron dense while that of human is generally more variable and includes crystalline patterns of scrolls, particles, crystals, as well as the electron-dense matrix material similar to that seen in the rat; and these patterns may coexist in the same cell or sometimes even in the same granule [124, 127].

With the exception of solid bone and cartilage, the mast cell, with its specific plasma membrane receptors (FcεRI) which specially bind the Fc portion of IgE antibody with high affinity [128, 129], can be found in every tissue and organ in the body of human as well as other vertebrates. It is the major source of tissue histamine [130] and is positioned in particularly large number at the sites where potentially noxious materials are likely to enter the body, such as the lungs, airways, skin, and gastrointestinal tract as well as generally in the connective tissue around the venules [127, 131]. Circulating mast cells are rare occurrence that happens almost exclusively in the neoplastic disorder such as systemic mastocytosis.

1.6.1.2. Role of mast cells

Due to the ubiquitous distribution throughout the body as well as their responsiveness to diverse stimuli and collection of potent mediators, mast cells have been implicated in most, if not all, inflammatory responses and pathological conditions. Unfortunately, to some extent, the classical view of mast cell function in IgE-mediated immediate hypersensitive or allergic reactions has been a conceptual blinker that has
Chapter 1

inhibited a more holistic view of mast cell biology. Nevertheless, mast cells also appear to participate in a wide variety of biological responses in which IgE has no demonstrable role [132]. Evidence is accumulating rapidly to indicate that mast cells are critically involved in not only immediate hypersensitivity reactions, but also in delayed hypersensitivity, immunologically non-specific inflammatory reactions, direct cytotoxicity, potentiation of cytotoxicity of eosinophils and macrophages, cell proliferation, differentiation and activation in a range of target cells, angiogenesis, tissue remodelling and wound healing, and immunoregulation [33, 133-136]. However, the precise functions of mast cells in such responses remain largely unknown.

Intestinal mucosal mast cells are often found near nerve fibres of the lamina propria, and it has been observed that nerve terminals appear to be in direct contact with the plasma membrane of mast cells in the rat ileum [137, 138]. The possible implications of bidirectional communication between the enteric neuroendocrine and immune system for intestinal functions and IBD have been suggested [53-55, 139]. In addition to its action via receptors (probably histamine receptor H1) on epithelial cells, histamine-mediated intestinal functions via neural H2 and H3 receptors have been observed in the small intestine and colon of the guinea pig [140, 141]. In addition to histamine, the mediators that alter epithelial transport include adenosine, 5-hydroxytryptamine, prostaglandins, leukotrienes, and platelet-activating factor, as well as others [139, 142-144], most of which, if not all, are mast cell mediators.

1.6.1.3. Mast cells and inflammatory bowel disease

Mast cells are found in the lamina propria of the normal small and large bowel. The mean population density seen in the intestinal mucosa depends on the biopsy location and is much higher in the ileum than in the lamina propria of the colon and rectum [145]. Mast cell hyperplasia is a consistent feature of UC and CD [145-149]. In UC, the mast cells are increased diffusely in the involved area, predominantly in the lamina propria [146], whereas in CD, the mast cells are also increased in the thickened submucosa and muscular layers of actively diseased segments, with the distribution reflecting the discontinuous lesions in this condition [148, 149]. This increase in number has led investigators to speculate on the important role mast cells play in IBD. It is certain that mast cells with their diverse mediators — including histamine, prostaglandins, sulphidopeptide leukotrienes, proteases, proteoglycans, chemotactic factors, platelet activating factor, and cytokines — are capable of contributing significantly to the various aspects of IBD [123, 135, 136, 150-152], such as induction,
maintenance, and extension of inflammatory lesions in the intestine. They may also play a role in tissue repair and hence may be important in remission; or play a role in tumour development and hence may be implicated in incidence of intestinal cancer in IBD [133]. Therefore, in view of these diverse and multifunctional mast cell mediators, it appears that there is a critical balance in the diverse actions of mast cells.

Another piece of evidence that supports the involvement of mast cells in IBD is the ultrastructural [149, 153, 154] and biochemical evidence [155-158] for local mast cell activation. In the ultrastructural and quantitative studies by Balázs et al [153], the investigators stressed the significance of an accumulation and degranulation of mast cells in the active stages of UC while in remission no significant increase in the total number of mast cells was observed. In a kinetic study of histamine release using endoscopically obtained specimens, Raithel et al [155] showed that spontaneous histamine release in specimens of active UC and CD was significantly higher than that in specimens of both disorders without active disease as well as that in specimens of normal control subjects not suffering from IBD. It was also shown in the same study that the spontaneous release in specimens of UC and CD without active disease was lower than that in normal control specimens. In a more recent study on the histamine release from gut mast cells derived from enzymatically dispersed endoscopic forceps biopsy specimens, Nolte et al [156] found significant differences between patients with IBD and normal controls, with the discharge of histamine upon challenge with anti-IgG\* being confined to patients with IBD, especially mast cells from inflamed mucosa, while polyclonal anti-IgG did not induce any histamine release in any subjects. They also found an increased mast cell count in normal gut mucosal tissue from patients with UC, but not from those with CD, when compared with patient control subjects; and the mast cell count in inflamed tissue was greater than that in normal tissue. Furthermore, it was confirmed in the same study the unresponsiveness of basophils to anti-IgG\*. These investigators suggested the presence of functional Fc receptors and the presence of IgG\* (in addition to IgE) in gut mucosal mast cells. They also suggested an increase in the amount of IgG\* in the mast cells of IBD patients without the increase in IgE when compared with the normal subjects. In view of the increase in the local proportion of IgG-producing cells in IBD as reported previously [42] and the histamine release effect of anti-IgG\*, these investigators implicated the possible pathogenetic role of IgG\* antibodies in IBD, and suggested that passive sensitization with subclass IgG\* antibodies should be performed to further elucidate their finding.
Chapter 1

The knowledge of modulation of mast cell (from different sources) activities as well as activities of its circulatory counterpart, basophil, by drugs used in the therapy of IBD such as corticosteroids [133, 159, 160] and sulphasalazine (SASP) [71, 161, 162] lends its support to the involvement of mast cells in IBD. In one of the studies using corticosteroids, inhibition in IgE- but not IgG-mediated histamine release from guinea pig lung was observed [159]. It has also been shown that steroid treatment can reduce mast cell number in particular tissues, such as the study in which a single steroid treatment led to a remarkable reduction in mast cell number in the rat intestine but not elsewhere within 4 - 24 hours [163]. The other drug known to affect intestinal mucosal mast cell number is the immunosuppressant cyclosporin A (CsA) [98]. In this case the significant reduction in cell numbers (by half) as well as reduction in tissue rat mast cell protease II (RMCP II) in the jejunum were noted within 2 weeks after daily subcutaneous injection of CsA at 50 mg/kg for 3 days as a loading dose, then at the same dose on alternate days; while a rapid drop of serum RMCP II was detectable 3 hours after intravenous administration (50 mg/kg). The authors suggested that the observations of slow reduction in cell number and in the tissue content of RMCP II were due to the suppression of T cell-mediated regulatory stimuli to proliferation of mast cell precursors and/or their migration by CsA, an explanation which is not applicable to the observations by King and co-workers because the time of onset is different; while the effect on serum RMCP II was due to the suppression of mediator secretion [98]. Furthermore, chronic topical steroid treatment reduced human skin mast cells [133]. In a study with SASP, the drug inhibited histamine secretion by rat peritoneal mast cells and mouse cultured mast cells but potentiated anti-IgE-induced histamine secretion by human blood basophils [164]. This potentiating effect by SASP on anti-IgE-mediated histamine release from human basophils as well as human intestinal mast cell was also observed in a recent study in which 5-aminosalicylic acid (5-ASA, a metabolite of SASP) was also studied and shown to inhibit stimulated histamine release from intestinal mast cell and basophils as well as production of prostaglandin D₂ by the intestinal mast cells [162]. These functional differences indicated by a drug having different effects on mediator release from different mast cell populations warrant further investigations of the effects of drugs for IBD treatment on mast cell subpopulations and differential mediator release.

The mechanism responsible for mast cell degranulation in IBD is unknown. Although in most of the above-mentioned functional studies of mast cells, anti-IgE was used as the secretagogue, yet the number of local IgE-containing immunocytes generally
Chapter 1

remains normal in UC or slightly decreased in CD patients [145, 165]. Therefore, the involvement of IgE as a mast cell secretagogue in IBD is questionable. The marked increase in IgE-containing cells in IBD reported by O'Donoghue & Kumar [166] has been shown to reflect allergic proctitis which is a distinct clinical and pathological entity different from UC and CD [167]. It is most likely that other secretagogues, such as reactive oxygen metabolites [168, 169] and bile acids (see Section 1.6.2.), may play a more significant role.

1.6.1.4. Mast cell heterogeneity

There is a body of evidence which firmly indicates that mast cells do not represent a homogeneous cell population but are heterogeneous. Mast cells from different species, from different tissues or organs within the same animal, and even within a single organ, may exhibit marked variations in their morphological, biochemical, and functional properties [123-124, 170-172]. The best example of heterogeneity lies in the comparison between intestinal mucosal (MMC) and peritoneal (connective tissue-type mast cells, CTMC) mast cells in the rat. These two subsets of mast cells, MMC and CTMC, are anatomically restricted to their respective sites, and differ in their morphological, cytochemical, and functional properties.

As generally recognized, the first observation that rat mast cells from different anatomical sites exhibited variations in their histochemical staining characteristics was made in 1906 by Maximow [124, 127, 131]. Beginning in the 1960s, Enerbäck [131] greatly extended the early observations of mast cell heterogeneity and defined in detail the conditions of fixation and histochemical staining which discriminated between the MMC observed in the intestinal lamina propria and the CTMC of the skin, peritoneal cavity, and other sites in the rat. Different conditions of both fixation and staining may be required to demonstrate metachromasia in rat mast cells, and conventional methods of fixing and staining CTMC do not apply to MMC. Carnoy's solution and a low concentration of a formaldehyde-acetic acid mixture are required to preserve rat MMC while normal aldehyde fixatives reversibly block the staining of rat MMC. By contrast, the staining of rat CTMC is affected little by the method of fixation. While rat CTMC are readily stained by toluidine blue at pH 4, optimal staining of rat MMC by the same dye requires a pH of 0.5. Also rat CTMC granules stain red with copper phthalocyanine dyes, such as astra blue or alcian blue, in a staining sequence with safranin, while rat MMC granules stain blue. Similarly, while rat CTMC are strongly stained by berberine, rat MMC show no reaction. The diversity of staining properties between these two mast
cell types is due to their different granule glycosaminoglycans. Large amounts of the highly charged and highly sulphated acidic proteoglycan heparin which is responsible for the granules' propensity to take up cationic dyes are present in rat CTMC [124, 171]. By contrast, rat MMC contain no heparin, but do contain predominantly a more soluble, less highly charged, and lower sulphated (compared with heparin) proteoglycan chondroitin sulphate di-B [124, 170, 171] which has weaker affinity for cationic granule stains.

Rat MMC and CTMC can be distinguished by morphological criteria. In the resting state, rat CTMC is densely packed with numerous granules which are 0.2-0.4 μm in diameter and appear homogeneous by electron microscopy [123-126]. Ultrastructurally, compared with rat CTMC, rat MMC are smaller and more variable in shape, contain fewer and smaller granules of more variable size and shape, often show cytoplasmic processes and projections, and may have a migratory capacity [131, 170]. Upon stimulation, rat CTMC undergo degranulation by a process of sequential exocytosis while MMC exhibit swelling of granules but no exocytosis [124, 172]. The number and density of high affinity IgE receptors on rat CTMC surface is significantly higher than that on rat MMC surface, with rat MMC but not rat CTMC exhibiting cytoplasmic or internalized IgE in parasite (e.g. *Nippostrongylus brasiliensis*)-infected rat [133, 170, 172]. With respect to mast cell ontogeny and development, differences may exist between these two mast cell types (see Section 1.6.1.5.). The half-life of rat MMC is about 40 days, while rat CTMC have a very long lifespan with half-life of more than 6 months [131].

Different mast cell populations vary qualitatively and quantitatively in their preformed mediators and mediators that are synthesized de novo following cell activation. Apart from the above-mentioned proteoglycans, the predominant serine protease (chymotryptase) of rat peritoneal mast cell (RPMC, CTMC type), rat mast cell protease I (RMCP I), has been shown to differ from that of rat MMC in which rat mast cell protease II (RMCP II) dominates [123, 124, 131, 136]. RPMC also contain carboxypeptidase. Both rat mast cell types contain histamine, though different quantitatively — 1.3 pg per rat MMC and 15 pg per RPMC [125]. Rat MMC and RPMC may also differ in their ability to produce a variety eicosanoids which are synthesized de novo from the oxidative metabolism of arachidonic acid following stimulation. Partially purified rat intestinal MMC produce both prostaglandin D₂ and leukotriene C₄ and B₄, while RPMC produce predominantly prostaglandin D₂ but little or no leukotrienes.
Interspecies mast cell heterogeneity can be exemplified by the presence of the mediator 5-hydroxytryptamine in rodent mast cells, but apparently not in man [124].

RPMC and rat MMC also respond differently to secretagogues [33, 124, 125, 173] and inhibitory pharmacological agents [124, 174, 175]. RPMC respond with histamine secretion not only to IgE related stimuli and calcium ionophores but also to a wide range of basic compounds including compound 48/80, bee venom peptide 401, polyamines, a number of neuropeptides and opiates; in contrast, rat intestinal MMC do not respond to compound 48/80, polymyxin, peptide 401, or neuropeptides (except substance P), and are less responsive to the rest of the secretagogues mentioned. Furthermore, many drugs, such as disodium cromoglycate, theophylline, and isoprenaline, inhibit RPMC histamine secretion but without effect on rat intestinal MMC, while doxantrazole and quercetin inhibit antigen-induced histamine release by both mast cell subtypes. Table 1.2 and 1.3 summarize some functional responses of mast cell subsets from different species to various secretagogues and inhibitors.

Mast cells in other species, including human, also exhibit heterogeneity in multiple aspects of their phenotypes, but the situation is less circumscribed [123, 124, 127, 136, 171, 172]. For example, like rats, humans have mast cell subpopulations which differ in neutral protease content — subpopulation MC$^{\text{MC}}$ which contains measurable levels of both tryptase and chymase, and subpopulation MC$^{\text{CT}}$ which contains tryptase but no detectable chymase. However, while MC$^{\text{CT}}$ predominate in lung and small intestinal mucosa and MC$^{\text{MC}}$ predominate in skin and small intestinal submucosa, all these sites contain representatives of both mast cell subsets, and hence human mast cells cannot be classified as MC$^{\text{CT}}$ and MC$^{\text{MC}}$ based on tissue location alone [127]. Also it was found that guinea pig mast cells from cardiac, lung, mesenteric, kidney, and intestinal tissues were adversely affected by formalin fixation; and in addition, they were all alcian blue positive/safranin negative and berberine negative irrespective of whether they were from connective tissue or mucosa sites [176]. These findings highlight the interspecies mast cell heterogeneity.

To summarize, it is apparent that, while mast cell populations exhibit heterogeneity it may not be possible to identify one or two key phenotypic characteristics that can accurately predict many others. Hence, one should bear such heterogeneity in mind when studying drug effect on mast cell functional responses, and be cautious in extrapolating the findings.
Chapter 1

1.6.1.5. Origin of mast cells

In contrast to blood basophils which differentiate and mature in the bone marrow and circulate in the blood as mature and functionally active cells, mast cell precursors migrate into and circulate within the blood, and give rise to localized, tissue-bound mast cell precursors whose differentiation may be regulated by local microenvironment [124, 127]. It seems likely that, based on tissue culture studies and experiments in mast cell deficient mice, mast cells are derived from multipotent bone marrow haemopoietic precursor cells [124, 127]. Similar observations and conclusions have also been drawn from studies of rat mucosal mast cells [170]. In both rats and mice, intestinal mucosal mast cell proliferation has been shown to be dependent on the integrity of the T cell system of the host, in that no proliferation occurs in nude mice [177] or in severely T cell-depleted rats [178]. Based on in vitro studies with rodent cells, the major properties of T cell-derived factor(s) for mast cell proliferation are expressed by multipotential colony-stimulating factor interleukin-3 (IL-3), and coculture with fibroblasts appears to be critical in the maintenance and/or development of peritoneal or connective tissue mast cells [123, 127, 171, 172]. More recently, in vitro studies have shown that, other than IL3, stem cell factor and IL10 are involved in the proliferation or maturation/differentiation of murine mast cells [136].

In human, similar approaches as those for rodents have been employed to study mast cell origin and development [123, 124, 127, 170, 179]. Similar to RMCP II containing rodent mucosal mast cells, human MC\textsuperscript{c} are dependent on the presence of a factor(s) from T cells for their differentiation and maintenance, while the population of human MC\textsuperscript{c} (the counterpart of RMCP I containing rat CTMC) found in the intestinal submucosa and smooth muscles do not seem to be dependent on the same factor(s).

Mast cell phenotypic variation theoretically might reflect several mechanisms acting alone or in combination: (a) the existence of distinct mast lineages for different mast cell subtypes; (b) the process of cellular maturation/differentiation; (c) the functional status of the cell; and (d) the acquisition of molecules derived from other cell types [127]. While there is no convincing evidence that distinct mast cell lineages exist, the last three mechanisms actually occur [127]. It is believed that microenvironmental factors contribute to the mast cell heterogeneity [127, 124]. Nevertheless, the existence of distinct mast cell lineages has by no means been ruled out. There are many possible sources of variation in microenvironmental factors: such as constitutive variation based on anatomical site; acquired variation in association with inflammatory, immunological, or disease processes, hormonal fluctuations, aging, and others which
might induce changes in anatomical microenvironments; and mast cell dependent variation through mast cell degranulation and the production of cytokines [127]. It has been shown that, at least in the mouse, in the appropriate microenvironment transdifferentiation between mast cell subpopulations is possible [180].

1.6.1.6. Mast cell mediators

Mast cells represent a major source of tissue histamine and other potent chemical mediators implicated in a wide variety of inflammatory and immunological processes. Upon appropriate activation, either by immunological or non-immunological stimuli, mast cells release both preformed mediators stored in the cytoplasmic granules and mediators synthesized de novo, such as prostanoids, leukotrienes, and/or platelet activating factor. The preformed and stored mediators can be divided into those which are soluble when the cell is activated (including histamine, serotonin, certain proteinases, lysosomal enzymes, and chemotactic factors) and those which maintain a close association with extruded granules (including other proteinases, proteoglycans, inflammatory factors, and enzymes such as peroxidase and superoxide dismutase) [124, 133].

1.6.1.6.1. Histamine

Histamine is closely associated with mast cells in almost all tissues [181] and it is present in large amounts all along the intestinal tract of mammals [182]. Histamine in mast cells is formed via the decarboxylation of L-histidine by a pyridoxal phosphate-dependent cytoplasmic enzyme, histidine decarboxylase, and is bound to the anionic side chains of proteoglycan in the granule matrix [183]. Human intestinal mucosa mast cells contain 1-3 pg of histamine per cell [126, 184, 185], while rat mucosal and serosal mast cells contain 1-2 pg/cell and 10-30 pg/cell respectively [125, 131, 142]. Once released from mast cells, histamine is metabolized via methylation by histamine N-methyl transferase and oxidation by diamine oxidase to form methyl histamine and imidazole acetic acid respectively. Methyl histamine is normally oxidized by monoamine oxidase to methylimidazole acetic acid, while imidazole acetic acid may conjugate with ribose to form imidazole acetic acid riboside. The histamine catabolites are then excreted in the urine, sometimes with a small amount of intact histamine [183].

There are three classes of histamine receptors, $H_1$, $H_2$, and $H_3$, through which histamine exerts its biological effects [134, 181]. One or more of the three classes are expressed on many tissues and cell types. $H_1$ receptors mediate the contraction of
gastrointestinal and respiratory smooth muscles and the increased vascular permeability. Histamine increases vascular permeability via the H$_1$ receptors on the endothelial cells of the postcapillary venules, resulting in extravasation of plasma protein and fluid as well as leucocytes into the extracellular spaces and the formation of oedema [134]. The activation of endothelial cells is accompanied by the synthesis and release of other mediators such as prostacyclin, platelet activating factor, and endothelium-derived relaxant factor nitric oxide [134], all of which are proinflammatory and have been implicated to play a role in IBD [186-189].

Histamine, via H$_2$ receptor activation, stimulates gastric acid secretion, increases heart rate, and modulates the activity of inflammatory and immunocompetent cells [134, 136, 190, 191]. With respect to modulation of inflammatory and immunocompetent cells, the effects of histamine mediated via H$_2$ include the inhibition of basophil and mast cell mediators release, lysosomal enzyme release from granulocytes, and production of complement and acute phase proteins; the inhibition of basophil and neutrophil chemotaxis; the downregulation of mitogen- and antigen-induced lymphocyte proliferation, T cell colony formation, and T cell-mediated cytotoxicity; the inhibition of immunoglobulin production; the marked enhancement of natural killer cell activity; and the involvement in the cytokine network, both as a modulator of certain cytokine-receptor interactions and as a target of cytokine action. In addition, the suppression of the function of Fc receptors and complement receptors in neutrophils, and the direct chemotactic effect of histamine on eosinophils are mediated through both H$_1$ and H$_2$ receptors.

Histamine H$_2$ receptors are found in central and peripheral nervous system, and they are thought to regulate the release and synthesis of neural histamine as well as the release of other neurotransmitters [134]. Hence, physiologically, H$_2$ receptors may control cerebral circulation and may produce vasodilation by inhibition of sympathetic tone, and the activity of parasympathetic nervous system may also be regulated [134]. However, histamine is rarely found in enteric neurons, and it appears not to be a neurotransmitter in the local circuits but, rather, a signal substance released by intestinal mast cells in the process of neuroimmune communication [140].

As alluded to above (Section 1.6.1.2.), histamine has a role in the regulation of colonic transport function. It augments colonic secretion [140, 141, 192], resulting in diarrhoea which is one of the clinical manifestations of IBD. Histamine may interact with the neutrophil-derived oxidant hypochlorous acid to form reactive N-chlorinated derivatives, histamine chloramines, which are highly lipophilic and reported to retain histamine agonist properties [193]. These histamine chloramines have been proposed
to represent a novel form of mediators of gastrointestinal pathophysiology [194]. Histamine has also been shown to enhance the catalytic activity of the free radical-generating enzyme xanthine oxidase and increase the production of reactive oxygen radicals which damage endothelial cells [195].

It appears that the biological relevance of histamine extends beyond its well-characterized role in mediating allergic reactions.

1.6.1.6.2. 5-Hydroxytryptamine

As a mast cell mediator, 5-hydroxytryptamine (5-HT) or serotonin occurs in mast cells of the rat and mouse, but not in man [124], though it is found in man in the central nervous system, the enterochromaffin cells of the gut, and platelets. It can induce bronchoconstriction, vasoconstriction, and increase vascular permeability. The function of 5-HT in the intestine is probably concerned with motility and secretion, and infusion of 5-HT in man produces spasm of the intestine and diarrhoea [139].

1.6.1.6.3. Proteoglycans

As noted previously, the metachromatic staining properties of mast cells are rendered by the proteoglycans present in the granules. There are two major types of glycosaminoglycans found in mast cells — heparin and chondroitin sulphates — which are attached within the mast cell to small peptide cores produced by a single gene, and hence the heterogeneity must lie in the glycoaminoglycan side chains attached to this peptide core [133]. The function of proteoglycans is uncertain. However, by virtue of their polyanionic character and macromolecular structure, they interact with other preformed basic mediators, and consequently may facilitate the uptake, storage, and regulation of stability and activation of other mediators in the mast cell. Furthermore, apart from the ability to stabilize proteases, both heparin and chondroitin sulphates can inhibit certain aspects of the complement cascade, with only heparin possessing anticoagulant properties [124]. It has been suggested that the extent of dissolution of the various secreted mast cell granules in extracellular fluid may largely depend on their interaction with proteoglycans. Apart from having important physiological consequences for the interaction of the proteases with substrates and inhibitors and for enzyme regulation, the degree to which secreted mast cell granules dissolve in extracellular fluids (thereby releasing soluble enzymes and proteoglycans) may determine the distance...
Chapter 1

from the cells of origin over which the mast cell enzymes exert their action [196].
Heparin has also been suggested to be involved in the modulation of vasculature [133].

1.6.1.6.4. Neutral protease

As indicated above, mast cell subsets differ in neutral protease content. In the
rat, CTMC from skin, muscle, lung parenchyma, and peritoneal cavity possess RMCP I and carboxypeptidase, whereas MMC from gastrointestinal mucosa possess a distinct RMCP II. RMCP II is readily soluble and can be detected in the intestinal lumen and in the circulation during anaphylactic reaction. In murine mast cells, five chymases — mouse mast cell protease (MMCP)-1, -2, -3, -4, and -5, one mast cell carboxypeptidase, and two tryptases — MMCP-6 and -7 — have been reported [136]. MMCP-3, -4, -5, and -6, and mast cell carboxypeptidase are present in CTMC, whereas MMCP-1 and -2 are present in MMC. As mentioned above (Section 1.6.1.4.), two types of proteases, chymase and tryptase, have also been identified in human mast cells, one population of which contains a tryptase (MC^T), whereas another contains a tryptase and chymase (MC^C). MC^C also contain mast cell carboxypeptidase and cathepsin G like protease [136]. Although neutral proteases catalyse the cleavage of peptide bonds at neutral pH, their precise biological roles are unknown. However, based on their natural substrates which include C3 forming C3a, fibrinogen, procollagenase, HMW kininogen, fibrinogen, and type IV collagen of the basal lamina underlying epithelia [142, 171, 197], on theoretical grounds neutral proteases might work in concert with acid hydrolases in modifying tissue ground substance. Human chymase has been shown to activate the precursor of IL-1B, and it is also potentially important in activating angiotensin I [136]. In vitro human chymase hydrolyzes neurotensin, kinetensin, and leu^4-enkaphalin [136]. Human tryptase activates metalloproteinase III and inactivates fibrinogen, and more recently it has been shown to degrade calcitonin gene related peptide [136].

1.6.1.6.5. Preformed chemotactic mediators

Apart from histamine as a chemotactic mediator, mast cells also release a variety of preformed chemotactic mediators which include eosinophil chemotactic factor of anaphylaxis, neutrophil chemotactic factor of anaphylaxis, and inflammatory factor of anaphylaxis [142, 150, 198]. These mediators among others facilitate the migration of neutrophils, eosinophils, basophils, and mononuclear cells to the site of mediator release. In gut lesions of patients with UC, increased number of eosinophils together with
Chapter 1

secreted eosinophil cationic protein have been reported, and these cells are thought to play a role in the inflammatory process of the disease [22]. Neutrophils have been implicated in the pathogenesis of tissue damage at the inflammatory site [51].

1.6.1.6.6. Other preformed mediators

There are also various other enzymes, which are in much smaller amounts, present in mast cell granules. To list a selected few, they are acid hydrolases (β-glucuronidase, acid phosphatase, and N-acetyl-β-glucosaminidase), arysulphatases, kallikrein, and antioxidant enzymes such as peroxidase and superoxide dismutase [142, 150, 198].

Certain mast cell types have been shown to contain vasoactive intestinal polypeptide which is a neuropeptide [124], and this finding thus further supports the notion of communication between immune system and neuroendocrine system as alluded above.

1.6.1.6.7. Newly synthesized mediators

Following appropriate immunological or non-immunological stimulation, mast cells are capable to synthesize de novo a variety of mediators via the metabolism of arachidonic acid. Liberated from membrane phospholipids by the actions of phospholipases, arachidonic acid undergoes oxidative metabolism via the cyclooxygenase pathway to prostaglandins (PGE₂, PGF₂α, PGI₂, and PGD₂) and thromboxanes (TXA₂ and TXB₂) or the lipoxygenase pathway to leukotrienes (LTB₄, LTC₄, LTD₄, and LTE₄) and hydroxyeicosatetraenoic acids (HETE) [142, 150, 198]. However, as indicated above, there is heterogeneity in the ability of mast cells from different species and tissues to synthesize these eicosanoid mediators. In general, the predominant cyclooxygenase product generated by mast cells through the initial formation of unstable cyclic endoperoxides PGG₂ and PGH₂ is PGD₂, while main lipoxygenase products generated are sulphidoepetide leukotrienes LTC₄, LTD₄, and LTE₄ (formerly known as slow reacting substance of anaphylaxis) via the intermediate leukotriene LTA₄ which may also be converted to LTB₄ [124, 126, 151]. LTA₄ is in turn formed from the unstable intermediate 5-hydroperoxyeicosatetraenoic acid which may also be metabolised to HETE.

The mentioned eicosanoids constitute the most diverse family of inflammatory mediators and immunomodulators with respect to the many different cellular sources, pathways of generation, and biological effects, and they have been implicated in playing an important role in IBD [186-188, 190, 199-202].

29
Chapter 1

Platelet activating factor (PAF) is an endogenous phospholipid mediator which is synthesized and released by endothelial and inflammatory cells, including mast cells [203-205]. It mediates a myriad of biological responses such as stimulation of platelet and neutrophil aggregation, chemotaxis, degranulation, production of other mediators such as arachidonic acid metabolites as a result of stimulating arachidonic acid release and metabolism, generation of free radicals, bronchoconstriction, vasodilatation, and induction of vascular permeability [206-209], and hence it has been implicated as a mediator of inflammation and anaphylaxis. In the gastrointestinal tract, it is a potent ulcerogenic agent that it is regarded as a mediator of gastric ulceration as well as induces ischaemic bowel necrosis, and has been implicated in playing an important role in IBD [210-214].

More recently, mast cells have been demonstrated to secrete newly generated and preformed multifunctional cytokines (subcellular localization of the latter requires further clarification) that modulate IgE production and inflammation [136]. They include tumour necrosis factor-α, IL-4, IL-5, and IL-6. Some of these cytokines have been implicated in IBD [49].

1.6.1.7. Mast cell activation

Mast cells as well as basophils express high-affinity plasma membrane receptors (FcεR1) which can be cross-linked by multivalent anti-FcεR1 antibody, or bind Fc portion of IgE molecule with the Fab available for cross-linking by specific multivalent antigen [128, 129]. The binding of IgE to these specific mast cell receptors is attainable by active or passive sensitization. After sensitization, cross-linking of IgE molecules by specific multivalent antigen during subsequent exposure to the antigen triggers mast cells to undergo anaphylactic degranulation or exocytosis via an integrated, noncytolytic series of biochemical [128, 129, 142] and ultrastructural changes [215, 216]. Antibodies to IgE or to plasma membrane FcεR1 may also initiate the similar sequent of events. Lower-affinity receptors for certain IgG subclasses — IgG2a in the rat and IgG4 in human — are also present in mast cells, and upon appropriate stimulation can induce mediator secretion [217]. IgG4 has reaginic activity [46] and its possible role as a reaginic antibody for gut mast cells from patients with IBD has been shown [156].

Apart from the immunoglobulin mediated release, non-cytotoxic degranulation of mast cells may also be induced by any of a long and growing list of immunologically nonspecific agents including anaphylatoxins (complement C3a and C5a); cytokines (e.g. interleukin 1); many naturally occurring or synthetic basic peptides and polyamines (e.g.
polymyxin B, polylysine, bee venom peptide 401, and compound 48/80); bioactive peptides (e.g. bradykinin); leucocyte-derived polypeptides (e.g. neutrophil and eosinophil cationic proteins); selected neurpeptides, endogenous opiates, and hormones (e.g. substance P, vasoactive intestinal peptide, somatostatin, and β-endorphin); lectins (e.g. concanavalin A); polysaccharides (e.g. dextran); calcium ionophores (e.g. ionophore A23187 and ionomycin); chemical secretagogues (e.g. sodium fluoride); exogenous ATP; and others [133, 142, 218-220]. Though the sensitivity to these stimuli differs markedly according to species and mast cell population (see Section 1.6.1.4.), the fact that such a wide range of stimuli other than IgE and antigen can induce mast cell mediator release, implicates the potential roles of mast cells in a variety of biological responses and diseases not involving IgE [132].

Mast cell granular content may also be released in a nonselective and cytotoxic manner by a variety of physical and chemical agents, such as thawing, heating, and surface active compounds [221]. These agents indiscriminately and irreversibly disrupt the cell membrane, resulting in lysis (hence cytotoxic). As a result of lysis, intracellular cytoplasmic substances not associated with the granules (such as lactic dehydrogenase, ATP, and K⁺) as well as histamine are released; and in contrast, non-cytotoxic selective mast cell secretagogues, both immunologically or non-immunologically specific, induce histamine release via a process of exocytosis without the loss of the said cytoplasmic markers that are not associated with the granules [222]. It is well known that the non-cytotoxic histamine secretion from mast cells requires calcium (see Section 1.6.1.8.3.) and ATP, which may be supplied by oxidative metabolism or glycolysis [223-225]. In a glucose-free medium, the glucose analogue, 2-deoxyglucose (an inhibitor of glycolysis), and antimycin A (an inhibitor of oxidative metabolism), by blocking the generation of ATP, block the non-cytotoxic histamine release from mast cells. The non-cytotoxic release is also temperature-dependent [226]. In contrast, the cytotoxic histamine release in general is not affected by the factors mentioned for non-cytotoxic release.

1.6.1.8. Activation mechanism of mast cells

As mentioned above, mast cells release preformed granule associated mediators and newly synthesized lipid-derived mediators when high affinity receptors (FcεR1) are cross-linked by multivalent anti-FcεR1 antibody, or when multivalent antigen or multivalent anti-IgE cross-links IgE bound to FcεR1. Mast cells can also be stimulated via receptors for certain ligands, such as FMLP and C3a. The process is regulated by a mechanism
Chapter 1

which is not yet completely understood, and implicates many complex and different biochemical pathways [227-230].

1.6.1.8.1. Serine esterase activation

Early experiments using a number of protease inhibitors, including diisopropyl fluorophosphate (DFP), trypsin and chymotrypsin substrates or inhibitors, suggested that activation of a membrane-associated serine esterase played an important role in the early stages of antigen-induced histamine release from guinea pig lung, rat mast cells, and human lung [231-233]. These protease inhibitors were found to inhibit IgE-dependent phospholipid methylation, cyclic AMP rises, Ca\(^{2+}\) influx, and histamine release [234]. It was also found that the IgE-receptor mediated increase in phospholipid methylation and adenylate cyclase activation in isolated plasma membranes were blocked by these inhibitors. Inhibition by DFP, an inhibitor of enzymes bearing an activated serine, was found to require the presence of the inhibitor during antigen activation, and the inhibitor was not effective if it was added and washed out prior to stimulation. These findings raise the possibility that a serine esterase(s) is activated as the result of Fc\(_\gamma\)R1-stimulation and is the target of DFP. This serine esterase(s) in resting cells is present in an inactive form which is not affected by DFP. However, the specificity of DFP effects is questionable, as nucleophilic serine residues have also been demonstrated in active sites in a variety of hydrolytic enzymes outside the protease family, and DFP inhibition of mast cell ATP generation has also been reported [235]. Besides, the identity of the DFP-sensitive protease has remained elusive despite all these early observations.

1.6.1.8.2. Guanosine triphosphate (GTP)-binding proteins

Certain ligands interact with specific cell surface receptors and thereby trigger a series of molecular events that ultimately produce their biological effects. With certain receptors, this process was found to be controlled via GTP-binding regulatory proteins (G-proteins) which serve as signal transducers, linking extracellularly oriented receptors to membrane-bound effectors [236]. Several studies have suggested the involvement of G-proteins in the transduction mechanism of mast cell activation [223, 237-239]. In 1983, Gomperts [237] showed that the introduction of nonhydrolyzable GTP analogue (e.g. GTP\(_\gamma\)S) into permeabilized rat mast cell induced histamine release in response to the addition of extracellular calcium. Further studies provided evidence for the
participation of G-protein in the activation of phospholipase C (PLC) in mast cell exocytosis, resulting in the formation of second messengers inositol 1,4,5-triphosphate (IP$_3$) and 1,2-diacylglycerol (DAG) [223, 238] (see below). Cockcroft et al in 1987 [238] also observed in their system of streptolysin O-permeabilized RPMC that activation of PLC was not involved in triggering of the GTP$_\gamma$S-dependent histamine release, as the inhibition of PLC by neomycin, hence preventing the production of inositol phosphates, did not affect the histamine release. Hence, a G-protein (G$_{\gamma}$), which is distinct from G$_p$ (PLC-associated G-protein), has been proposed to be involved at a late stage in the secretory process [223].

In contrast, using ATP-permeabilized RPMC and bone marrow-derived mouse mast cells (BMMC) to introduce nonhydrolyzable guanosine phosphates GTP$_\gamma$S and GDP$_\gamma$S, it has been shown that in the transduction of IgE-mediated triggering signal to PLC in rodent mast cells may not involve a classic G-protein, though the possibility that a unique G-protein may exist in a special compartment which is not accessible to the experimental procedures used cannot be ruled out [240]. This finding is supported by the observations that pretreatment of rat mast cells and human basophils with pertussis toxin (PT), which inactivates PT-sensitive G-proteins, and BMMC with cholera toxin (CT), which inactivates CT-sensitive G-protein, affects neither IgE-mediated histamine release nor hydrolysis of inositol phospholipid [241, 242]. However, in the same studies by Saito et al, it was shown that histamine release by 48/80 or thrombin from rodent mast cells, and by FMLP from human basophil were sensitive to PT, while thrombin was also sensitive to CT. These data suggest that G-proteins may play a role in signal transduction by some agents (48/80, thrombin, and FMLP) which involve distinct signal transduction from that by IgE-dependent ligands.

Sodium fluoride has been reported to activate G-protein in a GTP-independent manner via fluoroaluminate ion AlF$_4^-$ which is formed by combination of fluoride ions from NaF with trace quantities of aluminium ions from glassware [243-245]. This action of NaF is not specific; however, it is the only compound available to activate G-protein without the recourse to using potentially artificial membrane preparation or cell permeabilization procedures for introducing nonhydrolyzable GTP analogue [246]. The histamine release from isolated rat mast cells induced by NaF has been shown to be insensitive to pertussis toxin [244].

The wasp venom peptide mastoparan has been demonstrated to activate G-proteins directly by mimicking agonist-bound receptors [247, 248]. Due to the striking similarities shared by 48/80, Sub P, and mastoparan in activating rat peritoneal mast
cells, it has been suggested that the action of 48/80 and Sub P may directly activate G-protein [249]. The involvement of G-protein $G_E$ in 48/80-induced histamine release has been suggested [223, 240].

1.6.7.8.3. Role of calcium

The importance of calcium in the secretion of many cell types has been known for some time. The indirect evidence of the requirement of extracellular $Ca^{2+}$ for optimal antigen-induced histamine release was first demonstrated in 1958 by Mongar & Schild [250] in isolated guinea pig lung fragments. The theory developed for the initiation of muscle contraction by a rise in cytosolic calcium and the information about the calcium dependence of secretory processes led to the hypothesis of calcium as the 'second messenger' for stimulus-secretion coupling [251]. Since then, several independent investigations have provided evidence that the requirement and the influx of extracellular calcium into the mast cell is linked to histamine release.

First, it was shown that there is a requirement for extracellular calcium in the range 0.1-1.0 mM for mast cells stimulated by IgE-directed agonists [252]. Using extracellular radioisotopic $^{45}Ca$, Foreman et al [253] demonstrated the accumulation of $^{45}Ca$ within mast cells that were stimulated with antigen, dextran, and Con A. This method of monitoring intracellular calcium levels has been used extensively by other workers in the same field, and similar findings have been reported with human lung mast cells, basophils, and rat and mouse mast cells [100, 225, 254, 255]. It was found that the increased accumulation of the isotope preceded or was concurrent with histamine release. Further studies using calcium-sensitive fluorescent probe quin-2 have demonstrated an increase in cytosolic calcium of mast cells upon anti-IgE, 48/80, and A23187 stimulations [256].

Second, lanthanum, which has similar ionic radius to that of calcium and a high affinity for cell membrane calcium binding sites but does not enter cells, is a very potent inhibitor of the calcium action in mast cell histamine release [257, 258]. This inhibition is reversible by increasing the extracellular calcium. The competitive effect for calcium binding sites by lanthanum is attributable to its valency which is higher than that of calcium.

Third, introducing calcium into the mast cell by direct microinjection [259] or calcium containing liposomes [260] can elicit an exocytotic response. The effect in both cases is unique to calcium, and neither magnesium nor potassium alone induced positive response.
A fourth piece of evidence showing that the influx of calcium into the mast cell initiates secretion has been provided by the action of calcium ionophores such as A23187 and ionomycin [261, 262]. Ionophores are lipid-soluble organic molecules that act by facilitating the transport of metal ions such as calcium from one aqueous phase across a lipid to a second aqueous phase (e.g., across cell membrane), hence raising cytosolic free calcium artificially by circumventing the cell surface ligand receptors. It was first shown with RPMC that, in the presence of an adequate level of extracellular calcium, A23187 produces marked histamine release accompanied by the uptake of calcium, though it has no histamine-releasing activity by itself [261]. Its action is calcium specific, since magnesium, which is likewise transported by A23187, will not induce secretion even when used in much higher concentrations [263]. Hence, the A23187-induced histamine release falls progressively as the extracellular calcium concentration is lowered [261, 264], and is completely abolished in conditions of rigorous calcium-deprivation [262]. As discussed by Douglas and Nemeth [265], the calcium-dependent secretory response of the mast cell induced by A23187 conforms to the classic exocytotic pattern not only in its dependence on metabolic energy and temperature, but also in its appearance in light and electron microscopy. It has been established that histamine release from various mast cell populations of different animal species can be achieved by A23187 bypassing the cell surface receptors [124, 174, 185, 266, 267].

Finally, calcium has been found to induce non-cytotoxic histamine release from mast cells permeabilized by ATP, Sendai virus, streptolysin-O, or β-escin [223, 268]. The cells are first permeabilized in the absence of divalent cations before the induction of release.

Calcium pools involved in histamine release

It is well known that the elevation of cytosolic free calcium ions may be due to an influx of extracellular calcium and/or mobilization of intracellular stores of calcium. This calcium entry and mobilization may be achieved through a number of mechanisms, for example through calcium channels operated by voltage, by receptors, or by second messengers [237, 269-272]. Unlike excitable cells, patch clamp studies of mast cell models have shown that mast cells do not seem to use voltage-dependent calcium channels [273, 274]. However, secretagogues can activate calcium channels by second messengers [271]. The calcium channel has been established as a glycoprotein [275], which may involve sialic acid residues in basophils and mast cells [276, 277].
Taking advantage of their calcium antagonistic effect and their different ionic radii (hence different abilities of penetrating cell membrane), lanthanum and other lanthanides have been used to show that there are at least two sources of calcium that can contribute to the elevation of cytosolic calcium upon stimulation [266, 278]. They are: (a) extracellular source of calcium which binds loosely to superficial sites of mast cell membrane and equilibrates with the extracellular environment; and (b) the intracellular source of calcium which is sequestered in cellular organelles or membrane-bound. The former is the main source used by IgE-mediated ligands in inducing histamine release, while the latter, by polybasic secretagogues.

As mentioned above, there is a requirement for extracellular calcium in the range 0.1-1.0 mM for mast cells stimulated by IgE-directed agonists [252]. It has been suggested that, upon stimulation, an influx of calcium ions from the said superficial sites on mast cell membrane gives rise to exocytosis [256, 269, 270, 278, 279]. Experimental evidence shows that when rat mast cells isolated in the presence of calcium are stimulated with selective secretagogues in the calcium-free media, there is a progressive decrease of histamine release with the increasing preincubation time of mast cells prior to stimulation [280]. This is attributable to the dissociation of calcium ions from the membrane progressively with time in absence of calcium in the extracellular medium.

The histamine release induced by NaF from isolated rat mast cells has also been shown to be dependent on the extracellular calcium [281].

It has been long known that extracellular calcium is not required for mast cell stimulation by 48/80 [282, 283]. However, stimulated histamine release is abolished in cells which have been treated to deplete internal calcium stores by prolonged incubation with calcium chelating agent, EDTA in the absence of added calcium [266, 278, 284]. Hence the substantial stimulated histamine release in the absence of extracellular calcium is attributed to the mobilization of intracellular stores of calcium ions. Other basic secretagogues having the same effects typified by 48/80 include peptide 401, polylysine, histone, eosinophil major basic protein, and Sub P [278, 285-288]. It has been shown that Sub P induces a transient increase in intracellular calcium which is independent of external calcium [289].

The fluorescent probe quin-2 has also been used to provide the evidence of calcium release from intracellular calcium store [256, 290].
Chapter 1

Role of calcium binding protein calmodulin

The importance of calcium in mast cell histamine release is apparent. However, the exact mechanism of how calcium initiates histamine release is not fully understood. From the studies on other cell systems, a calcium-binding regulator protein, calmodulin, is implicated as an intracellular ‘calcium receptor’ through which calcium exerts its action [291]. In smooth muscle, it is calmodulin that apparently acts as calcium receptor and functions to effect calcium-dependent protein phosphorylation [292]. Moreover, calcium-dependent protein phosphorylation has been observed in the mast cell [293]. Hence, apart from regulating the activation of calcium-dependent enzymes, including protein kinase C (PKC) (see section below), the increase in cytosolic calcium may also regulate enzymes controlled by calcium or calcium/calmodulin-dependent protein kinases, many of which are associated with enzymes which are potentially involved in the regulation of mast cell histamine release [278, 294, 295]. Calmodulin also autoregulates its own activity by controlling the cytosolic free calcium through activation of the calcium pumps [291, 296, 297]. More recently, with the aid of the immunosuppressant cyclosporin A and FK506, calmodulin and calcium/calmodulin-dependent phosphatase (calcineurin) have been discovered to be involved in the actions of calcium in the T-cell receptor-mediated transduction pathway [298].

Taken together the above-mentioned findings, the distribution of calmodulin in mast cells, and the inhibition by calmodulin antagonists on mast cell histamine release, it has been suggested that elevated cytosolic calcium may act through the activation of calmodulin [265, 299-301].

1.6.1.8.4. Role of phosphoinositide metabolism

The current concept of the mechanism of secretion envisages a complex process in which the breakdown of phosphatidylinositol (PI) in the plasma membrane plays a central role and leads to the generation of intracellular messengers [272, 302]. As stated above, receptor activation on the plasma membrane is coupled through a G-protein to polyphosphoinositide phosphodiesterase [223, 238]. This catalyses the hydrolysis of phosphatidylinositol 4,5-biphosphate (PIP$_2$) to the intracellular messengers: IP$_3$, which mobilizes stored calcium and may promote the influx of external calcium, perhaps working in conjunction with inositol-1,3,4,5-tetrakisphosphate [272, 303]; and DAG which stimulates PKC [302]. PIP$_2$ and phosphatidylinositol-4-phosphate (PIP) are formed as a result of PI phosphorylation by ATP-dependent specific kinases [304].
Chapter 1

Rat mast cell granules have been shown to contain active PI kinase and PIP kinase [305].

Using radiolabelled $^{32}\text{PO}_4$ and $^3\text{H}$-inositol, the importance of the PI response was first reported by Cockroft and Gomperts [306], and Kennerly et al [307], independently in 1979. It was found that mast cell showed marked receptor-initiated enhancement of $^{32}\text{PO}_4$ and $^3\text{H}$-inositol incorporation into PI and phosphatidic acid (PA), as well as unexpectedly phosphatidylcholine (PC). Similar findings were also reported by Schellenberg in 1980 [308]. Based on the negative results in a study of $^3\text{H}$-glycerol incorporation, the author emphasized that the enhancement of incorporation of $^{32}\text{PO}_4$ into PI, PA, and PC by various non-cytotoxic releasers was not due to the de novo synthesis of these phospholipids.

The DAG production from PIP$_2$ is normally transient and temporally corresponds to the formation of IP$_3$, and it is often followed by a more sustained increase in the amount of DAG [302]. There is increasing evidence that the DAG formation from PC via the initial conversion of PC to PA by phospholipase D (PLD) is the major pathway of DAG production during mast cell activation [309-313].

The simultaneous increase in inositol phospholipid breakdown and histamine release by 48/80, Sub P, and mastoparan, has also been demonstrated [314-317].

**Inositol triphosphate and calcium signalling**

The second messenger IP$_3$, which is released into the cytosol, has been shown to mobilize calcium via specific receptors from internal stores, such as endoplasmic reticulum (ER), and has also been suggested to induce influx of external calcium [268, 272, 318]. Using permeabilized rat peritoneal mast cells, it has been shown that the intracellular store of calcium is located in the ER, and IP$_3$ induces degranulation dose-dependently [268, 319].

The influx of external calcium may also require the presence of IP$_4$ [272, 303]. There is growing evidence that inositol phosphates may have direct effects on calcium channels that allow influx of calcium through the plasma membrane. For example, the IP$_3$-induced entry of calcium in lymphocytes may be mediated by a receptor which contains sialic acid and is localized in the plasma membrane [320]. In addition to the direct effects, IP$_3$ may also induce calcium influx indirectly through a more complex mechanism involving ER. Putney in 1986 [321] used the term "capacitative entry" to introduce the idea that the influx of external calcium seemed to be regulated by the calcium content of a portion of the ER lying close to the plasma membrane. It has
been shown in the mast cell that calcium entry is stimulated when the ER stores are artificially emptied by applying the calcium pump inhibitor thapsigargin or the calcium ionophore ionomycin [318]. When the ER is fully charged, the entry is prevented, but as soon as IP$_3$ drains calcium out, the influx switches on automatically. This mechanism is thought to account for the entry of calcium into mast cells [318]. A possible explanation for this capacitative mechanism is that IP$_3$ receptor might function to communicate information between the ER and plasma membrane [272]. The concept is that part of the IP$_3$ receptor communicates with two calcium channels, one in the ER and the other in the plasma membrane. The conformational state induced by IP$_3$ binding opens the ER channel, and as the pool loses its calcium, the receptor may alter its conformation, leading to the opening of the plasma membrane calcium channel.

1,2-diaclylycerol and the activation of protein kinase C

PKC is widely distributed in the cytosol and membrane fractions of a variety of mammalian tissues, and there is evidence which suggests that PKC-mediated protein phosphorylations play a central role in stimulus-effect coupling in these tissues [302, 322]. The presence of PKC in RPMC and rat basophilic leukaemia cells has been reported, although the activity is much lower than that in the cerebral cortex or in the other peritoneal cells [323]. DAG, which is retained within the membrane environment, has been shown to activate PKC which in turn leads to phosphorylation of serine and threonine residues in a subset of proteins in various cell types, including mast cells [302, 305, 324-327]. Translocation of PKC into cell membrane is a cytosolic calcium concentration-dependent process that may be crucial for mast cell histamine release [326]. PKC, with its activation, has been proposed as a possible modulator for extracellular calcium-dependent stimuli as the result of the observations of enhanced calcium uptake and the potentiation of the response to antigen, A23187, and NaF [323-325, 327] The enhanced calcium uptake also reflects that PKC may contribute to the replenishment of the intracellular calcium stores after the secretory response [325]. Moreover, it has been reported that histamine release induced by antigen (which is calcium-dependent), but not that by 48/80, is inhibited by pretreatment with PKC inhibitors, implicating the involvement of PKC in IgE-mediated release, but not in 48/80-induced release [328]. However, it has also been reported that the activation of PKC is not essential for mediator release induced by antigen stimulation in rat basophilic leukaemia 2H3 cells [329, 330]. Furthermore, findings from a study using permeabilized rat mast cells has suggested that the PKC activation is not required for exocytosis to
Chapter 1

occur [331]. Thus the relationship between PKC activation and histamine release is not yet clear. It has been shown that there are more than one species of PKC molecule with multiple discrete subspecies (10 subspecies), and not all of them are calcium- and/or DAG-dependent for their activation [302].

The synthetic diacylglycerol, 1-oleoyl-2-acetyl-glycerol (OAG) and the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) can interact at the same site as DAG and activate PKC [332, 333]. OAG or TPA (ie. activation of PKC) alone induces slow histamine release [325, 334]. Depending on the concentrations and incubation periods of OAG or TPA, synergistic and inhibitory effects of both compounds on histamine release induced by secretagogues have been reported [324, 325, 335]. Low concentrations and short incubations of OAG/TPA show potentiation in histamine release, whereas high concentrations and long incubations show inhibition. It has been shown that the inhibition of histamine release during PKC activation by phorbol esters is mediated through inhibition of PI breakdown and calcium mobilization, perhaps by phorbol esters [310, 335]. Alternately, it may be the true effects of PKC. Thus, the potentiation suggests that PKC is involved in the secretory process by the secretagogues, while the inhibition indicates a regulatory function [325] (possibly desensitization). The slow histamine release induced by PKC activation may suggest that PKC may be important but not sufficient in mast cell activation.

1,2-Diocanoyl-sn-glycerol (Dic8) is a synthetic 1,2-diglyceride that has been reported to activate PKC [336, 337].

1.6.1.8.5. Role of cyclic AMP

The exact role of adenosine 3', 5'-cyclic monophosphate (cAMP) in mast cell activation is not fully understood. The IgE-mediated activation of mast cells and basophils produces a striking, rapid, and transient rise in levels of intracellular cAMP [338-340]. However, with non-IgE-mediated secretagogues, such as 48/80 and A23187, serosal mast cell activation is not accompanied by an increase in cAMP levels, but in fact is accompanied by a decrease [341, 342]. Moreover, pharmacological interventions that raise the cellular levels of cAMP either inhibit or have no effect on IgE-mediated histamine release from isolated guinea pig and human lung tissue, dispersed human lung mast cells, human basophils, and rat mast cells [341, 343-346]. These findings suggest that the function of cAMP is more likely to negatively regulate IgE-mediated histamine release (ie. inhibition).
cAMP activates protein kinase A (PKA) [305], and in general, this activation is considered to be terminated by the action of phosphodiesterases [347]. Similar to IgE-mediated release, NaF-induced histamine release from rat mast cells is also accompanied by an elevation of cellular cAMP level [348]. It has been shown that, in intact human platelets, activation of G-protein by NaF leads to the activation of adenylate cyclase and PLC activities, resulting in the increase of PKC activity and inositol phosphate formation [349]. As stated above, NaF will activate all G-proteins, and it has been shown that it stimulates adenylate cyclase activity at lower concentrations and inhibits at higher concentrations in various tissues [350, 351]. The NaF-induced histamine release from RPMC has been shown to be inhibited by the elevation of cAMP, hence the suggestion that PKA negatively regulates (ie. inhibition), while PKC, which is dominant, positively regulates (ie. stimulation) the histamine release induced by NaF [327].

There is an extensive heterogeneity in the signal transduction pathways used by cells, and many variations exist from tissue to tissue. Most tissues seem to involve two basic pathways: one depends on the formation of cAMP as a second messenger, while the other relies on the rapid turnover of PI as well as calcium mobilization. It has been suggested these control systems may operate in a monodirectional or bidirectional manner [352]. In the monodirectional systems, the activation of one pathway may potentiate the other, whereas in the bidirectional systems, the activated pathways may counteract each other. There is some evidence to indicate that cAMP inhibits PI kinase and PIP kinase in rat mast cells [305].

1.6.1.8.6. Role of tyrosine kinase

Apart from G-protein-linked receptors mentioned above, there are receptors linked by tyrosine kinase either directly or indirectly. Recently, there is increasing evidence for an important role of protein tyrosine kinases (PTK) in IgE-dependent mast cell degranulation [312, 353-358]. Following receptor aggregation, a number of proteins have been reported to be phosphorylated on their tyrosine residues [357]. Some of these proteins have been identified, including the 140 kDa PLCγ [354], a 72 kDa tyrosine kinase [360], and the β (33 kDa) and γ (9-11 kDa) chains of the IgE receptor [357]. Tyrosine kinase inhibitors, including tyrphostins, genistein, and lavandustin A, block IgE-dependent histamine release [353, 356, 358], suggesting the participation of protein tyrosine phosphorylation in IgE-dependent mast cell histamine release. The role of this tyrosine phosphorylation in regulating mast cell activation may explain the above-mentioned findings (Section 1.6.1.8.2.) by Saito et al [240], indicating the classic G-
protein may not be involved in the transduction of IgE-mediated triggering signal to PLC in rodent mast cells. Protein kinase phosphatases which control protein tyrosine phosphorylation in conjunction with PTK have also been implicated in the control of FcR1 signalling cascade [361].

1.6.1.8.7. Phospholipase activation and arachidonic acid liberation

As mentioned above, mast cells are capable of synthesizing de novo a variety of mediators via the metabolism of arachidonic acid (AA) upon stimulation. It has been well documented that the liberation of AA as a precursor of eicosanoid generation in stimulated mast cells is due to the direct hydrolytic action of phospholipase A2 [362-365]. Exogenously added PLA2 or the PLA2 activator melittin has been shown to induce mast cell degranulation [366]. Moreover, PLA2 inhibitors, including 2,4'-dibromoacetophenone, mepacrine, specific anti-PLA2 antibody, a degradation product of complement C3, and the fungal product thiocin A1, have been shown to inhibit histamine release induced by IgE, calcium ionophore A23187, con A, 48/80, and substance P [364]. Some of the free fatty acids and lysophospholipids liberated by the action of PLA2 may also involved in the activation of PKC [302]. Lysophospholipids have also been reported to be fusogenic and to have potential importance in the secretory process [367, 368]. There is also ample evidence that PKC may be involved in the activation of PLA2 (intracellular PLA2) [302]. PLA2 is involved in the formation of PAF by deacylating 1-O-alkyl,2-acyl-PC to form the critical PAF precursor 1-O-alkyl,2-lyso-PC which is then acetylated by the acetyltransferase reaction to form PAF [206, 369]. These observations have led to the proposal that PLA2, especially the low molecular weight secretory PLA2, may have an essential role in the regulation of mast cell degranulation process [364].

However, in some instances, stimulated histamine release is not accompanied by the AA metabolism to generate lipoxygenase products [370, 371]. In addition, in the above-mentioned studies of PLA2 inhibitors, inhibition of histamine release is not always accompanied by the inhibition of eicosanoid generation. These findings could be attributed to the observations that there is more than one phospholipid pool of AA mobilize within mast cells during activation, suggesting that each pool of AA may use a distinct PLA2 [365]. Several discrete phospholipases A2 have been demonstrated and identified in mammalian tissues, including mast cells [364, 365, 372]. Therefore, degranulation and eicosanoid generation in mast cells appear to be regulated independently by discrete phospholipases A2 [364, 365].
AA may also be liberated by the action of PLC from phospholipid-derived DAG by the sequential action of DAG lipase and monoacylglycerol lipase [309]. More recently, it has been reported that AA may be liberated by DAG lipase action on DAG which is generated by the sequential action of PLD and phosphatidate phosphohydrolase [309-313] (Fig. 1.3).

1.6.2. Bile acids

Since CD and UC, despite the significant pathological and clinical differences, are chronic diseases with recurrent exacerbations and remissions, or sometimes they are active continuously, it is reasonable to expect that the agent or agents responsible should be present in the lumen of the gut regularly. Bile acids which have been implicated in gastric inflammation and ulceration [373-375] seem to fit this requirement. It is possible that under appropriate conditions bile acids manifest their damaging effects and expose the gut to luminal antigen(s) which may be regularly present, and which could be an altered bile acid metabolite brought about by altered bacterial metabolism. The epithelial damage may also be brought about by the impaction of inspissated faeces. Consequently, possibly facilitated by undefined genetic influences, a sensitization to luminal antigen may take place, after which an immunological sequence accountable for the inflammatory responses may be initiated. It has been proposed that IBD could be the result of an altered bacterial metabolism of bile acids in genetically susceptible individuals [376, 377].

1.6.2.1. Bile acids in the intestine

Bile acids which are biological detergents when secreted by the liver are completely conjugated with either glycine or taurine. Deconjugation and conversion of hepatic cell-synthesized primary bile acids (e.g. cholic acid) to related secondary bile acids (e.g. deoxycholic acid) by bacterial enzyme action occurs during the passage through the intestine. Tertiary bile acids (e.g. ursodeoxycholic acid) results from further hepatic alteration. When entering the duodenum, these secreted bile acids are reabsorbed via enterohepatic circulation and returned to the liver for re-secretion. Under normal conditions, only about 5% of the bile acids in the enterohepatic cycle, or up to 30% of the total pool per day (= about 6 enterohepatic cycle), enters the large intestine (in which further reabsorption takes place) through the ileoocaecal valve [378]. Hence, very little of the bile acids is lost in the faeces. The detergent effect of bile acids
allows the formation of a stable solution or emulsion of many fatty materials, and is essential to the digestion and absorption of fat, fatty acids, cholesterol, and fat-soluble vitamins.

1.6.2.2. Bile acids and colonic function and integrity

1.6.2.2.1. Transport and permeability

Diarrhoea is one of the cardinal symptoms in patients with UC and CD. The feature of diarrhoea is the secretion of water, electrolytes, and mucus (especially in UC) by the intestine. In addition, loss of plasma and blood through the inflamed ulcerated mucosa may occur. Perfusion of colons of a number of species, including human, with bile acids (secretory dihydroxy bile acids: chenodeoxycholate and deoxycholate) or using a more physiological model of ileal infusion of bile acid in human subjects with an uncleansed large intestine has been demonstrated to bring about marked morphological changes in the colonic mucosa which are often associated with changes in permeability and in fluid and electrolyte secretion [378-380]. Evidence has been produced to dissociate the secretion-promoting activity of bile acids from their action on colonic permeability [378]. The implication of increased mucosal permeability has already been mentioned above (Section 1.3.4.); and the increased colonic permeability induced by bile acids has been shown to increase mucosal sensitivity to antigen in sensitized guinea pigs [381].

Secretory bile acids are potent stimuli for mucus secretion by the large intestine [378, 379, 382]. Depletion of mucosal mucus with the loss of cytoprotective effect of mucus may permit mucosal damage through detergent effects of bile acids [378] among other agents or increased antigen presentation. Depletion of goblet cell mucus is a striking histological feature of active UC (see Section 1.3.7.).

1.6.2.2.2. Degeneration, proliferation, and dysplasia

UC and CD patients with long standing disease have a higher risk of developing intestinal cancer [10, 383]. Intra-colonic instillation of secretory bile acids have been shown to cause mucosal injury, surface cell loss [379, 384, 385], and increases in epithelial proliferative activity [384, 386]. While the mucosal damage could account for the altered permeability and DNA loss [379], the increased epithelial proliferative activity have been implicated in the risk of tumour growth [380, 387]. A relationship between mucosal damage, as caused by bile acids, and development of colon cancer has also
been suggested [388], though the epithelial proliferative activity has been reported to be independent of the loss of colonic surface epithelium [380]. Evidence has also accumulated from studies of the epidemiology and of experimental animal models of colorectal cancer, and the finding of specific bile acid receptors in colorectal cancers in man [389] seem to incriminate bile acids in the promotion colorectal neoplasia [376, 390, 391]. An association of high faecal bile acid concentrations with dysplasia and carcinoma in UC has also been suggested [392].

1.6.2.3. Mechanisms underlying interaction of bile acid with colonic mucosa

The alterations of morphology and function of colonic tissue by bile acids probably relate to some properties which also damage mucosal epithelial cells, such as detergency, or the capacity to penetrate lipid bilayers or solubilize membrane phospholipids and protein components with subsequent change in membrane structure and permeability [378]. The effects of bile acids on colonic tissue may also be due to their capabilities to enhance the release of cyclooxygenase and lipoxygenase products from the colon [387], to induce increases in reactive oxygen species [380], to release colonic vasoactive intestinal polypeptide [393], and to activate protein kinase C indirectly by increasing diacylglycerol [380].

1.6.2.4. Bile acid and mast cells in present study

Histamine, one of the mediators of mast cells, can also induce colonic events analogous to those induced by bile acids [134, 141]. Mast cells are found in the lamina propria of the normal large bowel as well as other tissues, and mast cell hyperplasia is a consistent feature of UC and CD [145-149, 155]. In view of the fact that the effects of bile acids may be mediated by histamine, it would be interesting to see whether bile acids interact with mast cells to induce histamine release.

1.6.3. Reactive oxygen metabolites

Oxygen-derived free radicals and other reactive oxygen metabolites have emerged as a common pathway of tissue injury in a wide variety of otherwise disparate disease processes. Reactive oxygen metabolites such as hydroxyl radical, superoxide, hydrogen peroxide, and hypochlorous acid have been implicated to play an important role in the pathogenesis of inflammation and in mediating the cellular injury associated with gastrointestinal disease, including UC and CD [51, 58, 394-397]. It is thought that the
primary mediators of tissue damage are the secondarily derived oxidants such as hydroxyl radical and hypohalous acid, though superoxide or hydrogen peroxide may be injurious to tissue directly [398, 399]. Although cells in the gastrointestinal tract, like all other cells, possess numerous antioxidant enzymes and scavengers to protect themselves from these injurious agents [400, 401], the rate of production of reactive oxygen metabolites may exceed the capacity of the antioxidant defences, thereby resulting in substantial tissue damage. It has been shown that the colonic mucosa is relatively deficient in antioxidant enzymes when compared to liver, and most of the protective enzyme activity is localized within the epithelium and not the mucosal interstitium [401]. Furthermore the gastrointestinal tract is particularly well endowed with the enzymic machinery necessary to form large amounts of reactive oxygen metabolites and chlorinated oxidants. Oxidases found in resident phagocytic leucocytes (macrophages, neutrophils, eosinophils), and mucosal epithelium appear to be the major sources of reactive oxygen metabolites in the intestine [402].

1.6.3.1. Production of reactive oxygen metabolites

1.6.3.1.1. Endogenous sources

Free radicals and other reactive oxygen metabolites are formed continuously as byproducts of normal cellular metabolism as a result of partial reduction of oxygen [51, 58, 403, 404]. The cytochrome oxidase-catalysed reduction of molecular oxygen (O\textsubscript{2}) (approximately 90% of total consumed in mammalian cells) to water by the addition of four electrons is the major source of energy for aerobic organisms. The addition of one electron to O\textsubscript{2} yields the superoxide anion (O\textsubscript{2}\textsuperscript{-}) which is formed nonenzymatically in virtually all aerobic cells as a result of the autooxidation of the constituents of the mitochondrial electron transport chain. In addition, superoxide anions are generated enzymatically by a number of oxidases (other than cytochrome oxidase), which include mucosal oxidases such as xanthine oxidase, xanthine dehydrogenase (either converted to oxidase form through the limited proteolysis — a probable mechanism during ischaemia, or by a reversible oxidation of sulphhydryl side groups) [51], NADPH oxidase, amine oxidase, and aldehyde oxidase as well as NADPH-dependent oxidase found in the resident phagocytic leucocytes (macrophages, neutrophils, eosinophils) of the lamina propria [58, 399]. Another potential source of free radicals is the cell membrane, including the endoplasmic reticulum and nuclear envelope. This is because the metabolism of the membrane-derived arachidonic acid by cyclooxygenase, lipoxygenase,
and epoxygenase to produce prostaglandins, leukotrienes, and epoxides, respectively, represents a source of free radicals [51]. The arachidonic acid cascade can also be initiated by free radicals generated from other sources.

The cellular damaging effect associated with superoxide is usually attributed to its role as a precursor to more reactive oxygen radical species [51, 58, 404]. Two molecules of superoxide dismutate spontaneously, or enzymatically as a consequence of the activity of superoxide dismutase to form $O_2$ and hydrogen peroxide ($H_2O_2$). Hydrogen peroxide is almost always formed under any circumstance where there is the generation of superoxide anions:

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

Hydrogen peroxide is also formed by the direct two electron reduction of $O_2$ via a number of oxidases such as monoamine or diamine oxidases:

$$O_2 + 2e^- + 2H^+ \rightarrow H_2O_2$$

Unlike superoxide, hydrogen peroxide is not a true free radical species, and it has a longer half-life and thus persists longer in the tissue to exert its deleterious effects to tissue components, either directly (if present in very high concentrations) or through the secondary generation of other oxidants such as hydroxyl radical ($OH^-$). Under the catalytic influence of transition metals such as iron, superoxide and hydrogen peroxide can interact via the iron-catalysed Haber-Weiss reaction to yield the potent oxidant, hydroxyl radical [58, 403, 404]:

$$O_2^- + Fe^{3+} \rightarrow O_2 + Fe^{2+}$$

$$H_2O_2 + Fe^{3+} \rightarrow OH^- + OH^- + Fe^{2+}$$

Sum: $O_2^- + H_2O_2 \rightarrow OH^- + OH^- + O_2$

The identity and availability of the naturally occurring iron-containing compound that can catalyse hydroxyl radical formation in vivo remains unknown. However, there are several potential sources of iron which may account for the physiological origin of the iron pool. These sources include the autophagic degradation of ferritin, haemoglobin, modification of iron-binding proteins (e.g. transferrin and lactoferrin) by micobrial protease, nonprotein-bound iron (e.g. iron salts such as iron citrate or potentially iron-citrate acetate), and local iron-binding compounds (siderophores) produced by microbial pathogens [403, 405].
Recent evidence suggests that OH' could also be generated in phagocytes by either the myeloperoxidase (MPO) or eosinophil peroxidase system in a transition metal-independent manner [405]:

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$
$$H_2O_2 + Cl^- + H^+ \xrightarrow{MPO} HOCl + H_2O$$
$$HOCl + O_2^- \rightarrow HO^- + Cl^- + O_2$$

The NADPH-dependent oxidase system located on the surface membrane of phagocytes, including neutrophils, eosinophils, monocytes, and other macrophages, is a highly efficient source of superoxide radical generation [51]. In addition to the production of superoxide, hydrogen peroxide, and hydroxyl radical, activated phagocytes secrete myeloperoxidase into the extracellular medium where it catalyses the oxidation of chloride ion (Cl') by H_2O_2 to yield hypochlorous acid (HOCl) which is the most reactive oxidant produced by neutrophils in appreciable amount [406]. Hypochlorous acid which is more selective for its biological targets as an oxidant than hydroxyl radical, may further react with endogenous amine to form chloramines, powerful oxidizing agents, which are responsible for much of neutrophil toxicity [407]. The cytotoxicity associated with HOCl and chloramines is a result of sulphydryl oxidation, haemoprotein and cytochrome inactivation, chlorination of purine bases on DNA, and degradation of amino acids and proteins [399]. They may also mediate tissue injury indirectly via their ability to activate collagenase and gelatinase, and inactivate antiproteases [407].

1.6.3.1.2. Oxidized metabolites of nitrogen

Nitric oxide radical biosynthesized from the metabolism of L-arginine catalyzed by two distinct types of nitric oxide synthase is generated by neutrophils [408], macrophages [409], and mast cells [410], in addition to endothelial cells. It has been suggested that under certain circumstances, hydroxyl radical might be produced by the reaction of nitric oxide and superoxide radical [405, 415]:

$$NO^+ + O_2^- \rightarrow ONOO^-$$
$$ONOO^- + H^+ \rightarrow ONOOH \rightarrow NO_2^- + OH^-$$

Nitric oxide has also been implicated as a potential pathophysiological mediator in IBD [189, 411-413]. It may be involved in the epithelial permeability effect of mast cells [414]. Recently, a possible role of nitric oxide in regulating the above-mentioned "capacitative entry" of Ca" has been suggested [416]. However,
1.6.3.1.3. Exogenous sources

Free radicals can also be produced in biological systems by a number of exogenous agents such as xenobiotics, carbon tetrachloride, cigarette smoke, and gamma radiation [51, 403]. Evidence continues to accumulate that the toxicity of many drugs and other xenobiotics is mediated by oxidative stress, rather than, as has been held for many years, by the cellular interactions of the reactive metabolites derived from the parent compounds [403]. There appears to be a relationship between smoking and IBD [51, 10, 383].

1.6.3.2. Cell and tissue injury by reactive oxygen metabolites

The toxic reactive oxygen metabolites may cause tissue injury and destruction if the rate of their production exceeds the antioxidant defences of the cells, and/or the antioxidant defences of the cells are weakened. There are a number of conditions in which either case may happen; namely, hyperoxia, reactions to xenobiotics, reactions during arachidonic acid metabolism, respiratory burst activity during phagocytosis, and reaction during ischaemia/reperfusion [51, 58, 396, 417]. Being highly reactive, hydroxyl radical, or an equivalently reactive species, generally reacts with the first structure of the cell it encounters — often the lipid and/or protein components of cell or organelle membrane, and initiates those events that lead to the eventual cell death [51]. Though free radicals can attack any biochemical component of the cell, the most important targets are the phospholipids of cellular membranes with the initiation and propagation of lipid peroxidation; the mitochondrial inner membrane with a loss of energization and cellular stores of ATP; and the DNA with the appearance of single-strand breaks and their subsequent repair [403].

It has been suggested that superoxide anions and hydrogen peroxide injure cells as a result of the generation of more potent oxidizing species (Fig. 1.4). In addition to superoxide anions and hydrogen peroxide, the third essential component of the complex that mediates the lethal cell injury is a cellular source of ferric iron which is reduced to ferrous iron by superoxide anions (see above). As mentioned before, the ferrous iron then reacts with hydrogen peroxide to produce a more potent oxidizing species, like hydroxyl radical, which may also be formed by other reactions, or an
equivalently reactive species. In turn, the hydroxyl radical initiates the autocatalytic peroxidative decomposition of the phospholipids of the cellular membranes. Alternately, the hydroxyl radical damages the inner mitochondrial membrane. Upon mitochondrial de-energization, a sequence of events is initiated and similarly leads to the loss of viability [4031. The DNA damage does not always result in cell death, and it can be mutagenic [51, 4031. Proteins are also vulnerable to free radical-mediated attack. The denaturation of enzymes results in loss of their catalytic function, while denaturation of structural proteins, or of complex sugars in the intracellular matrix, may result in destruction of the architecture of cells and intercellular structures such as basement membranes [51].

Neutrophils which accumulate in response to the tissue injury generate more superoxide as well as other toxic mediators such as elastase, collagenase, and other proteases [4071, thus contributing to the tissue damage. Reactive oxygen metabolites are capable of inducing mast cell histamine release [168, 169], perhaps together with protease and other mediators. Histamine has been shown to be a potent stimulator of the free radical-generating enzyme xanthine oxidase [195], and it may also interact with neutrophil-derived HOCI to generate reactive N-chlorinated derivatives [418], and thus contribute to the pathophysiology of IBD.

Since the gastrointestinal tract is constantly exposed to dietary lipids, drugs, variety of pro-oxidants, and transition metals such as iron which may be present in free form in the lumen, its mucosa is very vulnerable to peroxidative damage. Furthermore gut lumen is rich in various types of bacteria and their metabolism may result in the production of a variety of pro-oxidants. One of the chemotactic bacterial products, FMLP which is produced by *E. coli*, is capable of activating neutrophils to produce superoxide anions, hydrogen peroxide, and hypochlorous acid [58, 419].

1.6.3.3. Anti-oxidant defense mechanisms

1.6.3.3.1. Endogenous-enzymatic antioxidants

The enzymes that provide the first line of defense against reactive oxygen metabolites are superoxide dismutase, catalase, and glutathione peroxidase (Fig. 1.4). Superoxide dismutase, which is found in the cytosol and mitochondria, represents a family of metalloproteins that catalyses the dismutation of superoxide anion free radical to hydrogen peroxide and molecular oxygen [51, 58]. Catalase, which is found in the cytosolic or peroxisomal fractions of most mammalian cells, is a haem protein that
catalyses the reduction of hydrogen peroxide to molecular oxygen and water [51, 58]. Glutathione peroxidase catalyses the reduction of peroxides, including hydrogen peroxide and lipid hydroperoxides, to water via the glutathione redox cycle, using glutathione as a substrate [51, 58]. The oxidized glutathione can then be regenerated by glutathione reductase, an energy-consuming reaction requiring the oxidation of NADPH [51]. Glutathione peroxidase is localized in the cytosol and mitochondria of most cells [58].

The mitochondria-based cytochrome oxidase system, upon which aerobic organisms now depend to generate ATP, probably evolved primarily as a highly efficient antioxidant mechanism by virtue of the efficient ability of ATP to effectively scavenge four electrons a time and could be regarded as such [51].

1.6.3.3.2. Endogenous-nonenzymatic antioxidants

The second line of defense against reactive oxygen metabolites-mediated injury is provided by nonenzymatic antioxidants. They can be divided into those compounds which act in the lipid phase and those which act in the aqueous phase. α-Tocopherol (vitamin E) and β-carotene (a precursor to vitamin A) are both lipophilic antioxidants which directly scavenge free radicals, thus protecting cell membrane from lipid peroxidation [51]. Ascorbate, urate, cysteine, ceruloplasmin, billirubin, and even albumin are all aqueous phase compounds which also appear to be able to act as primary scavengers of toxic oxidants of physiological importance [420]. Mucin overlaying the gastrointestinal mucosa has also been suggested to play a role in protecting the epithelium from reactive oxygen metabolites generated within the lumen, due to their sugar content which can be an effective radical scavenger [51].

1.6.3.3.3. Exogenous antioxidants

Numerous exogenous agents which may prevent free radical-mediated injury have been found by studying their effects on mechanisms analogous to those of the endogenous antioxidant defences. These agents may ameliorate tissue injury by interrupting free radical generation or propagation at any of several points along the cascade to tissue damage such as inhibition of xanthine oxidase (e.g. allopurinol, folic acid) or NADPH oxidase (e.g. adenosine, diphenylene iodonium, monoclonal antibody), functioning as superoxide dismutase (e.g. CuDIPS) or directly scavenging free radicals (e.g. mannitol), augmentation of glutathione peroxidase activity (e.g. glutathione), and
Chapter 1

inhibition of neutrophil adhesion (e.g. monoclonal antibody against CD11/CD18 complex which mediates neutrophil adherence to endothelial cells) [51].

1.6.3.4. Focus of study

UC and CD have received relatively less attention in relation to reactive oxygen metabolites as well as histamine release from mast cells. It will be of interest to see how the histamine release induced by reactive oxygen system can be modulated by established and novel drugs used for the treatment of IBD.

1.6.4. Glutathione

The tripeptide glutathione, \( \gamma \)-glutamylcysteinylglycine, is the most abundant endogenous intracellular nonprotein thiol which is a major constituent of living cells. It may be present as the thiol-reduced form (GSH) and as the disulphide-oxidized form (GSSG). Free glutathione \textit{in vivo} is mostly present as GSH rather than GSSG, but up to a third of the total cellular content can be present as mixed disulphides with compounds that contain SH group such as cysteine, coenzyme A, and the SH group of the cysteine residues of several proteins [404]. GSH is biologically active and serves several independent functions. Due to its binding to proteins and by thiol/disulphide exchange, it stabilizes protein structure and plays a role in the regulation of enzyme activities as cofactor with several enzymes, including glyoxalase, maleylacetoacetate isomerase, and prostaglandin endoperoxide isomerase [404, 421]. It is a specific substrate for the enzymes GSH peroxidase [422] and glutathione S-transferase [423] that play a critical role in detoxification and antioxidation processes of cells. Also together with glutathione S-transferase, it participates in the synthesis of leukotriene \( C_4 \) [424], which is further metabolized via \( \gamma \)-glutamyl transpeptidase and dipeptidase to leukotriene \( D_4 \), then \( E_4 \) [425]. It has also been shown that GSH is important in the activation of human lymphocytes [426].

1.6.4.1. Role of glutathione against free radical toxicity

As mentioned previously, glutathione peroxidase is one of the endogenous enzymatic antioxidants against reactive oxygen metabolites that requires GSH as its specific substrate. Due to its physical and chemical properties, GSH achieves its reducing power against oxidative species through the sulphhydryl group of cysteine. In fact, it is regarded as one of the most important and efficient defense mechanisms in
living cells against oxidative stress-induced peroxidation of membrane polyunsaturated fatty acids as well as other macromolecules. GSH participates in detoxification of electrophilic compounds to form nontoxic conjugates either spontaneously or via catalysis of glutathione-S-transferases [423]; as a reductant in the reduction of peroxides (hydrogen peroxide and lipid peroxides) to water catalyzed by GSH-peroxidase-GSSG reductase system; and direct scavenging of free radicals or quenching of excited state [51, 404, 422]. Depletion of GSH potentiates cell injury from the types of processes involving GSH just indicated in the foregoing text, and stimulation of processes that support maintenance of intercellular GSH protects against injury [427].

There is considerable evidence that GSH is effective against lipid peroxidation and other reactive species-induced damage [403, 404]. Apart from the important role of GSH in protecting hepatocytes against reactive oxygen compounds and free radicals [403], it has been shown that isolated cells from rat small intestine [427], lungs [428], and kidneys [429] are substantially protected by exogenous GSH, which is transported intact into the cells, against chemical-induced oxidative injury. Inhibition of GSH redox cycle by nitrofurantoin has been shown to increase rat hepatocyte susceptibility to the xenobiotic [404]. Also impairment of GSH redox cycle, but not of catalase, has been demonstrated to dramatically increase susceptibility of pulmonary artery endothelial cells to polymorphonuclear cell-induced hydrogen peroxide attack [404]. Moreover, transient depletion of GSH in the rat lungs by injection of diethyl maleate has been reported to enhance oxygen toxicity in lungs exposed to hyperoxia [404]. Altered kidney function has also been observed in rat with GSH depleted by diethyl maleate injection [430]. One of the interesting aspects of the role of GSH is its beneficial effect on inflammatory diseases. GSH has been shown or implicated to ameliorate ischaemia/reperfusion-induced oxidative injury in the heart [431, 432] and the kidneys [433]. Considerable evidence also shows a critical role for intracellular GSH in protecting against chemical carcinogenesis [434], and radiation-induced injury [404].

1.6.4.2. Free radical production during thiol group oxidation

Although GSH is generally regarded as a free radical scavenger, yet as a result of autooxidation, thiol compounds, including cysteine and GSH, have been shown to produce superoxide, hydroxyl radicals, hydrogen peroxide, and thyl radicals [404]. GSH was found to enhance (at low concentration) or inhibit (at high concentration) hydroxyl radical formation [404].
1.6.4.3. **Interest of study**

In view of the known protective role of GSH against carcinogens and oxidants, its role in gastrointestinal mucosa cytoprotection is of interest. There are relatively few studies of large bowel in relation to GSH. Some controversy exists with regard to the exact role of GSH in gastric mucosal cytoprotection. Contradictive results have been shown in the experimental model of ethanol-induced gastric haemorrhagic gastritis and necrosis in rats [435-437]. However, exogenous GSH has been shown to significantly protect isolated rat small intestinal epithelial cells against injury induced by t-butyl hydroperoxide or menadione, and the protection was found to depend on the uptake of intact GSH [427, 438]. Loss of GSH has been demonstrated with a concurrent dramatic increase in neutrophils into the small intestinal mucosa of the cat during ischaemia and reperfusion [439]. Decreased GSH content has been reported in inflamed colonic mucosa from patients with IBD [440].

It will be interesting to study rectocolonic GSH content with regard to inflammation and its modulation by drugs in animal models, and also to see how exogenous GSH may affect certain events involved in inflammation and cell damage. *In vivo*, the liver releases GSH [441] and contributes to the maintenance of circulating GSH in the plasma [442].

1.6.5. **Cell membrane stabilization**

The most fundamental task of the biological membrane is to separate the special physical properties and chemical composition of a living organism from its environment. Membranes also form barriers between various intracellular compartments with their specialized chemical contents. While membranes have been bestowed with many functions, such as carrier-mediated transport, enzyme activity, and ligand binding to surface receptors and their functional coupling to effector systems, their selective performance in this isolatory respect remains crucial for the living cell. There are occasions, such as during the inflammatory process, that the efficiency of membrane’s isolatory capacity may be overwhelmed, resulting in eventual cell death. Drug compounds that stabilize the cell membrane could enhance cellular resistance to membrane perturbation and preserve cell membrane integrity, hence cell survival, as well as modulate membrane functions.
1.6.5.1. **Erythrocyte membrane stabilization**

Due to their ready availability and the ease of handling, erythrocytes are widely used in the studies of membrane structure and function, including the pharmacological property of membrane-stabilizing activity by different drugs. Moreover, the mature mammalian erythrocyte is devoid of nucleus, mitochondria, and all other subcellular structures, hence, any surface active effects of a drug will mainly be due to its action on the plasma membrane.

The anti-allergic drug — disodium cromoglycate [443], many anti-inflammatory drugs [444-446], or some tranquilizers [447, 448] and antihistamines [447, 449] have been shown to stabilize the membrane of erythrocytes. Non-steroid anti-inflammatory drugs potently inhibit heat-induced haemolysis, and the correlation between their membrane stabilizing and anti-inflammatory activities is thought to be significant [446, 450].

1.6.5.2. **Erythrocyte membrane stabilization and inflammation**

Erythrocytes are rich in endogenous anti-oxidant enzymes, and they may metabolize reactive oxygen metabolites such as superoxide radical and hydrogen peroxide which can cross the erythrocyte membrane rapidly by diffusion via membrane anion channels in the case of former and by free diffusion in the latter [451]. It has been shown that erythrocytes scavenge extracellular hydrogen peroxide and inhibit the formation of hypochlorous acid and hydroxyl radical [452]. Moreover, it has been demonstrated that erythrocytes, either in intact or lysed form, can protect isolated lungs and pulmonary artery endothelial cells [453], and isolated heart [432] against endogenous or exogenous hydrogen peroxide-mediated injury. However, intact cells are preferential, as haemolysis releases haemoprotein-associated oxidant or haemoglobin which will interact with hydrogen peroxide or lipid hydroperoxides to yield further haemoprotein-associated oxidant that is capable of peroxidizing a variety of polyunsaturated fatty acids, phospholipid, and cellular membrane [454-457]. As active episodes of intestinal mucosal inflammation and injury coincide with interstitial haemorrhage [458] and lipid peroxidation [459], it has been suggested that haemoglobin-catalyzed reactions may play an important role in mediating and/or exacerbating inflammatory tissue injury such as that of IBD [58]. The presence of haemoglobin indeed has been proposed to mediate and/or exacerbate oxidative injury in tissues such as the central nervous system [460], retina [461], kidney [462], and
synovial cavity [463]. Therefore, apart from being a general membrane model for studying pharmacological activity, stabilizing erythrocyte membrane and maintaining its integrity in its own right may have direct relevance to the pathophysiology of oxidative injury.

1.6.3. Focus of study

It will be interesting to see whether the drugs used in the treatment of IBD stabilize erythrocyte membrane and prevent haemolysis induced by membrane labilizers such as heat, bile acid (deoxycholic acid), and enzyme (phospholipase C).

1.7. AIMS OF THE PRESENT STUDY

In the present study, using intact animal models and isolated cell preparations, the in vivo and in vitro pharmacological actions of balsalazide (a novel sulphasalazine-like drug), sulphasalazine, and the in vitro actions of cyclosporin A on some aspects of the IBD, with emphasis on mast cell histamine release, were assessed and compared. Using the rat model of ethanol-induced colitis, the relationship between rectocolonic GSH, inflammation, and lesion formation was studied. The effects of the oral treatment with BSZ and SASP on chronic and active rat colitis induced by ethanol+2,4,6-trinitrobenzene sulphonic acid, and the topical treatment with the same drugs on acute rat colitis induced by ethanol using the in situ perfused rat rectocolon as a model were assessed. Histamine release induced by a bile acid, peroxidase/H₂O₂/halide system (free radical generating system), and some other more conventional secretagogues from rat peritoneal mast cells and dispersed guinea pig rectocolonic mucosal cells was compared. The effects and the mode of actions of BSZ, SASP, and their metabolites, as well as CsA on mast cell histamine release were studied. Finally, using rat erythrocytes as a membrane model, the hypothesis that the drugs might exert their inhibitory and protective action by cell membrane stabilization was tested.
## Chapter 1

<table>
<thead>
<tr>
<th>Feature</th>
<th>Ulcerative colitis</th>
<th>Crohn’s disease</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>History</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diarrhoea</td>
<td>Bloody</td>
<td>with or without blood</td>
</tr>
<tr>
<td><strong>Examination</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clubbing</td>
<td>Rare</td>
<td>Common</td>
</tr>
<tr>
<td>Abdominal mass</td>
<td>Very rare</td>
<td>Common</td>
</tr>
<tr>
<td>Colour of peritoneum</td>
<td>Pink</td>
<td>Violaceous</td>
</tr>
<tr>
<td>Large fleshy tags</td>
<td>Absent</td>
<td>Uncommon</td>
</tr>
<tr>
<td>Multiple fistulae</td>
<td>Absent</td>
<td>Uncommon</td>
</tr>
<tr>
<td><strong>Sigmoidoscopy</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&quot;Normal&quot; rectal mucosal</td>
<td>Absent</td>
<td>Present in half the patients</td>
</tr>
<tr>
<td><strong>Radiology</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Small bowel disease</td>
<td>Backwash ileitis</td>
<td>Common</td>
</tr>
<tr>
<td>Continuity</td>
<td>Continuous</td>
<td>Discontinuous</td>
</tr>
<tr>
<td>Symmetry</td>
<td>Symmetrical</td>
<td>Asymmetrical</td>
</tr>
<tr>
<td>Ulceration</td>
<td>Shallow</td>
<td>Deep</td>
</tr>
<tr>
<td>Fistulae</td>
<td>Absent</td>
<td>Occur</td>
</tr>
<tr>
<td>Distribution</td>
<td>Decreasing from left to right</td>
<td>Areas of relative or absolute sparing distal to macroscopic disease</td>
</tr>
<tr>
<td><strong>Histology</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Distribution</td>
<td>Mucosal (and submucosal)</td>
<td>Submucosal and mucosal</td>
</tr>
<tr>
<td></td>
<td>Diffuse</td>
<td>Focal</td>
</tr>
<tr>
<td>Goblet cell population in active phase</td>
<td>Depleted</td>
<td>Not depleted</td>
</tr>
<tr>
<td>Length of tubules in chronic disease</td>
<td>Diminished</td>
<td>Normal</td>
</tr>
<tr>
<td>Diagnostic</td>
<td>None</td>
<td>Granulomata, giant cells</td>
</tr>
</tbody>
</table>

Table 1.1. Differences between ulcerative colitis and Crohn’s disease (adapted from ref. 3).
Figure 1.1. Structures of (a) Sulphasalazine and (b) Balsalazide.
Figure 1.2. Summary of the sites at which sulphasalazine (SASP), 5-aminosalicylic acid (5-ASA), and sulphapyridine (SP) have been reported to inhibit inflammatory cell function, mediator release, and to block prostaglandin metabolism. Only effects observed (regardless of species or preparation) at 2.0 mM or below are indicated. FMLP = formyl-methionyl-leucyl-phenylalanine, TNFα = tumour necrosis factor α, MPO = myeloperoxidase, HETE = hydroxyeicosatetraenoic acid. (Adapted from ref. 71).
Table 1.2. The response of mast cells from different species to compound 48/80 in vitro. (Adapted and modified from ref. 123, 124, 185).

++ = marked release, + = moderate release, — = negligible release.
Chapter 1

A.

<table>
<thead>
<tr>
<th>Secretagogue</th>
<th>Rat</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PMC</td>
<td>MMC</td>
</tr>
<tr>
<td>Antigen/Anti-IgE</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Calcium ionophores</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Compound 48/80</td>
<td>+</td>
<td>—</td>
</tr>
<tr>
<td>Substance P</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Morphine</td>
<td>+</td>
<td>—</td>
</tr>
</tbody>
</table>

B.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Rat</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PMC</td>
<td>MMC</td>
</tr>
<tr>
<td>DSCG</td>
<td>+</td>
<td>—</td>
</tr>
<tr>
<td>Theophylline</td>
<td>+</td>
<td>—</td>
</tr>
<tr>
<td>Isoprenaline</td>
<td>±</td>
<td>—</td>
</tr>
<tr>
<td>Quercetin</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Doxantrazole</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

**Table 1.3.** Functional characteristics of human and rat mast cell populations. A: Responses to various secretagogues. B: Inhibition of histamine release by anti-allergic drugs. (Adapted from ref. 124 and 185).

PMC = peritoneal mast cells, MMC = mucosal mast cells, LMC = lung mast cells, CMMC = colonic mucosal mast cells, CS/MSMC = mast cells from colonic submucosa/muscle, SMC = skin mast cells, DSCG = disodium cromoglycate.

+ = response observed, ± = some variable response of small magnitude, — = no effect, NA = data not available.

a Mast cell populations studied were of various purities.
b Effect on parenchymal cells obtained by enzymatic dispersion.
c Effect on cells recovered by broncho-alveolar lavage.
Figure 1.3. Release of arachidonic acid (AA) via the hydrolysis of phosphatidylcholine (PC) by phospholipases. PKC = protein kinase C, PLA₂ = phospholipase A₂, PLC = phospholipase C, PLD = phospholipase D, PA-PAase = phosphatidic acid phosphohydrolase, DL = diacylglycerol lipase, MGL = monoacylglycerol lipase, DAG = diacylglycerol, MG = monoacylglycerol, PA = phosphatidic acid, Lyso-PC = lysophosphatidylcholine, FA = free fatty acid, P = phosphate, Ch = choline, PG = prostaglandin, LT = leukotriene, HETE = hydroxyeicosatetraenoic acid, PAF = platelet activating factor.

(Adapted from ref. 230)
Figure 1.4. The mechanisms of cell injury by activated oxygen species. (Adapted and modified from ref. 403). GSH = reduced glutathione, GSSG = oxidized glutathione, GSH Red = glutathione reductase, GSH Pox = glutathione peroxidase, SOD = superoxide dismutase, O$_2^-$ = superoxide, OH$^+$ = hydroxyl radical, LH = polyunsaturated fatty acids, L$^*$ = lipid radicals, LOO$^*$ = lipid peroxyl radicals, LOOH = Lipid hydroperoxides.
CHAPTER 2
MATERIALS AND METHODS

2.1. MATERIALS AND SOURCES

4-Aminobenzoyl-β-alanine
Gift from Biorex Laboratories, London

N-(4-aminobutyl)-5-chloro-1-naphthalenesulphonamide
Sigma

N-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide (W7)
Sigma

5-Aminosalicylic Acid
Gift from Biorex Laboratories, London

Antimycin A
Sigma

L-Ascorbic acid
Sigma

Balsalazide
Gift from Biorex Laboratories, London

n-Butanol
BDH

Calcium chloride
BDH

Collagenase, Type 1A
Sigma

Compound 48/80
Sigma

Cyclosporin A
Gift from Sandoz

Deoxycholic acid, sodium salt
Sigma

Diamine oxidase
Sigma

o-Dianisidine dihydrochloride
Sigma

Diethyl maleate
Sigma

Dimethyl sulphoxide
Sigma

2,4-Dinitrophenol
Sigma

1,2-Dioctanoyl-sn-glyceral
Sigma

Disodium hydrogen phosphate
BDH

Disodium cromoglycate
Sigma

5,5’-Dithiobis-(2-nitrobenzoic acid)
Sigma
Chapter 2

<table>
<thead>
<tr>
<th>Chemical Name</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA (ethylenediaminetetraacetic acid)</td>
<td>Sigma</td>
</tr>
<tr>
<td>EGTA (ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid)</td>
<td>Sigma</td>
</tr>
<tr>
<td>Ethanol, absolute</td>
<td>BDH</td>
</tr>
<tr>
<td>Ether, anaesthetic</td>
<td>May &amp; Baker</td>
</tr>
<tr>
<td>Foetal calf serum</td>
<td>Gibco</td>
</tr>
<tr>
<td>d-Glucose</td>
<td>BDH</td>
</tr>
<tr>
<td>Glutathione, reduced form</td>
<td>Sigma</td>
</tr>
<tr>
<td>Heparin</td>
<td>CP Pharmaceuticals</td>
</tr>
<tr>
<td>HEPES (N-2-hydroxyethyl piperazine-N'-2-ethane sulphonic acid)</td>
<td>BDH</td>
</tr>
<tr>
<td>n-Heptane</td>
<td>BDH</td>
</tr>
<tr>
<td>Hexadecyltrimethylammonium bromide</td>
<td>Sigma</td>
</tr>
<tr>
<td>Histamine diphosphate salt</td>
<td>Sigma</td>
</tr>
<tr>
<td>Histamine RIA 50 kit</td>
<td>Pharmacia</td>
</tr>
<tr>
<td>Horseradish peroxidase, Type 1</td>
<td>Sigma</td>
</tr>
<tr>
<td>House-dust mite antigen</td>
<td>Bencard</td>
</tr>
<tr>
<td>Hydrochloric acid</td>
<td>BDH</td>
</tr>
<tr>
<td>Hydrogen peroxide (30% solution)</td>
<td>Sigma</td>
</tr>
<tr>
<td>Ionophore A23187</td>
<td>Sigma</td>
</tr>
<tr>
<td>Lactate dehydrogenase assay kit (colorimetric method)</td>
<td>Sigma</td>
</tr>
<tr>
<td>Magnesium chloride</td>
<td>Fisons</td>
</tr>
<tr>
<td>Magnesium sulphate</td>
<td>Fisons</td>
</tr>
<tr>
<td>Melittin</td>
<td>Sigma</td>
</tr>
<tr>
<td>Methanol</td>
<td>BDH</td>
</tr>
<tr>
<td>Nembutal</td>
<td>Abbott</td>
</tr>
<tr>
<td>Perchloric acid (70%, sp. gr. 1.70)</td>
<td>BDH</td>
</tr>
<tr>
<td>Phenylbutazone</td>
<td>Sigma</td>
</tr>
<tr>
<td>Phospholipase C, Type 1</td>
<td>Sigma</td>
</tr>
<tr>
<td>o-Phthalaldehyde</td>
<td>Sigma</td>
</tr>
<tr>
<td>Picumast (BM 15,100)</td>
<td>Gift from Boehringer Mannheim</td>
</tr>
<tr>
<td>Platelet activating factor</td>
<td>Sigma</td>
</tr>
<tr>
<td>Polyethylene glycol 4000</td>
<td>BDH</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>BDH</td>
</tr>
<tr>
<td>Potassium dihydrogen phosphate</td>
<td>Fisons</td>
</tr>
</tbody>
</table>
Chapter 2

Sodium bicarbonate  BDH
Sodium carboxymethylcellulose  Sigma
Sodium chloride  BDH
Sodium hydroxide solution, 4 M  BDH
Sodium dihydrogen orthophosphate  Fisons
Sodium fluoride  Sigma
Sodium iodide  Sigma
Substance P  Sigma
Sulphapyridine  Gift from Biorex Laboratories, London
Sulphasalazine  Gift from Biorex Laboratories, London
5-Sulphosalicylic acid  BDH
Toluidine blue  Sigma
Trifluoperazine  Sigma
Trinitrobenzene sulphonic acid  Sigma
Tris (hydroxymethyl) aminomethane hydrochloride  Sigma
Tris (hydroxymethyl) aminomethane  Sigma
Triton X-405  BDH
Trypan blue  Gibco

2.2. ANIMALS

Both male and female Dunkin-Hartley guinea pigs (500-1200g) and male Sprague-Dawley rats (150-350 g) were used in the majority of the experiments. In some experiments, male Wistar rats (180-200 g) were used. However, the gender and strain were always kept consistent in the same series of experiments.

2.3. BUFFERS

2.3.1. HEPES-buffered Tyrode solution (HBTS)

Histamine release experiments with dispersed guinea pig rectocolonic and rat peritoneal mast cells were carried out in a modified Tyrode solution (TS) buffered with N-2-hydroxyethyl piperazine-N'-2-ethane sulphonic acid (HEPES); the solution from now
called HBTS. HBTS was also used as perfusion medium to remove blood from guinea pig tissues during the preparation of dispersed guinea pig rectocolonic mucosal cells. Further modifications of that buffer were made according to the design of the individual experiment. The compositions of the modified HBTS were as follows:

<table>
<thead>
<tr>
<th></th>
<th>Full-HBTS</th>
<th>CMF-HBTS</th>
<th>CF-HBTS</th>
<th>GF-HBTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium chloride (mM)</td>
<td>137</td>
<td>137</td>
<td>137</td>
<td>137</td>
</tr>
<tr>
<td>D-Glucose (mM)</td>
<td>5.6</td>
<td>5.6</td>
<td>5.6</td>
<td>---</td>
</tr>
<tr>
<td>Potassium chloride (mM)</td>
<td>2.7</td>
<td>2.7</td>
<td>2.7</td>
<td>2.7</td>
</tr>
<tr>
<td>Calcium chloride (mM)</td>
<td>1.0</td>
<td>---</td>
<td>---</td>
<td>1.0</td>
</tr>
<tr>
<td>Magnesium chloride (mM)</td>
<td>1.0</td>
<td>---</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Sodium dihydrogen orthophosphate (mM)</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td>HEPES (mM)</td>
<td>10.0</td>
<td>10.0</td>
<td>10.0</td>
<td>10.0</td>
</tr>
</tbody>
</table>

CMF = Calcium-Magnesium Free, CF = Calcium Free, GF = Glucose Free.

The pH of the solution was adjusted to pH 7.4 with 2 M NaOH.

2.3.2. Hank's balanced salt solution (HBSS)

For the isolation of guinea pig rectocolonic mucosal mast cells, HBSS without NaHCO₃ and with or without calcium and magnesium, as appropriate, were used. The compositions were as follows:

<table>
<thead>
<tr>
<th></th>
<th>HBSS</th>
<th>EDTA-HBSS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium chloride (mM)</td>
<td>137.0</td>
<td>137.0</td>
</tr>
<tr>
<td>D-Glucose (mM)</td>
<td>5.6</td>
<td>5.6</td>
</tr>
<tr>
<td>Potassium chloride (mM)</td>
<td>5.4</td>
<td>5.4</td>
</tr>
<tr>
<td>Disodium hydrogen phosphate (mM)</td>
<td>0.34</td>
<td>0.34</td>
</tr>
<tr>
<td>Calcium chloride (mM)</td>
<td>1.26</td>
<td>---</td>
</tr>
<tr>
<td>Magnesium chloride (mM)</td>
<td>0.49</td>
<td>---</td>
</tr>
<tr>
<td>Magnesium sulphate (mM)</td>
<td>0.41</td>
<td>---</td>
</tr>
<tr>
<td>Potassium dihydrogen phosphate (mM)</td>
<td>0.44</td>
<td>0.44</td>
</tr>
<tr>
<td>EDTA (mM)</td>
<td>---</td>
<td>0.13</td>
</tr>
<tr>
<td>HEPES (mM)</td>
<td>5.96</td>
<td>5.96</td>
</tr>
</tbody>
</table>

EDTA = Ethylenediaminetetraacetic Acid.
2.3.3. Krebs solution

The solution was used in perfusion studies. The pH of the solution was 7.4, and it consisted of sodium chloride (118 mM), potassium chloride (4.7 mM), magnesium sulphate (1.18 mM), potassium dihydrogen phosphate (1.18 mM), D-glucose (11.1 mM), sodium bicarbonate (25 mM), and calcium chloride (2.5 mM). The solution was gassed with a mixture of 95% of oxygen and 5% carbon dioxide for at least one-half hour before use.

2.3.4. TRIS-buffered electrolyte solution (TBES)

The electrolyte solution was based on that used by Rafter et al (1986) [385]. This solution was used in the studies of in situ perfused rat rectocolon preparations. It consisted of KCl (19 mM), NaCl (83 mM), polyethylene glycol (PEG) 4000 (3 g/l), and TRIS (10 mM) adjusted to pH 7.9.

2.3.5. Phosphate buffer

\( \text{Na}_2\text{HPO}_4 \) and \( \text{NaH}_2\text{PO}_4 \) were used to prepare phosphate buffers of various pH and concentrations which were used in the measurement of myeloperoxidase activity and glutathione content, and also in the experiments on erythrocyte membrane stabilization. The pH, concentration, and modification of the buffer will be indicated under the appropriate procedures.

2.3.6. Bicarbonate buffer

Bicarbonate buffer of 1.39% (pH 8.1) was used in the erythrocyte stabilization experiments.

2.3.7. Isotonic saline

153 mM of NaCl (9 g/ml).

2.3.8. Heparinized saline

Saline + heparin (10 U/ml).
2.4. PREPARATION OF CHEMICAL AND BIOCHEMICAL SOLUTIONS

All compounds used were finally diluted with buffer used in the corresponding experiment.

For in vitro experiments, aliquoted stock solutions (5 mM) of sulphasalazine (SASP), balsalazide (BSZ), and their metabolites 5-aminosalicylic acid (5-ASA), sulphapyridine (SP) and 4-aminobenzoyl-L-alanine (4-ABA) were made in saline and kept at -20 °C. Dissolution of SASP was facilitated by increasing the pH of saline with 2 M NaOH, then the pH of the solution was adjusted immediately to 7.4 with HCl. Solutions of 5-ASA were discarded if discoloured (due to decarboxylation with the formation of meta-aminophenol). In some experiments, stock solutions of 10 mM were also prepared. The drugs for oral administration were dissolved (balsalazine) or suspended (sulphasalazine) in 0.25% sodium carboxymethylcellulose (20 mg/ml) and kept at 4 °C during the period of treatment. In the in situ perfusion experiments, drugs were prepared in the buffer solution used, and in the EtOH-challenge experiments, in the 30% EtOH prepared in buffer.

Stock solutions of cyclosporin A (CsA) (100 mM), ionophore A23187 (10 mM), and platelet activating factor (PAF) (4 mM) were prepared in absolute ethanol and stored at -20 °C. Antimycin A stock solution (200 μM) was freshly prepared in absolute ethanol (incubating ethanol concentrations <0.001%). Dimethyl sulphoxide (DMSO) was used to prepare the stock solution of 1,2-dioctanoyl-sn-glycerol (Dic8) (30 mM) which was also kept at -20 °C. Calmodulin antagonist stock solutions (10-50 mM) were freshly prepared in DMSO (incubating DMSO concentrations <0.1%). In some experiments, trifluoperazine was dissolved in saline. The use of DMSO or EtOH in drug solutions (up to 0.3% v/v and 0.1% v/v, respectively) did not show any significant effect on the tests carried out.

Stock solutions of the enzyme phospholipase C (1 mg/ml = 3.8 U/ml) and horseradish peroxidase (500 U/ml) were prepared in saline, aliquoted and stored at -20 °C. Substance P and compound 48/80 were dissolved in distilled water as concentrated stock solutions (1 mM and 120 μg/ml, respectively) and were stored at -20 °C as aliquots. NaF stock solution (200 mM) was freshly prepared in distilled water.

Phenylbutazone was dissolved in saline with appropriate small amount of 1 M NaOH and warming to give 5 mM stock solution which was adjusted to pH 7.4 with 1 M HCl.
All other compounds, unless otherwise stated were prepared in the appropriate aqueous buffer solutions. Polypropylene containers and pipette tips were used whenever necessary to minimize binding of the compounds to plastic surfaces.

2.5. ISOLATION OF MAST CELLS

2.5.1. Rat peritoneal mast cells

The animal was killed by ether overdose or anoxia with 95% CO₂, and the skin was removed to expose the abdominal wall. Heparinized saline (10 ml with heparin at 10 U/ml) was injected into the peritoneal cavity and the abdomen was gently massaged for 2 minutes before opening the wall. The abdominal wall was opened by a careful incision along the linea alba to avoid any blood vessels, and the peritoneal fluid was collected carefully using a plastic or siliconised pipette and was placed in a plastic tube. Any blood-contaminated samples were discarded. The recovered peritoneal fluid was centrifuged at room temperature (RT) for 5 minutes (150 g), washed twice and resuspended in the appropriate buffer according to the experiment to be carried out.

The recovered cells were used without further purification. On an average, 6.2% (range 5-9%) of the cells in the suspension were stained metachromatically with toluidine blue (0.005%, w/v) when counted by means of an improved Neubauer Haemocytometer. Viability assessed by Trypan blue exclusion test (0.1%) was found to be greater than 90% of the total cell number, and each sample contained approximately 0.5 x 10⁶ viable cells.

2.5.2. Guinea pig rectocolonic mucosal mast cells prepared by scraping (non-enzymic dispersion)

Guinea pigs were given heparin (2000 U/kg) intraperitoneally and killed 30-45 min later by cervical dislocation. The abdomen was opened and the animal was perfused with HBTS in situ via the ventral aorta at a location before branching of the inferior mesenteric artery to remove the blood. The rectocolon was then dissected out, flushed with HBTS to remove any faecal matter from the lumen, and cut open longitudinally and divided into pieces of approximately 5 cm long. After removing the mesentery, loose mucus, and lymphatic patches, the rectocolonic pieces were washed 3 times in pre-warmed EDTA-HBSS (in 100 ml for 10 min each time at 37 °C with stirring) to remove the epithelium. Then the mucosa was scraped from the muscular layer with a blunt
spatula, suspended in HBSS, expressed through a syringe, and the cell suspension was then filtered through moistened gauze (100 μM pore size). The residue was further disrupted by expression in HBSS and filtered as before. The cells from the filtrate were pooled, recovered by centrifugation (150 g, room temperature, 5 min), and washed twice in HBTS. The cells obtained in this manner (modification of the method of Befus et al., 1982 [125]) were used without further purification.

2.5.3. Guinea pig rectocolonic mucosal mast cells prepared by enzymic dispersion

The initial preparation of the animal was similar to that described above (Section 2.5.2.). The rectocolon was first cut into 3 cm pieces for EDTA-HBSS washing, then was further cut into 2x2 mm pieces after washing. The finely cut tissue (2x2 mm) was then dispersed twice in HBSS containing foetal calf serum (FCS, 20%) and collagenase (25 U/ml) with stirring at 37 °C for 45 min, without first separating the mucosa from the muscular layer. After each digestion, the mixture was filtered through moistened gauze as above, then through a wool column containing loose nylon scrubbed wool (0.5 g) packed in a 10 ml syringe with HBSS added to remove any air trapped. Cells were recovered by centrifugation (5 min, room temperature, 150 g), resuspended, and kept in HBSS containing 5% FCS. The final digested tissue residues were further expressed through a syringe and cells were recovered as before. The three portions of cells were then pooled, washed twice in HBTS, and used without further purification.

The mixed cells showed 37% viability by Trypan blue exclusion test and each sample used in experiments contained approximately 2.4x10⁶ viable cells.

2.6. HISTAMINE RELEASE FROM ISOLATED MAST CELLS

Triplicate samples were used for each treatment in all the experiments. Also for each treatment, triplicate blank samples (cell-free control samples), which were devoid of cells but otherwise identical in every respect to the test samples, were used for the interference test.

2.6.1. Effect of histamine liberators

Aliquots of cell suspension (0.45 ml per aliquot) were dispensed into test tubes according to experimental protocol and left to equilibrate in a water bath (37 °C) for
5 min. The test secretagogue (0.05 ml) at 10-fold the required incubating concentration was added to the cell samples. After the pre-determined time (depending on the nature of the experiment), the reaction was stopped by the addition of 1.5 ml of ice-cold HBTS, and the tubes were kept in an ice bath whenever possible until the completion of further preparatory procedures for sample storage or assay. The supernatant, obtained after centrifugation (800 g, 6 min, 4 °C), was separated from the cell pellet. The cell pellet was resuspended in HBTS (2 ml), and the suspension was either boiled for 10 min in a water bath (for radioimmunoassay, Section 2.7.1.) or treated with perchloric acid (final concentration of 0.4 M in 2 ml) to release cellular histamine. The supernatant was treated in a manner similar to the cell pellet suspension, except where the supernatant was destined for lactate dehydrogenase (LDH) assay (Section 2.11.). Samples for LDH assay were kept at 4 °C without further treatment and assayed within a day. In most experiments, the samples for histamine measurements (Section 2.7.2.) were frozen and stored at -20 °C until the assay.

Histamine release was expressed as a percentage of the total cellular content of amine:

\[
\text{Histamine Release (\%) = \left\{ \frac{HS}{HS+HCP} \right\} \times 100}
\]

Where, \( HS = \) histamine content in the supernatant,
\( HCP = \) corresponding histamine content in cell pellet.

In all experiments, the value of histamine release was corrected for the spontaneous release occurring in the absence of any inducer or any interference with the assay procedure (see Section 2.7.3.).

2.6.1.1. Time-course of histamine release

The incubation time of cells with inducers was varied depending on the nature of the experiment, and the reactions were stopped after the pre-determined incubation period with ice-cold HBTS.

2.6.1.2. Liberator concentration-dependence of histamine release

Cells were incubated with various concentrations of inducers for a pre-determined periods of time, and the reaction was terminated by addition of ice-cold HBTS.
Chapter 2

2.6.1.3. Temperature-dependence of histamine release

Cells were allowed to equilibrate at the temperatures studied (0, 10, 25, 37 °C) for a period of 5-10 min. The releaser was added to induce histamine release for a further period of pre-defined time, after which the reaction was terminated with ice-cold HBTS.

2.6.1.4. Calcium-dependence of histamine release

Washed cells were apportioned equally and resuspended in HBTS with various concentrations of calcium (0-4 mM). After being equilibrated for 5 min at 37 °C, cell aliquots with appropriate buffer were challenged with inducer, and the reaction was stopped with ice-cold HBTS after a pre-determined time.

2.6.1.5. pH-dependence of histamine release

Cells were resuspended in prewarmed HBTS of different pH values immediately prior to the commencement of test. After equilibration (5 min, 37 °C), the releaser was added to induce histamine release. The reaction was terminated by the addition of ice-cold HBTS.

2.6.1.6. Effect of metabolic inhibitors on histamine release

Cells were resuspended in GF-HBTS and preincubated with one of the metabolic inhibitors (antimycin A or 2,4-dinitrophenol) of various concentrations for 10 min. Test histamine releaser was added to induce release for a pre-defined time period, and the reaction was terminated with ice-cold HBTS.

2.6.2. Effect of modulators on induced histamine release

Aliquots of cells were equilibrated as mentioned above, and were then preincubated with the modulator for a pre-defined period of time before the addition of secretagogue. Concurrent addition of modulator and releaser was carried out in some cases. The reaction was terminated as before. The results were expressed as the percentage inhibition of the induced histamine release in the absence of modulator (control release). Hence,

\[
\text{Inhibition (\%) = \left\{(Ra-Rp)/(Ra)\right\} \times 100}
\]

Where, Ra = release in the absence of modulator,
Chapter 2

\[ Rp = \text{release in the presence of modulator.} \]

Values of inhibition are given as mean±sem. Negative values of inhibition indicates potentiation or augmentation of histamine release.

Similar to that mentioned above, values of histamine release were corrected for the spontaneous release or any interference with the assay procedure. The effect of modulator on spontaneous histamine release was also examined. As mentioned above, control samples devoid of cells were used for checking the interference in histamine assay (see Section 2.7.3.).

2.7. HISTAMINE ASSAY

2.7.1. Histamine radioimmunoassay

Pharmacia Histamine RIA 50 radioimmunoassay kit was used to assay histamine content in samples from some of the experiments. The kit employs a fixed amount of \(^{125}\text{I}\)-labelled histamine-albumin complex that competes with histamine/methyl-histamine in the sample for the binding sites of the specific monoclonal antibodies. The assay was performed in duplicate for each test sample and standard. The bound histamine was separated from free histamine by addition of a second antibody immunoadsorbent followed by centrifugation and decanting. The radioactivity in the pellet was then measured using a gamma counter. From the radioactive count obtained, a standard curve was constructed, and the histamine content of test samples was determined from the standard curve.

2.7.2. Spectrofluorometric assay of histamine

The perchloric acid-treated samples were first centrifuged (1700 g, 5 min, RT) to remove protein precipitated by perchloric acid. The supernatants were then assayed for histamine by the automated double-extraction procedure, based on the principle of Shore et al (1959) [464] using a Technicon Autoanalyser II.

The procedure involves the extraction of histamine into salt-saturated butanol from the sample after alkalinization, followed by the separation of butanol-organic phase and aqueous phase (wash). The organic phase was made less polar by mixing with n-heptane and the histamine was re-extracted into a stream containing diluted HCl. Finally the amine was condensed with o-phthaldialdehyde (OPT) under alkaline conditions to
yield a highly intense fluorophore which was stabilized by acidification. The intensity of the fluorescence, as a measure of the histamine concentration in the sample, was read by a fluoronephelometer and recorded on a Technicon pen recorder. The sensitivity of this procedure is 0.5 ng/ml of histamine, and a minimal volume of 0.8 ml is required.

Test samples were passed through the system preceded by known standards and with marker standards of histamine at intervals of 9-12 samples.

In some cases, the authenticity of histamine was further tested by incubating samples with either heat-inactivated diamine oxidase (10 min, 100 °C) or 0.1 U/ml diamine oxidase (60 min, 37 °C). The samples were then assayed as before.

2.7.3. Interference test of fluorometric assay of histamine

Using known quantities of histamine, the cell-free control samples (see above) were employed to check the possible interference during fluorometric assay. Some compounds or drugs give fluorescence readings in the absence of histamine. Sometimes the reading of histamine samples in the presence of these compounds cannot be corrected by simple subtraction, for these compounds may behave differently in the presence of histamine, or any changes in their environment for that matter. In this case any compound that quenches histamine fluorescence reading produces an apparent inhibition.

It was found in the study that one of the drugs being investigated, 5-aminosalicylic acid (5-ASA), showed a fluorescence reading in the absence of histamine but quenched the histamine fluorescence reading drastically in some cases under the experimental conditions used. The magnitude of interference varied with the secretagogues and/or experimental conditions employed. For each defined experimental condition, a full interference test was usually carried out more than once, especially with 5-ASA. In a full test, the effect of cell-free control samples on standard histamine readings was tested over a range of histamine standards (3 concentrations) which covered the range of test samples. This was achieved by adding known amounts of histamine into cell-free control samples, then comparing the readings of these samples (denoted as apparent histamine readings) with those of the histamine standards of the same concentrations. When there was interference, an interference curve was constructed by plotting apparent histamine readings against standard histamine readings. From the curve, the interference on test sample readings was corrected.
In repeat experiments, if a full interference test for the same defined experimental conditions had been carried out previously, normally only one concentration of histamine standard was used. The concentration of histamine used was similar to that of the control histamine release induced by secretagogue without drug treatment for the particular experiment. The magnitude of interference was expressed as a ratio from which correction might be assessed in conjunction with the full interference test carried out previously.

2.8. ANIMAL MODELS WITH ETHANOL-INDUCED RECTOCOLONIC INFLAMMATION IN RATS

2.8.1. In vivo model

The method of inducing inflammation by ethanol (EtOH) plus the hapten 2,4,6-trinitrobenzene sulphonic acid (TNBS) was based on that described and characterized by Morris and co-workers [120, 465, 466].

2.8.1.1. Induction of inflammation

Male Wistar rats (190 ± 10g) were fasted overnight but allowed water ad lib, and randomized into treatment groups. After being lightly anaesthetized with ether, animals were each given a single intra-colonic instillation (via enema) of 0.25 ml of 30% (v/v) ethanol (acute inflammation) or 0.25 ml 30% ethanol containing 20 mg of TNBS (chronic inflammation). Control animals were given aqueous enema. All animals were allowed recover and allowed food after the enema. Depending on the experimental protocol, the animals were killed either at the end of drug treatment as in the studies of drug effect, or at various time intervals as in the kinetic studies. The animals were killed by cervical dislocation. The rectocolon was removed, opened by a longitudinal incision, cleaned with ice-cold saline, blotted dry, and divided longitudinally into two portions which were as closely identical as possible with regard to the damage and/or inflammation. The two portions of each rectocolon were then weighed separately and stored at -70 °C until the assay for MPO activity (Section 2.9.) and GSH content (Section 2.10.). In some cases, when gross morphological damage of the rectocolon was to be scored (Section 2.8.1.3.), the rectocolon was cleaned with tap water after longitudinal opening, pinned out on a wax bed flooded with saline, and assessed immediately by an independent observer using a stereomicroscope; then samples were taken and kept at -70 °C as above.
2.8.1.2. Effect of drugs on the induced inflammation

Drugs were given either orally or subcutaneously. The general grouping of animals was as follows:

<table>
<thead>
<tr>
<th>Group</th>
<th>Enema</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Water</td>
<td>Oral dose of drug vehicle.</td>
</tr>
<tr>
<td>EtOH-Induced</td>
<td>EtOH or</td>
<td>Oral dose of drug vehicle.</td>
</tr>
<tr>
<td>Control</td>
<td>EtOH + TNBS</td>
<td></td>
</tr>
<tr>
<td>Drug + EtOH-Induced</td>
<td>EtOH or</td>
<td>Oral dose of drug solution</td>
</tr>
<tr>
<td>EtOH-Induced</td>
<td>EtOH + TNBS</td>
<td>or suspension.</td>
</tr>
</tbody>
</table>

In some cases, a group of animals without induced inflammation but treated with the drug constituted an additional control.

2.8.1.3. Damage score

As the gross morphological features of rectocolonic mucosa from EtOH- and EtOH+TNBS-induced models were vastly different, two damage scoring systems were used.

System 1 — for EtOH-induced model (acute inflammation):

<table>
<thead>
<tr>
<th>Score</th>
<th>Gross Morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Normal.</td>
</tr>
<tr>
<td>1</td>
<td>Slight hyperaemia and vascular congestion with diffuse hyperaemic pin-points.</td>
</tr>
<tr>
<td>2</td>
<td>Moderate hyperaemia and congestion with denser hyperaemic pin-points and small erosions and/or haemorrhagic streaks.</td>
</tr>
<tr>
<td>3</td>
<td>Severe hyperaemia and congestion with haemorrhagic spots in addition to dense hyperaemic pin-points, and longer hyperaemic/haemorrhagic streaks/erosions.</td>
</tr>
<tr>
<td>4</td>
<td>As score 3, but with very severe hyperaemia, congestion, haemorrhage, and long erosions with width &gt; 1 mm.</td>
</tr>
</tbody>
</table>

System 2 [120, 465] — for EtOH+TNBS-induced model (chronic inflammation):
Chapter 2

Score | Gross Morphology
--- | ---
0 | No damage.
1 | One region of localized inflammation or thickening, but no ulcer.
2 | Linear ulceration, but no significant inflammation.
3 | Linear ulceration with inflammation at one site.
4 | Two or more sites of ulceration and/or inflammation. Ulcers present in at least one site.
5 | Two or more sites of ulceration and inflammation or one major site of ulceration and inflammation extending > 1 cm along the length of the rectocolon.

"Inflammation" denoted the presence of hyperaemia and bowel wall thickening.

2.8.2. In situ ethanol (EtOH)-perfused rat rectocolon model

The methodology of Rafter et al (1986) [385] was followed with modification.

2.8.2.1. Preparation of animal

After being fasted overnight, groups of 3-4 male Sprague-Dawley rats (200-250 g) were anaesthetized with Nembutal. Via laparotomy (incision made along linea alba to avoid blood vessels), the colon was flushed clear of faecal residue with Krebs solution using a 20 ml syringe through an incision made at the caecocolonic junction. This junction was then cannulated with silicon rubber tubing (id 1/16"), while another tubing was inserted through the anus into rectum, giving a perfusion segment of approximately 10 cm length. The abdomen was then closed with Michel clips and the wound covered with moist cotton wool. The animals were kept under anaesthesia and warmed with radiant heat of an electric lamp throughout the perfusion procedure. At the end of the experiment, the animals were killed by cervical dislocation, the rectocolon of each animal was removed, blotted dry, weighed, and stored at -70 °C until the assay for tissue myeloperoxidase activity (Section 2.9.) and glutathione content (Section 2.10.).
2.8.2.2. **Perfusion procedure**

The rectocolonic lumen was perfused isoperistaltically at a constant rate of 0.5 ml/min with TBES. The perfusate collection commenced after the animals were allowed to settle for 30-45 min following the operative procedure, and the time of commencement was designated as time zero. The perfusion, drug treatment, and perfusate collections were carried out according to the following protocol:

<table>
<thead>
<tr>
<th>Perfusion Fraction</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Period (min)</td>
<td>0-20</td>
<td>20-40</td>
<td>40-55</td>
<td>55-75</td>
<td>75-95</td>
<td>95-115</td>
</tr>
<tr>
<td>Ethanol Control</td>
<td>buffer</td>
<td>buffer</td>
<td>30% EtOH</td>
<td>buffer</td>
<td>buffer</td>
<td>buffer</td>
</tr>
<tr>
<td>Drug (500 μM)</td>
<td>buffer</td>
<td>drug</td>
<td>30% EtOH</td>
<td>drug</td>
<td>drug</td>
<td>drug</td>
</tr>
</tbody>
</table>

Drug and 30% EtOH were prepared in the buffer solution used, and at the EtOH-challenge period (40-55 min), the drug was dissolved in the 30% EtOH prepared in buffer. At the end of the 40-55 min period, after the removal of EtOH from the perfusion solution, a short burst of fast perfusion with the respective solution was carried out to eliminate luminal EtOH, and then the original rate was resumed.

The perfusate fraction for each period was collected on ice and divided into 3 portions. One portion was boiled for 10 min and kept at -20 °C until histamine radioimmunoassay (Section 2.7.1.); one portion was kept at -20 °C directly until glutathione assay (Section 2.10.); while the third portion was kept at 4 °C for lactate dehydrogenase assay (Section 2.11.) within 48-72 hours.

2.9. **DETERMINATION OF MYELOPEROXIDASE (MPO) ACTIVITY**

The method used was based on that described by Bradley *et al* (1982) [467] and Krawisz *et al* (1984) [468], utilizing hydrogen peroxide as a substrate for the myeloperoxidase. It was as follows (Section 2.9.1. and 2.9.2.).
Chapter 2

2.9.1. Preparation of tissue homogenate

A tissue sample (200-400 mg) was minced in a beaker containing 1 ml of hexadecyltrimethylammonium bromide (HTAB) buffer (0.5% HTAB in 50 mM phosphate buffer, pH 6.0) on ice, followed by homogenization in a test tube with a Polytron homogenizer (three times for 30 seconds each, on ice). After homogenization, the homogenizer was rinsed twice with 1 ml of HTAB buffer. The pooled homogenate and wash were sonicated for 10 seconds, freeze-thawed three times. The freeze-thawed sample was centrifuged at 40,000g (4 °C, 15 min). The supernatant was then assayed for MPO activity.

2.9.2. MPO activity assay

The MPO activity was measured spectrophotometrically. A 0.1 ml aliquot of the supernatant was mixed with 2.9 ml of 50 mM phosphate buffer (pH 6.0) containing 0.167 mg/ml o-dianisidine hydrochloride and 0.0005% hydrogen peroxide. The change in absorbance at 460 nm was measured with a spectrophotometer, and the MPO activity was expressed as either optical absorbance per gramme tissue or rate of change of optical absorbance per gramme tissue (absorbance/min/g tissue).

2.10. DETERMINATION OF GLUTATHIONE (GSH)

The spectrophotometric method used was adapted from that of Ellman (1959) [469], who reported that 5,5'-dithiobis-(2-nitrobenzoic acid) is reduced by SH groups of reduced glutathione (GSH) to form 1 mole of 2-nitro-5-mercaptobenzoic acid per mole of SH. The nitromercaptobenzoic acid anion has an intense yellow colour and has been used to measure SH groups, hence to determine GSH. The procedure was as in the following sections (Section 2.10.1.- 2.10.3.).

2.10.1. Preparation of tissue homogenate

Tissue homogenate was obtained either from rectocolonic mucosa or intact rectocolonic tissue including muscular layers. A mucosal sample from each animal was obtained by scraping and put into 1.0 ml of cold 100 mM phosphate buffer (pH 7.4) of known weight, and the sample weight was determined (usually 300-500 mg) before homogenization. The rectocolonic sample that included muscular layers (200-400 mg)
was first minced in a beaker containing 1.0 ml of cold 100 mM phosphate buffer (pH 7.4). After the initial preparation, samples from either sources were then treated in the same way. The sample was homogenized in a test tube with a Polytron homogenizer (three times for 30 seconds each, on ice). After homogenization, the homogenizer was rinsed twice with 1.0 ml of 100 mM phosphate buffer (pH 7.4), and the washings were pooled with the homogenate, hence a total volume of 3 ml of homogenate was obtained for each rectocolonic sample. An equal volume (3.0 ml) of 4% (w/v) 5-sulphosalicylic acid (5-SSA) in distilled water was added to the homogenate and mixed thoroughly, and the mixture was then centrifuged for 10 minutes at 2100 g (4 °C). The supernatant was then used for GSH assay.

2.10.2. Preparation of perfusate

The procedure was similar to that mentioned above, except that no homogenization was required. Also to each 1.0 ml perfusate sample, an equal volume (1.0 ml) of 4% (w/v) 5-SSA was added, instead of 3 ml used above.

2.10.3. Glutathione assay

The assay was carried out in duplicate samples and standards. To 0.2 ml of supernatant, 0.02 ml of 5,5'-dithiobis-(2-nitrobenzoic acid) (DNTB, 4.0 mg/ml in 50 mM phosphate buffer, pH 7.0) was added, followed immediately by 1.8 ml of 100 mM phosphate buffer of pH 8.0. The mixture was vortexed and allowed to stand at room temperature for 2 min. The absorbance was then determined at 412 nm with a spectrophotometer. Reagent blanks consisting of 3.0 ml 100 mM phosphate buffer, pH 7.4 and 3.0 ml 5-SSA were assayed in duplicate in the same manner as described for test samples. Calibration curves of 0.01 μmol/ml to 2.0 μmol/ml of GSH (dissolved in 100 mM phosphate of pH 7.4) were prepared, and from the curve the GSH content of the samples was determined. The value of GSH was expressed as nmol/g wet tissue, μmol/g wet tissue, or nmol/ml where appropriate.

2.11. DETERMINATION OF LACTATE DEHYDROGENASE (LDH)

A Sigma LDH assay kit (colorimetric method) was used to detect LDH in tissue perfusates and samples from mast cell histamine release studies. The method is based on the LDH-catalyzed reversible reaction of pyruvic acid and lactic acid in the presence
of nicotinamide adenine dinucleotide (reduced form, NADH; and oxidized form NAD). At a rate proportional to the amount of LDH, the reaction equilibrium strongly favours the reduction of pyruvate to lactate. Moreover, pyruvic acid reacts with 2,4-dinitrophenylhydrazine to form an intensely coloured "hydrazone" that has peak absorbance over the broad wavelength range of 400-500 nm, while lactic acid, NADH, and NAD do not absorb significantly in this range. Hence, it is possible to measure the change in pyruvic acid content (the change of colour intensity) as a function of LDH activity. The amount of pyruvate remaining after the reaction is inversely proportional to the LDH activity in the sample which can be calculated from the calibration curve.

The test sample was added to a prewarmed (37 °C) mixture of Sigma pyruvate substrate and NADH, mixed gently, and incubated in a water bath of 37 °C for 30 min. At exactly 30 min the sample mixture was removed from the water bath, and the Sigma colour reagent was added to stop the reaction and to start colour development at room temperature. At 20 min after addition of the colour reagent, 0.4 M sodium hydroxide was added, and the mixture was left for at least 5 min but not more than 30 min. Then the absorbance at 440 nm was read and recorded versus water as reference. A calibration curve was prepared using the same wavelength and instrument as test samples. The value of LDH was expressed as U/ml.

2.12. ERYTHROCYTE MEMBRANE STABILIZATION

The methods used were based on those of Mizushima et al (1970) [446].

2.12.1. Preparation of erythrocytes for haemolysis experiments

Human blood was obtained by venipuncture, using a heparinized syringe, and placed into a beaker. The final concentration of heparin was 50 U/ml. Heparinized rat blood was collected from ether-anaesthetized male Sprague-Dawley rats, via the abdominal aorta, into a syringe containing 1/10th volume of heparin of 1000 U/ml. The animal was eventually killed by exsanguination. The beakers containing blood sample were rotated gently at a slight angle to oxygenate the blood as well as to mix it thoroughly with heparin. The blood was then centrifuged (1200 g, 10 min, room temperature) and the sedimented red blood cells were washed three times in saline. After each spin, the buffy coat of leucocytes was carefully removed if present, and the
packed erythrocytes were resuspended by repeated inversion of the centrifuge tube. The packed cells were finally suspended in 125 mM sodium phosphate buffer (pH 7.4) or in 1.39% sodium bicarbonate buffer (pH 8.1) to the required concentration of 5% (v/v) or 5.6% (v/v).

2.12.2. Procedure of heat-induced haemolysis

2.12.2.1. Tests with unmodified erythrocytes

To 0.2 ml of the drug solution or vehicle, 1.8 ml of 5% (v/v) suspension of the erythrocytes was added. After standing at room temperature (20-25 °C) for 10 min, the mixtures were heated in a water bath for 20 min at a temperature which produced 50 ± 10% of haemolysis in the absence of drug solution. The exact temperature used was within the range of 50-55 °C, and it was determined beforehand for each experiment. The tubes were shaken gently several times during the heating. At the end of 20 min, samples were cooled in ice and promptly centrifuged for 10-15 min at 1200 g (room temperature). The haemoglobin content of the clear supernatant was then assayed. Two extra sets of tubes (unheated control tubes and blank control tubes) following the same treatment protocol were also prepared. The unheated control set was kept in ice water instead of being heated, and was used to check whether the drug itself was haemolytic or the process of standing at room temperature induced any significant haemolysis. The blank control set (erythrocyte-free), for which the erythrocyte suspension was replaced with phosphate buffer, was subjected to the same heating process as experimental set and was used to set meter zero.

2.12.2.2. Tests with modified erythrocytes

In some experiments, rat erythrocytes were modified by the addition of 2,4,6-trinitrobenzene sulphonic acid (TNBS), phospholipase C (PLC), or deoxycholic acid (DA), as described below, and then resuspended in 125 mM sodium phosphate buffer (pH 7.4) before the procedure of Section 2.12.2.1. was carried out. The purpose of pretreating erythrocytes with the said agents was to see whether the alterations in erythrocyte membrane components would affect the effect of test drugs on heat-induced haemolysis.
Chapter 2

2.12.2.2.1. Modification by TNBS

To 36 ml of 5% suspension of washed erythrocytes in 1.39% bicarbonate buffer at pH 8.1, 4 ml of 40 mM TNBS in saline was added. The treated suspension was centrifuged after 90 min incubation at room temperature, and the treated erythrocytes were washed twice with saline before resuspension.

2.12.2.2.2. Modification by PLC

To 36 ml of 5% suspension of washed erythrocytes in 125 mM phosphate buffer at pH 7.4, 4 ml of 20 μg/ml PLC in saline was added. After incubation for 30 min at 37 °C the mixture was centrifuged and the treated erythrocytes were washed twice with saline before resuspension.

2.12.2.2.3. Modification by DA

To 36 ml of 5% suspension of washed erythrocytes in 125 mM phosphate buffer at pH 7.4, 4 ml of 1 mM or 5 mM DA in saline was added. After incubation for 30 min at 37 °C the mixture was centrifuged and the treated erythrocytes were washed twice with saline before resuspension.

2.12.3. Haemolysis induced by other membrane labilizers

To 0.2 ml of the drug solution or vehicle, 1.6 ml of 5.6% (v/v) suspension of washed erythrocytes in 125 mM sodium phosphate buffer at pH 7.4 was added. After leaving the mixture for 10 min at room temperature, haemolysis was induced by adding 0.2 ml of a predetermined concentration of the inducer in saline with gentle shaking, followed by further incubation at 37 °C for 60 min in the experiment using PLC or DA, and 30 min in the case of melittin. Further procedures were as described in Section 2.12.2.1.

In some of the experiments, half the aliquot volumes mentioned above (including the volumes of drug solution or vehicle, erythrocyte suspension, and inducer) were used with the volume ratio being kept constant. This change did not seem to affect the end results. Pilot experiments were carried out to predetermine the inducer concentrations used. The inducer concentrations that produced about 50% haemolysis in the absence of test drug (drug solution was replaced with drug vehicle) were used for testing the effect of the drugs.
2.12.4. Assay of haemoglobin content and expression of haemolysis

The haemoglobin content of the clear supernatant was measured colorimetrically by recording the absorbance at 540 nm using a Beckman DU 50 Spectrophotometer, and the zero was set with the corresponding blank. When the haemolysis was too strong, the supernatant was diluted before measurement. This was in fact the case in all the experiments performed, and 6-fold dilution was carried out. Since the unheated control tubes kept in ice water showed minimal haemolysis, the values were ignored.

The total haemolysis of the erythrocyte sample used was achieved by hypotonic disruption of erythrocytes together with vigorous shaking. The total haemoglobin content was represented by the absorbance of the supernatant of the totally haemolyzed sample. Hence the % haemolysis is expressed as follows:

\[
\text{Haemolysis (\%)} = \left\{ \frac{\text{AS}}{\text{AT}} \right\} \times 100
\]

Where \( \text{AS} \) = absorbance of experimental sample,
\( \text{AT} \) = absorbance of totally haemolyzed sample.

Values of haemolysis are given as mean±sem.

In the experiments of testing the effect of drugs, the mean haemoglobin content of the inducer control (drug-free) tubes was expressed as 100% of haemolysis. The relative haemolysis of each test sample was expressed as the % of haemolysis calculated with respect to the inducer control (drug-free). Hence,

\[
\text{Relative Haemolysis (\%)} = \left\{ \frac{\text{AD}}{\text{Mean AlC}} \right\} \times 100
\]

and,

\[
\text{Inhibition (\%)} = 100 - \text{Relative Haemolysis (\%)}
\]

\[
= \left\{ \frac{\text{Mean AlC} - \text{AD}}{\text{Mean AlC}} \right\} \times 100
\]

Where \( \text{AlC} \) = absorbance of inducer control (drug-free),
\( \text{AD} \) = absorbance of drug-treated sample.

Values of both relative haemolysis and inhibition are given as mean±sem.

2.12.5. Procedure of heat-induced protein coagulation (denaturation)

To 0.2 ml of the drug solution or vehicle, 1.8 ml of 1% (w/v) bovine serum albumin in 0.125 M phosphate buffer (pH 6.0) was added. After standing at room temperature for different time periods (0 to 60 min), the mixture was heated for 3 min in a water bath of 80 °C. The heated tubes were cooled in water and the turbidity of the solution was measured spectrophotometrically at 660 nM. The result was expressed
Chapter 2

as relative coagulation (or relative denaturation, %) which was calculated with respect
to drug-free heated control (which was expressed as 100 %) by the same method
described in the haemolysis test above.

2.13. TREATMENT OF RESULTS

Results were calculated as the mean±sem. Statistical comparison of results was
carried out by using Student’s t-test or Mann-Whitney U-test where appropriate. The
level of significance was taken as p<0.05.
CHAPTER 3

ANIMAL MODELS OF ETHANOL- AND HAPTEN-INDUCED
RECTOCOLONIC INFLAMMATION

3.1. INTRODUCTION

Inflammatory bowel disease (IBD) which is of unknown aetiology and precise pathogenesis is characterized by the presence of acute and chronic inflammation of the gastrointestinal tract, brought about probably by the interactions between host immunological responses, genetic influences, and environmental agents [2, 5, 10, 470]. Reactive oxygen metabolites (ROM) have been implicated in mediating mucosal inflammation in active IBD [51, 58, 394-97]. Glutathione (GSH) is important in the detoxification of oxygen radicals and the inhibition of lipid peroxidation [403, 404], and it is possible that its deficiency in mucosa may diminish the capacity of mucosa to protect itself from sustained oxidative insult. Cytoprotective agents may mediate their activity, in part, by an elevation or preservation of cellular GSH level. It was shown in the treatment of rheumatoid arthritis that the increase in intracellular GSH correlated to clinical improvement [471]. However, there are relatively few studies of large bowel in relation to GSH, and its cytoprotective role in the gastrointestinal tract is not clear [435-437]. Myeloperoxidase (MPO) is an enzyme found in neutrophils and, at much lower concentration, in monocytes and macrophages [472, 473]. Its activity is directly proportional to neutrophil number and has been used as a marker for polymorphonuclear leucocyte infiltration (quantitative index of inflammation) in a diverse number of tissues including human skin [467], rabbit eye [474], dog heart [475] as well as in models of intestinal inflammation in the rat [120, 466, 468]. Activated phagocytes secrete MPO into the extracellular medium where it catalyses the oxidation of chloride ion by hydrogen peroxide to yield hypochlorous acid which is the most reactive oxidant produced by neutrophils in an appreciable amount [406]. As an oxidant, hypochlorous acid is more selective in its action than the hydroxyl radical, and it may further react with endogenous amines to form chloramines — powerful oxidizing agents, which are responsible for much of the neutrophil toxicity [407].
Ethanol (EtOH) is believed to stress the peroxidative balance of the cell toward autooxidation, either acting as pro-oxidant or by lowering the cellular antioxidant level, leading to the production of ROM and lipid peroxidation, hence cellular injury [476, 477]. It has been used as an inducer in the studies of inflammation in gastrointestinal, including stomach [478], small intestine [479, 480], and colon [119].

With the incorporation of a hapten, 2,4,6-trinitrobenzene sulphonic acid (TNBS), ethanol is able to induce a chronic model of IBD in rats [120, 465].

In the present work, the relationship between rectocolonic GSH, inflammation, and lesion formation was studied in the rat models. Experiments were carried out to measure the GSH content (index of oxidant stress), MPO activity (index of inflammation), and lesion formation in rat rectocolon treated with ethanol, ethanol together with subcutaneous injection of diethyl maleate (DEM, depletor of GSH content), or ethanol + TNBS. Also studied were the effects of sulphasalazine (SASP) and balsalazide (BSZ) — a new drug as effective as SASP for the treatment of ulcerative colitis [79] — on the acute and chronic rectocolonic inflammation in the rat models. With the chronic model, the effects of both drugs were evaluated on the GSH content, MPO activity, and lesion formation in rat rectocolon following ethanol+TNBS-induced damage. The anti-inflammatory and cytoprotective effects of the drugs on the acute inflammation induced by ethanol using the in situ perfused rat rectocolon as a model were examined by measuring the GSH content, MPO activity, histamine release, lactate dehydrogenase (LDH) activity (index of tissue damage), and tissue weight (index of oedema).

3.2. MATERIALS AND METHODS

All materials and experimental procedures are outlined in the appropriate sections in chapter 2.

3.3. RESULTS

3.3.1. Determination of GSH content and MPO activity in rat rectocolon (ex vivo) following EtOH-induced damage
As shown in the table above, four groups of rats were given enemas: groups A and D were given 0.25 ml of vehicle (water), while group B and C, 0.25 ml of 30% EtOH; and group C and D received concomitant injection of diethyl maleate (DEM, 1 g/kg) subcutaneously. Further injections of DEM were given at day 3 and 6. Six animals from each group were sampled at 0.5, 4, 18 hour and 7 days. The results (Figure 3.1 and 3.2) are summarised as follows:

3.3.1.1. GSH content

As shown in Figure 3.1, when compared with control group, there was a significant decrease in GSH content in the rectocolon of animals given EtOH alone or EtOH+DEM at 30 min and 4 h after enema, with the maximum effect noted at 4 h and in EtOH+DEM-treated group. DEM (1 g/kg, s.c.) alone caused a small drop at 4 h, though the difference from the control group was not significant. The mucosal GSH content of EtOH-treated group returned to control level at 18 h, while that of EtOH+DEM- and DEM-treated groups appeared to return to a slightly higher level than that of control. As a function of time, there was a gradual increase in GSH content in the control group. This might be due to the fact that the animals were fasted overnight and allowed food immediately after enema. Fasting has been shown to reduce GSH level in rat liver, cardiac muscle, and stomach [481-483].

3.3.1.2. MPO activity

At 30 min after enema, EtOH-, EtOH+DEM, and DEM-treated groups showed small but significant increase in MPO activity when compared with the control (Figure 3.1). However, only the EtOH-treated group maintained an elevated MPO activity beyond 30 min after enema, while EtOH+DEM treated group returned to control level, and DEM-treated group showed marked decrease (Figure 3.1). There was an initial (at 30 min) low MPO activity in control animals. This could be due to fasting, and/or it was also possible that the higher MPO activity of control noted at other time periods.
was due to the inflammation caused by the experimental procedure.

### 3.3.1.3. Damage score

At 4 h after enema, there were significant increases in damage score in both EtOH- and EtOH + DEM-treated when compared with the control group (Figure 3.2). The multiple linear lesions with very little gross inflammation in the rectocolon given EtOH alone were accompanied by a decrease in GSH content and an increase in MPO activity; whereas EtOH + DEM treatment caused marked ulcerogenic response and some gross inflammation, together with a sharp drop in GSH content and MPO activity (Figure 3.2).

### 3.3.2. Effect of BSZ and SASP on chronic rectocolonic damage induced by EtOH + TNBS

At day 0, three groups (B, C, and D) of six rats each received an enema of 0.25 ml 30% EtOH containing 20 mg TNBS per animal, while control group A received an aqueous enema. Commencing at day 7, after the induction of rectocolonic damage, each rat was given a single oral dose of vehicle (0.25% carboxymethylcellulose, 5 ml/kg, groups A & B), BSZ (100 mg/kg, group C), or SASP (100 mg/kg, group D), daily for 14 days. At day 21, each animal was killed by cervical dislocation. The results (Table 3.1) are summarised as follows:

#### 3.3.2.1. Damage score

The results are shown in Table 3.1. EtOH + TNBS (group B) produced a marked and significant increase in rectocolonic lesion formation as compared with the controls (group A). BSZ treatment (100 mg/kg, group C) reduced the damage score when compared with the EtOH + TNBS control (group B), although not significantly. In contrast, SASP treatment (100 mg/kg, group D) produced a further increase in the score over EtOH + TNBS controls (group B), but the difference was not significant. However, the difference between BSZ (group C)- and SASP (group D)-treated groups was significant ($p<0.05$).
3.3.2.2. GSH content

The results are shown in Table 3.1. There were no significant differences between the groups in rectocolonic mucosal GSH content at 21 days after the enema administration.

3.3.2.3. MPO activity

The results are shown in Table 3.1. EtOH+TNBS treatment (group B) greatly increased mucosal MPO activity ($p<0.01$). BSZ treatment (100 mg/kg, group C) slightly decreased MPO activity, though the difference was not significant. However, this apparent small effect was due to one outlier which had mucosal MPO activity of $71.4 \times 10^{-2} \text{ absorbance unit min}^{-1} \text{ g}^{-1} \text{ tissue}$, while the rest in the same group had values ranging from 1.7 to $8.8 \times 10^{-2} \text{ absorbance unit min}^{-1} \text{ g}^{-1} \text{ tissue}$. When the outlier was omitted from the calculation, the effect of BSZ treatment changed from small inhibition (not significant) to total inhibition ($p<0.01$) of the MPO activity induced by EtOH+TNBS. SASP treatment (100 mg/kg, group D) produced no change from EtOH+TNBS-induced controls (group B).

3.3.3. Effects of BSZ and SASP on In Situ EtOH-perfused rat rectocolon

The drug (500 µM), either BSZ or SASP, was perfused before, during, and after the induction of acute inflammation in drug-treated group, while EtOH-induced control group was perfused with buffer only. The results are summarised as follows:

3.3.3.1. LDH release

The LDH release was measured as activity present in the perfusate. Both BSZ and SASP (both at 500 µM) treatment produced lower LDH release (Figure 3.3) and significant inhibition (48%, $p<0.05$ for BSZ; and 57%, $p<0.01$ for SASP) of total LDH release induced by EtOH (Figure 3.7.c).

3.3.3.2. Histamine release

Similar to LDH release, both BSZ and SASP inhibited EtOH-induced histamine release into the lumen at 500 µM (Figure 3.4 and Figure 3.7.d). The inhibition produced by BSZ was 90% ($p<0.01$) and that by SASP was 78% ($p<0.05$).
Chapter 3

3.3.3.3. MPO activity

The tissue MPO activity was expressed as degradation of H$_2$O$_2$ (absorbance unit/g tissue) (Figure 3.5), or rate of H$_2$O$_2$ degradation (absorbance unit/min/g tissue) (Figure 3.7.b). Both BSZ and SASP at 500 µM produced marked inhibition of MPO activity. The reduction by BSZ was 72% (p<0.05) and that by SASP was 74% (p<0.05).

3.3.3.4. GSH content

There was a higher tissue GSH content in both BSZ- and SASP-treated groups, while there was lower perfusate GSH content in both drug-treated groups; however the differences from the EtOH-induced control group were not significant (Figure 3.7.e and Figure 3.7.f). Efflux of GSH, which was indicated by perfusate GSH content, appeared to occur more readily from the drug-treated groups than EtOH control group; and the perfusate GSH content increased after the removal of EtOH, while that of both drug-treated groups started to decline (Figure 3.6).

3.3.3.5. Tissue weight

Tissue weight was measured as an index of oedema. The inhibitory effect of both BSZ and SASP at 500 µM on tissue oedema was moderate (35% by BSZ and 32% by SASP) and was not significant (Figure 3.7.a).

3.4. DISCUSSION

The results show that there was a correlation between the increase in MPO activity and lesion formation (damage score) in rat rectocolon treated with EtOH or EtOH+TNBS. MPO activity thus seems to be a useful index of inflammation as previously reported [4681, and it has been found to be maintained for up to 3 weeks in EtOH+TNBS-induced rectocolon damage in rats [4661. However, this trend was not shown in the concurrent treatment with DEM — a potent GSH depletor [484] (Figure 3.2). A possible explanation for this observation may arise from the role of GSH as antioxidant that a minimum level of it is required to maintain normal polymorphonuclear leucocyte (PMNL) functions. This is because, as reported, auto-reactive oxidants derived from the extracellular release of the MPO/H$_2$O$_2$/halide system during PMNL activation are extensively involved in the auto-oxidative inhibition of PMNL functions, including
motility, and this inhibition of motility is reversible in the presence of antioxidants such as ascorbate and cysteine [485, 486]. The maintenance of sulphydryl group (such as that of glutathione) in a reduced form at or above a critical minimal level may be important for motility [485]. In the present study, at 4 h, the marked depletion of mucosal GSH content induced by EtOH + DEM could render mucosal GSH level below the required minimum level for PMNL motility, thus leucocyte infiltration was inhibited. Consequently, MPO activity stayed at control level, even though high damage score was observed. The mucosal damage was derived from the initial lipid peroxidation and oxidative stress induced by EtOH + DEM. This initial damage might be prevented from perpetuating and propagating as a result of the initial inhibition of leucocyte infiltration, hence the subsequent absence of increased MPO activity even though the GSH content returned to normal or higher level (Figure 3.1). In this respect, depletion of GSH appears to give rise to beneficial effect. Furthermore, depletion of GSH has been shown to result in a marked increase in prostaglandin synthesis [487, 488]. Cyclooxygenase, the enzyme which converts arachidonic acid to prostaglandin endoperoxides, is known to require the presence of hydroperoxide to initiate its activity; and depletion of GSH may enhance the availability of hydroperoxide, hence promote prostaglandin synthesis [489]. DEM has also been shown to induce a significant increase in 6-keto-PGF1α (the degradation product of prostacyclin) and PGF2α in gastric mucosa [437]. Prostacyclin in turn has been shown to inhibit polymorphonuclear cell infiltration in vitro [490] and reduce leucocyte margination in the microcirculation [491], hence DEM may indirectly modulate the accumulation of inflammatory cells. Alternately, a non-specific action of DEM on protein synthesis [492] may account for the low MPO activity.

DEM conjugates with GSH and has a short duration of effect, since the mucosal GSH level increases rather rapidly after acute depletion [493]. This could account for the lack of depleting effect at 18 h and 7 day even though extra injections of DEM were given at day 3 and day 6 (Figure 3.1). This rapid recovery of GSH levels might be due to the increased synthesis, and/or the increased blood supply to the tissue (could be due to the effect of prostaglandins), as erythrocytes and plasma are rich sources of GSH. GSH is known to be released from the liver into the circulation to maintain inter-organ GSH homeostasis [494]. GSH synthesis is thought to be regulated by end-product feedback inhibition [495]. Depletion of GSH would be expected to cause a compensatory increase in synthesis, hence the slight increase over control level in EtOH + DEM- and DEM-treated groups at 18 h after enema (Figure 3.1). The absence
of this effect in the EtOH-treated group could be due to the elevated MPO activity at 18 h, hence, the oxidation of GSH (Figure 3.1).

In the EtOH+TNBS-induced model, the lack of change in mucosal GSH content is perhaps not surprising in view of the prolonged time since the original insult. However, BSZ did produce a modest increase (not significant) in mucosal GSH content (Table 3.1). The trend of higher tissue GSH content in drug-treated groups than EtOH control in the \textit{in situ} EtOH-perfused preparation (Figure 3.7.e) could be partly due to lesser GSH efflux (Figure 3.7.f) which in turn could be partly due to lesser blood loss (blood loss into the lumen, hence perfusate, as a result of mucosal damage) and lower oxidative stress in drug-treated groups. The former was as a consequence of protection of BSZ and SASP against cellular damage (Figure 3.3 & 3.7.c), while the latter, as a consequence of reactive oxygen metabolite scavenging effects of these compounds and a lower MPO activity (less inflamed) (Figure 3.5 & 3.7.b). SASP and its metabolite 5-aminosalicylic acid, and to lesser extent, sulphapyridine, have been shown to be free radical scavengers \[71\], but BSZ has yet to be been shown to be a free radical scavenger. As for the efflux of GSH in the \textit{in situ} perfused preparation (Figure 3.6 & 3.7.f), the early efflux in drug-treated groups might be the consequence of transcellular transport of GSH to the lumen in response to the presence of xenobiotics. Alternately, it is possible that in the presence of BSZ or SASP, the conjugation of GSH with EtOH or oxidation of GSH induced by EtOH was somehow prevented to different degrees. The low perfusate GSH content during and immediately after EtOH challenge in the EtOH control group did not necessarily indicate reduced efflux of glutathione, as the assay method used did not measure glutathione in oxidized form (GSSG) or conjugated form that could be induced by EtOH. In later perfusion periods after EtOH challenge (in the absence of EtOH), the profile of efflux might reflect the blood loss to the lumen as a consequence of tissue damage (Figure 3.3). However, as discussed by Halliwell \textit{et al} (1992) \[496\], free radicals may be derived from drugs such as anti-inflammatory drugs, hence, the possibility that the radicals derived from both drugs (BSZ and SASP) might have led to the oxidation of GSH should not be ruled out. It should be pointed out that, in cell/tissue free and enzyme free conditions, ethanol and both drugs did not affect the measurement of GSH (results not shown).

Besides scavenging free radicals, GSH has, as a result of autoxidation, been shown to produce superoxide and hydroxyl radicals, hydrogen peroxide, and thyl radical \[404\]. Depending on its concentration, GSH was found to enhance or inhibit hydroxyl radical formation \[497\]. Its role in inflamed rectocolon is complex, and its
Chapter 3

effects may reflect the balance of its two contrary actions. In the present study, as discussed above, it seems that the reactive oxygen species generated by leucocyte activity may be less susceptible to GSH than that generated by acute EtOH insult (i.e. GSH is depleted more readily by acute ethanol insult than by leucocyte activity indicated by MPO activity). Furthermore, the tissue GSH pool can be restored rather quickly.

The addition of the protein-denaturing TNBS to ethanol has produced an inflammatory bowel model of greater duration than for ethanol alone. Treatment of an established inflammatory state (day 7) resembles more closely the clinical position. It is surprising that SASP, in this test, had no ameliorating activity but in fact slightly aggravated the inflammation, while BSZ reduced both rectocolonic lesions and MPO activity induced by EtOH+TNBS. The lack of effect of SASP on rectocolon damage in this model is in agreement with the work of Boughton-Smith et al (1988) [465]. In contrast, both BSZ and SASP showed marked cytoprotective and anti-inflammatory effects in the acute inflammatory model represented by the in situ ethanol-perfused rat model. These findings are in agreement with the fact that both drugs are more successful in maintenance of remission [79, 80] rather than in treating severe active disease. BSZ has also been shown to have potent cytoprotective action on ethanol-induced gastric necrosis in rats [76].
Figure 3.1. Glutathione (GSH) content (μmol/g tissue) and myeloperoxidase (MPO) activity (x 10^{-2} absorbance unit/min/g tissue) in rat rectocolon with and without exposure to ethanol or ethanol + diethyl maleate (DEM). Animals were fed after ethanol challenge and killed without fasting, and extra doses of DEM were given at day 3 and 6 after ethanol challenge. Results are shown as mean±sem of 5-12 animals. Mann-Whitney U test: *p<0.05, **p<0.01, ***p<0.001, when compared with Control; *p<0.05, **p<0.01, ***p<0.001, when compared with Ethanol Control; **p<0.01, ***p<0.001, when compared with Ethanol + DEM.
Figure 3.2. Glutathione (GSH) content (µmol/g tissue), myeloperoxidase (MPO) activity (x10^2 absorbance unit/min/g tissue), and damage score in rat rectocolon with and without exposure to ethanol or ethanol+diethyl maleate (DEM) at 4 hours after induction. Results are shown as mean±sem of 6-12 animals. Mann-Whitney U test: *p<0.05, **p<0.01, ***p<0.001, when compared with Control; **p<0.01, ***p<0.001, when compared with Ethanol Control.
<table>
<thead>
<tr>
<th>Group</th>
<th>Enema</th>
<th>Damage Score</th>
<th>MPO Activity (x10^-2 abs unit/min/g tissue)</th>
<th>Mucosal GSH (µmol/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Control</td>
<td>Water</td>
<td>0.4 ± 0.4</td>
<td>7.1 ± 3.1</td>
<td>1.67 ± 0.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0 - 1.0)</td>
<td>(3.3 - 10.8)</td>
<td>(0.48 - 2.65)</td>
</tr>
</tbody>
</table>

| B. EtOH + TNBS Control | EtOH + TNBS | 2.5 ± 1.1*** | 19.2 ± 14.8** | 1.65 ± 0.15 |
|                        |             | (1.5 - 4.5)  | (8.4 - 48.4)  | (1.21 - 2.35) |

| C. BSZ (100) | EtOH + TNBS | 1.6 ± 0.9* | 16.9 ± 11.0 | 1.98 ± 0.19 |
|             |             | (0 - 2.5)  | (1.7 - 71.4) | (1.19 - 2.37) |

- One outlier of 71.4 was omitted from calculation.

D. SASP (100) | EtOH + TNBS | 3.5 ± 1.3*** # | 21.4 ± 6.7* | 1.45 ± 0.41 |
|              |             | (2.0 - 4.5) | (4.6 - 45.8) | (0.35 - 3.01) |

Table 3.1. Effect of balsalazide (BSZ) and sulphasalazine (SASP) on rat rectocolonic inflammation induced by ethanol (EtOH) and trinitrobenzene sulphanic acid (TNBS). Groups of 6 male rats were given an enema at day 0, and oral dosing of BSZ and SASP from day 7 to termination at day 21. Results are shown as means±SEM with range in parentheses. Mann-Whitney U test: *p<0.05, **p<0.01, ***p<0.001 -- significant difference from Group A; **p<0.05 -- significant difference from Group B; #p<0.05 -- significant difference from Group C. MPO = myeloperoxidase
Figure 3.3. Effect of balsalazide (BSZ) and sulphasalazine (SASP) on ethanol-induced lactate dehydrogenase (LDH) release into the lumen from the \textit{in situ} perfused (luminal) rat rectocolon. LDH was expressed as activity present in the perfusate. Results are shown as mean±sem of 3-4 animals. Student's \( t \)-test: *\( p < 0.05 \), **\( p < 0.01 \), when compared with Ethanol Control.
Figure 3.4. Effect of balsalazide (BSZ) and sulphasalazine (SASP) on ethanol-induced histamine release into the lumen from the in situ perfused (luminal) rat rectocolon. Results are shown as mean±sem of 3-4 animals. Student's $t$-test: *$p<0.05$, when compared with Ethanol Control.
Figure 3.5. Effect of balsalazide (BSZ) and sulphasalazine (SASP) on ethanol-induced tissue myeloperoxidase (MPO) activity in the \textit{in situ} perfused (luminal) rat rectocolon. MPO activity was measured as degradation of H$_2$O$_2$. Results are shown as mean±sem of 3-4 animals.
Figure 3.6. Effect of balsalazide (BSZ) and sulphasalazine (SASP) on ethanol-induced reduced glutathione (GSH) efflux into the lumen from the *in situ* perfused (luminal) rat rectocolon. Results are shown as mean±sem of 3-4 animals. Student's *t* test: *p* <0.05, **p** <0.01, when compared with Ethanol Control.
Figure 3.7. Summarized results of the effects of balsalazide (BSZ) and sulphasalazine (SASP) on in situ ethanol-perfused (luminal) rat rectocolon: (a) tissue weight (index of oedema); (b) tissue myeloperoxidase (MPO) activity (expressed as rate of H$_2$O$_2$ degradation); (c) total perfusate lactate dehydrogenase (LDH) activity; (d) total perfusate histamine; (e) tissue GSH content, and (f) total perfusate GSH. Results are shown as mean±sem of 3-4 animals. Student's $t$-test: *$p<0.05$, **$p<0.01$, when compared with Ethanol Control.
CHAPTER 4

THE EFFECT OF DEOXYCHOLIC ACID ON MAST CELLS
OF GUINEA PIG RECTOCOLONIC MUCOSA AND RAT
PERITONEAL CAVITY

4.1. INTRODUCTION

Since inflammatory bowel disease (IBD), including ulcerative colitis and Crohn’s disease, is a chronic disease with recurrent exacerbations and remissions, it is reasonable to expect that the agent or agents responsible may be present in the lumen of the gut regularly causing mucosal damage. Bile acids, which have also been implicated as a cause of gastritis, stress gastric ulceration, and chronic gastric ulcer (373-375) seem to fit this requirement. It has been proposed that IBD could be the result of an altered bacterial metabolism of bile acids in genetically susceptible individuals (376, 377). Intra-colonic instillation of secretory bile acids has been shown to cause mucosal injury, surface cell loss (379, 384, 385), and increases in epithelial proliferative activity (384, 386). While the mucosal damage could account for the altered permeability and DNA loss (a biochemical marker of exfoliated cells) of the colon (379), the increased epithelial proliferative activity of the colon has been implicated in the risk of tumour growth (380, 387). IBD patients with long standing disease have a higher risk of developing intestinal cancer (383). Evidence has accumulated from the studies of epidemiology and experimental animal models of colorectal cancer to incriminate bile acids in the promotion of colorectal neoplasia (376, 390, 391). An association of high faecal bile acid concentrations with dysplasia and carcinoma in ulcerative colitis has also been suggested (392). Excessive amounts of bile acids in the colon have also been shown to stimulate fluid, electrolyte, and mucus secretion and cause diarrhoea (379, 382) — one of the cardinal symptoms of IBD. Bile acid (such as cholic acid) is a potent releaser of colonic vasoactive intestinal polypeptide (VIP), a proposed mediator of diarrhoea (393).

Mast cells are found in the lamina propria of the normal large bowel as well as other tissues, and mast cell hyperplasia in the gastrointestinal tract is a consistent feature of IBD (145-149, 155). However, the role of mast cells and their mediators
remains unclear [133]. Histamine, one of the inflammatory mediators released from mast cells, can also induce colonic events analogous to those induced by bile acids, and these include enhancement of gut motility, colonic fluid and electrolyte secretion, the action and release of VIP, and tissue proliferation [134, 141]. Colonic histamine content is high in patients with ulcerative colitis [498]. It is thus of interest to investigate the relationship between bile acid, histamine, and mast cells in the colon.

The characteristics of histamine release by deoxycholic acid (DA) in vitro from the rectocolonic mucosal mast cells (RCMC) of guinea pigs was studied. Similar studies with DA were carried out on rat peritoneal mast cells (RPMC) for comparison.

4.2. MATERIALS AND METHODS

All materials and experimental procedures are outlined in the appropriate sections in chapter 2.

4.3. RESULTS

4.3.1. Effect of deoxycholic acid on histamine release from guinea pig RCMC

The cell suspension used was prepared from mucosal scraping without collagenase dispersion.

4.3.1.1. Effect of varying concentration of deoxycholic acid

Histamine release (HR, corrected for spontaneous release) induced by DA was marked (at 0.5 mM to 5 mM, 15 min incubation) and dose-related, exceeding 60% at 1 mM (Fig.4.1). In parallel with HR, there was also a dose-related release of lactate dehydrogenase (LDH) (Fig.4.2).

4.3.1.2. Effect of incubation time

The histamine releasing effect of DA was time-dependent (5 - 60 min), but reached a plateau and seemed to have a "self inhibitory" effect with longer incubating time (Fig.4.1).
Chapter 4

4.3.1.3. Effect of extracellular calcium

Varying Ca$^{2+}$ concentration (0 to 2.0 mM in the absence of Mg$^{2+}$) did not affect the histamine release induced by DA (Fig.4.3). The slight fluctuation and the shape of the curve might be due to the balance between the effects of free DA and free Ca$^{2+}$, as DA is a chelating agent.

4.3.1.4. Effect of temperature

The histamine release induced by DA (5 mM) was temperature-dependent, with low release at 0 and 10 °C and an increase between 22-37 °C (Fig.4.4).

4.3.1.5. Effect of pH

DA (5 mM)-induced histamine release peaked at pH 6.4, after which it started to decrease as pH increased (Fig.4.5).

4.3.1.6. Effect of metabolic inhibitors

Histamine release by DA was not inhibited by the metabolic inhibitors antimycin A (0.2-2.0 μM) and 2,4-dinitrophenol (50-200 μg/ml). On the contrary, there was slight potentiation (Fig.4.6).

4.3.2. Effect of deoxycholic acid on histamine release from RPMC

4.3.2.1. Effect of varying concentration of deoxycholic acid

DA (0.05 to 1.00 mM) produced a marked and dose-related release of histamine (38% at 0.5 mM and 86% at 1.0 mM, with 15 min incubation) together with a dose-related release of LDH (Table 4.1.A).

4.3.2.2. Effect of incubation time

At 0.5 and 1.0 mM of DA, histamine release was dependent on incubation time (up to 30 min) (Table 4.1.A).
4.3.2.3. *Effect of extracellular calcium*

The histamine releasing effect of DA (0.5 mM) was dependent on calcium concentration (0 to 4 mM) in the absence of Mg\(^{2+}\) (Table 4.1.B).

4.3.2.4. *Effect of pH*

Histamine release induced by DA (0.5 mM, at 15 min incubation) was dependent on pH (release of 89%, 87%, 38%, and 0% at pH = 5.5, 6.4, 7.4, and 7.9 respectively) (Table 4.2.A and 4.2.D).

4.3.2.5. *Effect of temperature*

With 15 min incubation time, histamine release induced by DA (0.5 mM) was temperature dependent (Table 4.1.C).

4.3.2.6. *Effect of metabolic inhibitors*

Histamine release induced by DA (1.0 mM) was not affected by the preincubation with antimycin A, 2,4-dinitrophenol, or 2-deoxyglucose (Table 4.2).

4.3.3. *Authentication of histamine released from cell preparation from guinea pig Rectocolonic Mucosal Scraping*

Histamine content was determined by the fluorometric method, using an Autoanalyzer with butanol extraction. As shown in one of the DA-induced release studies (Fig. 4.7), by using diamine oxidase, the authenticity of histamine released from guinea pig rectocolonic mucosal scraping preparation was confirmed. Any residual components which produced fluorescence after diamine oxidase treatment appeared to be the interference introduced by the treatment of diamine oxidase, either active or inactive.

4.4. *DISCUSSION*

It is known that mast cells in the mucosa of the gastrointestinal tract differ morphologically and functionally from mast cells in connective tissue in various organs
and in the body cavities, such as peritoneum [124, 127]. It is thus of interest to note that both rectocolonic mast cells of the guinea pig and rat peritoneal mast cells responded to DA. It was important to establish whether histamine release by DA was secretory or cytotoxic in nature. The lack of effect of metabolic inhibitors and the concomitant release of LDH by DA suggests that the release process is at least partly cytotoxic. This is compatible with the ability of DA to act as a biological detergent, to penetrate lipid bilayers or solubilize membrane phospholipids and protein components with subsequent change in membrane structure and permeability [378]. The observation that the LDH activities were very much higher for the guinea pig RCMC than for the RPMC could be explained by the difference in the number of viable cells per sample between guinea pig RCMC and RPMC samples. The viable cell number per guinea pig RCMC sample (2.4x10⁶ viable cells) was 5-fold higher than that of RPMC (0.5x10⁶ viable cells). It is conceivable that the cytolysis of other cell types present in the samples besides mast cells could also occur and contribute to the LDH activities. Alternately, it is possible that guinea pig RCMC preparation may be more susceptible to the cytotoxic effect of DA; or the LDH content per cell may be higher for RCMC than RPMC. However, the result of histamine release from RCMC in response to DA stimulation does not support the notion of higher RCMC susceptibility toward the cytotoxic effect of DA. The cytotoxic effect of DA has been shown previously for hepatocytes [499] and for intestinal epithelial cells in perfusion experiments [379, 385].

The results of the effects of pH on DA-induced histamine release from both RCMC and RPMC do not correlate with the results of Rafter et al (1986) from the perfusion study [385] which showed increasing damage caused to colonic epithelium by 5 mM deoxycholate with increasing pH. The discrepancy may be due to the fact that, in contrast to the present results, the results of Rafter et al were obtained by using calcium-free perfusion buffer. One would expect above the pKₐ of deoxycholate (6.58) [500], DA should exert larger cytotoxic effect as it is more soluble. Hence, it seems that the histamine release in the present case may be mainly due to the unionized DA. In the same study, Rafter et al also showed that calcium could prevent the damaging effect of DA, presumably by formation of non-irritating, non-soluble calcium soaps. Ionized bile acids can sequester calcium to form the calcium soaps mentioned above [501]. Therefore, at pH above the pKₐ and in the presence of calcium (as in the present case), as more DA is being ionized and forming calcium soaps, the effect of DA will be limited to the decreasing amount of unionized DA which will be present in larger amount at a pH below its pKₐ. As demonstrated and suggested by Batzri et al (1991)
in gastric mucosal cells, unionized DA can pass through the lipophilic lipid membrane into the cell, where it experiences higher pH (pH 7) and converts into ionized form, becoming relatively impermeable to the membrane and thus trapped. This mechanism of entrapment can result in intracellular DA concentrations that exceed the threshold for lipid solubilization, giving rise to cell injury by dissolution of cellular membranes. The rapid association of bile acids with gastric mucosal cells and their accumulation in these cells have been found to be independent of cellular energy, pH-dependent (larger accumulation at acidic pH), nonsaturable, and not animal species specific [502]. The observed effects of pH on DA-induced histamine release may be due to this entrapment mechanism together with the interaction of DA with calcium.

Despite its cytotoxic nature, the release of histamine by DA from colonic mucosal mast cells may be of relevance in allergic and inflammatory bowel conditions, DA at least being an enhancer of these processes, if not initiating an inflammatory reaction under certain conditions. Baird and Cuthbert (1985) [381] studied the influence of deoxycholate on the response of epithelial cells of colonic mucosa of sensitized guinea pigs to specific antigen (a model of food allergy). They showed that bile acid rendered these cells responsive to mucosally applied antigen (normally response occurs with serosal but not mucosal application), the response consisting of an electrogenic chloride secretion by the epithelial cells. It is thought that bile acid acts by increasing the permeability of the mucosal surface, allowing the antigen to reach sensitized immunocytes, possibly in the lamina propria. Bile acids have also been reported to enhance the release of cyclooxygenase and lipoxygenase products from the colon [386], to induce increases in reactive oxygen species [380], to release colonic VIP [393], to mobilize calcium from intracellular store [503, 504], and to activate protein kinase C indirectly by increasing diacylglycerol [380].

It may seem that the concentration of DA used in this study and that of Baird and Cuthbert (1985) [381] are too high to be of physiological significance. However, the lower concentration of bile acid that normally reaches the colon may lower the "threshold" for allergic and inflammatory reaction or vice versa, i.e. inflammation may lower the threshold for bile acid-induced histamine release. Furthermore, after small bowel surgical resection, DA concentration in the colon may be high enough (due to malabsorption) to cause release of histamine and other mediators, possibly leading to inflammation or disturbance in function (secretion of fluid, electrolytes, and mucus, and motility), and hence accentuate the diarrhoea caused by malabsorption [141, 505]. Finally, as suggested by Rafter et al (1986, 1991) [380, 385], it is not the total faecal
Chapter 4

bile acid concentration which is the most important factor in relation to mucosal damage in the colon, but rather the fraction of the total that is in solution in the aqueous phase of faeces (faecal water). It is possible that high concentration in faecal water may be achieved at a particular locality, as bile acid may not necessarily be distributed homogeneously.

In the pilot experiments, both BSZ and SASP as well as CsA were not effective in inhibiting histamine release induced by DA from both RPMC and RCMC (data not shown). Thus no further work was carried out.
Figure 4.1. Effects of dose and incubation time of deoxycholic acid (DA)-induced histamine release from dispersed guinea pig rectocolonic mucosal mast cells. Each point represents mean of 3 experiments. For clarity reason, values of sem are not plotted.
Figure 4.2. Deoxycholic acid (DA)-induced histamine release and LDH activity from dispersed guinea pig rectocolonic mucosal mast cells. Each point represents mean±sem of 3 experiments.
Figure 4.3. Effect of extracellular calcium concentration on deoxycholic acid (DA)-induced histamine release from dispersed guinea pig rectocolonic mucosal mast cells (in the absence of magnesium). Each point represents mean±sem of 3 experiments.
EGTA = ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid.
Figure 4.4. Effect of temperature on deoxycholic acid (1.0 mM)-induced histamine release from dispersed guinea pig rectocolonic mucosal mast cells. Each point represents mean±sem of 3 experiments.
Figure 4.5. Effect of pH on deoxycholic acid (1.0 mM)-induced histamine release from dispersed guinea pig rectocolonic mucosal mast cells. Each point represents mean±SEM of 3 experiments.
Figure 4.6. Effects of metabolic inhibitors antimycin A and 2,4-dinitrophenol on deoxycholic acid (DA, 1.0 mM)-induced histamine release from dispersed guinea pig rectocolonic mast cells. Results are shown as mean±sem of 3 experiments.
Table 4.1. Profile of deoxycholic acid (DA)-induced histamine release from rat peritoneal mast cells. (A) effects of dose, incubation time, and the corresponding lactate dehydrogenase (LDH) release; (B) effect of calcium; (C) effect of temperature; and (D) effect of pH. In all experiments, HBTS at pH 7.4, DA at 5x10^-4 M, 15 min incubation time, and temperature at 37 °C were used where applicable. Results are shown as mean±sem of 3 experiments, except (a). (a) Mean±sem of 2 experiments. (b) In the absence of Mg2+. (c) In the presence of 100 μM EGTA. nd = Not determined.
Table 4.2. Effects of metabolic inhibitors on deoxycholic acid (DA)-induced histamine release from rat peritoneal mast cells: (A) antimycin A, (B) 2,4-dinitrophenol, and (C) 2-deoxyglucose.

The cells were preincubated in the absence of DA with the inhibitor for 10 min, then followed by a further 10 min (in the case of 2-deoxyglucose) or 15 min (in the case of antimycin A and 2,4-dinitrophenol) incubation immediately after the addition of DA (10^{-5} M). Glucose was omitted from the buffer used (pH 7.4). Results are shown as means ± sem of 3 experiments.

<table>
<thead>
<tr>
<th></th>
<th>A. Antimycin A (µM)</th>
<th>B. 2,4-Dinitrophenol (µg/ml)</th>
<th>C. 2-Deoxyglucose (mM)</th>
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<td></td>
<td>0</td>
<td>0.05</td>
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<td></td>
<td>80.8 ± 0.9</td>
<td>80.7 ± 1.3</td>
<td>61.7 ± 3.7</td>
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<td>0.20</td>
<td>82.4 ± 0.8</td>
<td>75.7 ± 4.1</td>
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<td>0.50</td>
<td>82.7 ± 1.0</td>
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<td>0</td>
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<td>80.8 ± 0.9</td>
<td>84.8 ± 0.6</td>
<td>61.7 ± 3.7</td>
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<td>86.1 ± 0.3</td>
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<td>200</td>
<td>86.9 ± 0.9</td>
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Figure 4.7. Authentication of histamine release induced by deoxycholic acid (DA) from dispersed guinea pig rectocolonic mucosal cell preparation using diamine oxidase (DAO, 0.1 U/ml). Results are shown as mean±SEM of triplicate samples.
Chapter 5

CHAPTER 5

FREE RADICAL (PEROXIDASE/H$_2$O$_2$/HALIDE)-INDUCED HISTAMINE RELEASE FROM MAST CELLS OF GUINEA PIG RECTOCOLONIC MUCOSA AND RAT PERITONEAL CAVITY AND MODULATORY EFFECT OF SULPHASALAZINE, BALSALAZIDE, AND CYCLOSPORIN A

5.1. INTRODUCTION

As mentioned previously, mast cells and histamine have been implicated in the pathogenesis of inflammatory responses in inflammatory bowel disease (IBD). High colonic histamine content [498] and hyperplasia of mast cells in the gastrointestinal tract [145-149, 155] in patients with IBD have been observed. It is also known that histamine enhances gut motility, colonic fluid and electrolyte secretion, and the action of vasoactive intestinal polypeptide, a proposed mediator of diarrhoea, one of the symptoms in patients with IBD [141, 393, 505].

Inflammatory phagocytic leucocytes [506, 507] and reactive oxygen metabolites (ROM) [51, 58, 394-397] generated by them have been implicated in mediating mucosal inflammation in active IBD. Superoxide anion and hydrogen peroxide are produced by activated leucocytes. Hydrogen peroxide, though itself is not a true free radical, is the principal ROM which is formed by superoxide anion dismutation under any circumstance where there is the generation of superoxide anions. It has a longer half-life and thus can exist longer in the tissue to exert its deleterious effects on tissue components, either directly (if present in very high concentration), for example, on colonic tissue leading to cellular membrane damage and excess prostanoid production [508], or via the secondary generation of other oxidants. Activated phagocytes secrete myeloperoxidase (MPO) into the extracellular medium where it catalyses the oxidation of chloride ion (Cl$^-$) by H$_2$O$_2$ to yield hypochlorous acid (HOCl) [509-511] which is the most reactive oxidant produced by neutrophils in appreciable amount [406]. Free radicals have been suggested to be endogenous histamine releasers [168], and the peroxidase/H$_2$O$_2$/halide system has been shown to release histamine from isolated rat serosal mast cells [512, 513].
Chapter 5

It is thus of interest to investigate: (a) whether the peroxidase/H$_2$O$_2$/halide system could induce histamine release from enzyme-dispersed, isolated guinea pig rectocolonic mucosal cells (RCMC) which contain histaminocytes; and (b) whether drugs used for the treatment of IBD have any effect on histamine release induced by the system from guinea pig RCMC. In this study, horseradish peroxidase (HRP)/H$_2$O$_2$/Nal system was used to induce histamine release. Balsalazide (BSZ), sulphasalazine (SASP), and their metabolites 5-aminosalicylic acid (5-ASA, active moiety common to BSZ and SASP), 4-aminobenzoyl-$\beta$-alanine (4-ABA, carrier moiety for BSZ), and sulphapyridine (SP, carrier moiety for SASP) were tested. The effect of BSZ, SASP, and their metabolites was compared with two other agents, cyclosporin A (CsA) and reduced glutathione. CsA is an immunosuppressive agent shown to exhibit anti-allergic and anti-inflammatory effects [514] and to be useful in the treatment of IBD [83, 84, 86]. Reduced glutathione (GSH) is an antioxidant [404]. A similar study was also performed on rat peritoneal mast cells (RPMC) for comparison.

5.2. MATERIALS AND METHODS

All materials and experimental procedures are outlined in the appropriate sections in chapter 2.

5.3. RESULTS

5.3.1. Effect of H$_2$O$_2$/Nal/HRP system on histamine release from RCMC and RPMC

Cell samples in HBTS were incubated with inducer for 30 min at 37 °C and pH 7.4.

5.3.1.1 Effect on guinea pig rectocolonic mucosal cells (RCMC)

A series of experiments was carried out to study the effects of various combinations of hydrogen peroxide (H$_2$O$_2$), sodium iodide (Nal), and horseradish peroxidase (HRP) on histamine release from guinea pig rectocolonic mucosal cells. The results of some typical experiments are presented in Fig.5.1. At the doses tested, H$_2$O$_2$,
Nal, HRP, Nal+HRP, and H$_2$O$_2$+HRP caused little or no histamine release. Marked histamine release was induced by H$_2$O$_2$+Nal with or without HRP.

Using H$_2$O$_2$+Nal at various concentration ratios in the presence or absence of HRP, it was possible to obtain a dose-related stimulation of histamine release if the concentration ratio of H$_2$O$_2$/Nal was appropriate (Fig. 5.1.vi-viii, and Fig. 5.2). Increasing the concentration of H$_2$O$_2$ from 0.1 mM to 10 mM in combination with 10 mM of Nal and 0.25 U/ml of HRP, or increasing the concentration of Nal from 1.0 mM to 10 mM in combination with 5 mM of H$_2$O$_2$ and 0.25 U/ml of HRP induced histamine release dose-dependently (Fig. 5.2).

As expected, the addition of HRP did not cause significant difference in H$_2$O$_2$+Nal-induced histamine release (Fig 5.1.vi-viii). It was likely that the maximal effect of peroxidase might have been achieved by the presence of endogenous peroxidase in the cell preparation which contained large number of various cell types besides mast cells, viable or dead (on average, mixed cell viability was 37%, and viable cells per sample were 2.4x10$^6$).

5.3.1.2. Effect on rat peritoneal mast cells (RPMC)

Similar to those results observed with RCMC mentioned above, at the doses tested, H$_2$O$_2$, Nal, HRP, Nal+HRP, and H$_2$O$_2$+HRP did not induce histamine release from RPMC (Fig. 5.3.a.i & ii, and Fig. 5.3.b.v & vi). H$_2$O$_2$+Nal with or without HRP induced marked histamine release (Fig. 5.3.a.iii & iv, Fig. 5.3.b.vii & viii, and Table 5.1). However, in this case, though there was a dose-dependent stimulation of histamine release with increasing concentration of Nal (0.1 to 5.0 mM) and fixed concentration of H$_2$O$_2$, the stimulation of histamine release with increasing concentration of H$_2$O$_2$ (0.1 to 10 mM) and fixed concentration of Nal at 1.0 mM or below was inversely proportional to the concentration of H$_2$O$_2$ (Table 5.1 and Fig. 5.3.a.iii & iv). This inverse relationship diminished as the concentration of Nal increased (Table 5.1).

In general, H$_2$O$_2$+Nal-induced histamine release from RPMC was augmented with exogenously added HRP, and the effect was more profound at a certain H$_2$O$_2$ to Nal ratio (Table 5.1). The overall sensitivity toward added HRP might be due to the fact that less cells per sample ( >90% viability and 0.5x10$^6$ viable cells per sample) were used, and thus insufficient endogenous peroxidase for the reaction, depending on the amount of reactants (H$_2$O$_2$ and Nal) present.
5.3.2. Effect of BSZ, SASP, and metabolites on H$_2$O$_2$/Nal/HRP system-induced histamine release from guinea pig RCMC and RPMC

5.3.2.1. Effect on histamine release from guinea pig RCMC

The effect of BSZ, SASP, and their respective metabolites (all at 20-500 μM) on histamine release induced by the H$_2$O$_2$/Nal/HRP system was studied. Histamine release was induced by adding H$_2$O$_2$ (1 to 20 mM) to cell aliquots in HBTS containing Nal (10 mM) and HRP (0.25 U/ml) for 30 min at 37 °C and pH 7.4. In the studies with drug preincubation, the drug was preincubated with cell samples in the presence of Nal and HRP before the addition of H$_2$O$_2$; while in the studies without preincubation, the drug was added to cell samples containing Nal and HRP, and followed immediately by the addition of H$_2$O$_2$.

When histamine release from RCMC was induced by the combination of H$_2$O$_2$ at 10 mM, Nal at 10 mM, and HRP at 0.25 U/ml, 5-ASA, 4-ABA, and SP produced marked and dose-related inhibition of stimulated histamine release, whereas BSZ had weak activity (not significant) and SASP did not have any inhibitory effect in the dose range tested (Fig. 5.4). At 20 μM, 5-ASA showed a significant potentiation (Fig. 5.4). The degree of inhibition decreased with increasing concentration of H$_2$O$_2$ used (Fig. 5.5). The inhibitory effect was optimal when the drug was added at the time of challenge, and tachyphylaxis occurred as preincubation time was increased to 15 min (Fig. 5.6).

5.3.2.2. Effect on histamine release from RPMC

The effect of BSZ, SASP, and their respective metabolites (all at 20-500 μM) on histamine release induced by the H$_2$O$_2$+Nal was studied. Histamine release was induced by adding H$_2$O$_2$ (0.1 mM) to cell samples in HBTS containing Nal (0.1 mM) for 30 min at 37 °C and pH 7.4. The drug was preincubated for 5 min with cell samples in the presence Nal before the addition of H$_2$O$_2$.

At the concentrations tested (20-500 μM), the inhibitory effect of BSZ was weak, whereas that of SASP, 5-ASA, 4-ABA, and SP was marked and dose-related, especially the last three (Fig 5.7). At drug concentration of 100 μM, the corresponding inhibition (%) of stimulated histamine release for BSZ, SASP, 5-ASA, 4-ABA, and SP was 1, 8, 61, 72, and 106 % respectively.
Chapter 5

5.3.3. Effect of CsA on H₂O₂/Nal/HRP system-induced histamine release from guinea pig RCMC and RPMC

The procedure of induction of histamine release and drug treatment was similar to that outlined in section 5.3.2.1. Part of the results were obtained in collaboration with MNK Ho and presented at the 150th meeting of the British Pharmacological Society, 11th September, 1992.

5.3.3.1. Effect on histamine release from guinea pig RCMC

As shown in Table 5.2.a and Fig. 5.8, CsA (10-10000 nM) did not show any inhibitory effect on H₂O₂ (10 mM)/Nal (10 mM)/HRP (0.25 U/ml)-induced histamine release from guinea pig RCMC with various drug preincubation time (0-30 min).

5.3.3.2. Effect on histamine release from RPMC

The results are shown in Fig. 5.8. CsA (10-10000 nM) was found to produce a marked and dose-related inhibition of histamine release induced by H₂O₂ (0.1 mM) + Nal (1 mM) from RPMC. The IC₅₀ was between 1000 and 10000 nM.

5.3.4. Effect of GSH on H₂O₂/Nal/HRP system-induced histamine release from guinea pig RCMC and RPMC

5.3.4.1. Effect on histamine release from guinea pig RCMC

The procedure of induction of histamine release and drug treatment was similar to that outlined in section 5.3.2.1. At the drug concentration (50-500 µM) and preincubation time (0-15 min) tested, GSH did not show any inhibitory effect on histamine release induced by H₂O₂ (10 mM)/Nal (10 mM)/HRP (0.25 U/ml) from guinea pig RCMC (Table 5.2.a).

5.3.4.2. Effect on histamine release from RPMC

The procedure of induction of histamine release and drug treatment was similar to that outlined in section 5.3.2.2. GSH (20-500 µM) was tested in one of the experiments which showed inhibitory effect of BSZ, SASP, and their metabolites mentioned above. For comparison purpose, ascorbic acid at the same dose range
was also tested in the same experiment. GSH showed an inhibitory effect on H$_2$O$_2$ (0.1 mM) + Nal (0.1 mM)-induced histamine release from RPMC only at 500 µM, while ascorbic acid showed an inhibitory effect at 20 and 100 µM but not at 500 µM (Table 5.2.b).

5.4. DISCUSSION

The present results demonstrate clearly that the H$_2$O$_2$/halide/peroxidase system could induce marked release of histamine from guinea pig RCMC as well as rat peritoneal mast cells. Histamine release by H$_2$O$_2$ + Nal in the absence of exogenously added HRP could indicate that formation of hypoiodous acid (HOI) and iodination may also occur nonenzymatically and/or it could indicate the presence of endogenous peroxidase in the cell preparations used. The inverse relationship of histamine release from RPMC with increasing concentration of H$_2$O$_2$ (0.1 to 10 mM) and fixed concentration of Nal at 1.0 mM or below could be due to the reduction of HOI with the excess H$_2$O$_2$ (with respect to Nal and HRP) according to the reaction HOI + H$_2$O$_2$ → H$^+$ + I$^-$ + H$_2$O + O$_2$ [515]. That is, the product IO$^-$ (e.g. HIO) of HRP-catalysed reaction of H$_2$O$_2$ with Nal may be consumed by reacting with the excess H$_2$O$_2$ in a HRP-catalysed reaction shown as follows:

\[
\text{HRP} \quad \text{IO}^- + \text{H}_2\text{O}_2 \rightarrow \text{O}_2 + \text{H}_2\text{O} + \text{I}^- \quad [516]
\]

The net effect is the reduction of HOI content, hence, the decrease in histamine release. Alternately, it may be because the rate of HOI production is determined by the concentration of HRP, H$_2$O$_2$, or Nal, and the inactivation of HRP by the excess H$_2$O$_2$ may reduce the formation of HOI. H$_2$O$_2$ has been shown to inactivate peroxidases, including myeloperoxidase [517], lactoperoxidase [518], and chloroperoxidase [519]. The effect of inactivation of peroxidase by H$_2$O$_2$ on histamine release (decrease in release) will become apparent when peroxidase is limiting (i.e. when H$_2$O$_2$ is in excess) such that there is not enough peroxidase available to catalyse the formation of HOI, hence the decrease in histamine release. However, in the presence of excess peroxidase, partial inhibition of peroxidase by H$_2$O$_2$ need not affect the rate of HOI production. Thus, as previously suggested [520], only when the activity of peroxidase becomes limiting will HOI production start to decrease. The situation of excess H$_2$O$_2$ may arise when there is not enough halide to react with H$_2$O$_2$ (i.e. when the molar concentration ratio of H$_2$O$_2$ to Nal is greater than 1), and thus promotes the inactivation
Chapter 5

of peroxidase by H$_2$O$_2$. This may explain why the inverse relationship of histamine release with increasing H$_2$O$_2$ diminished as the concentration of NaI increased (Table 5.1). As to the effect of HRP on RPMC (Table 5.1), it is possible that H$_2$O$_2$ was limiting at concentrations below 5 mM such that the addition of exogenous HRP did not cause any difference. While with H$_2$O$_2$ at 10 mM, the added HRP was not enough to overcome the effect of inactivation of peroxidase by H$_2$O$_2$, hence no significant differences in histamine release were observed when exogenous HRP was added. These phenomena with RPMC were not observed with RCMC. This may be due to the fact that, with respect to H$_2$O$_2$, the endogenous peroxidase in the cell preparation and also the concentration of NaI used are optimal. In other words, H$_2$O$_2$ is limiting.

In the case of guinea pig RCMC, metabolites 5-ASA, 4-ABA, and SP produced marked and dose-related inhibition of histamine release, while BSZ and SASP did not show a significant inhibitory effect. The results are consistent with the findings of Molin and Stendahl (1979) [521] who studied the effect of SASP and its metabolites on human polymorphonuclear leucocyte (PMNL) function. They found that, at a dose range similar to that of the present study, SASP had no significant effect on H$_2$O$_2$/NaI/MPO system-mediated iodination and cytotoxicity, while 5-ASA and SP inhibited the iodination reaction and reduced the cytotoxic effect. Using reagent hydrogen peroxide and MPO in a cell-free system, Kettle and Winterbourn (1991) [522] found that SP was a potent inhibitor of the conversion of H$_2$O$_2$ to HOCl. SASP was also found to be effective but not as potent, while 5-ASA only effective at a much higher concentration. Also in the same study, via the spectral investigations, it was shown that the inhibitory drugs acted by trapping MPO as an inactive redox intermediate of the enzyme (designated as compound II) [522]. The potency of SASP and its metabolites in the present study and in the study by Molin and Stendahl with non-cell-free system was less potent than that shown with a cell-free system by Kettle and Winterbourn (1991) [522]. This may suggest that the drug effect may be affected by the cells present in the system. It has been suggested that superoxide generated by PMNL or by purified xanthine oxidase/xanthine system limits the inhibitory potency of the drugs on MPO by reducing compound II back to active enzyme which continues to produce HOCl [520]. The observed effect of 5-ASA (Fig. 5.4) could be explained by the suggestion that, at low concentration, 5-ASA is a good peroxidase substrate which converts rapidly any compound II formed to active enzyme, hence the production of HOCl; whereas at high concentration, it inhibits peroxidase irreversibly via a different mechanism [522]. Alternately, it could be the interaction of 5-ASA (at high concentration) with HOCl that prevents the effect of HOCl on
cellular constituents, hence the inhibition of histamine release at high concentration of 5-ASA. It has been shown that, by virtue of its being oxidized readily by HOCl and slightly less readily by chloramine NH2Cl (a chlorinated oxidant, see below), 5-ASA prevents the oxidation of l-cysteine by these chlorinated oxidants [523]. l-Cysteine is one of the important constituents of cell membrane protein and a precursor of GSH. The results with BSZ and SASP may have reflected the presence of 5-ASA moiety in the parent compounds. The lack of inhibition by SASP has also been demonstrated in other cell types. For example, SASP has been shown to enhance goat anti-human IgE-induced histamine release from isolated human intestinal mast cells and human blood basophils, whereas 5-ASA has been shown to be an effective inhibitor of stimulated histamine release in both cell types [162]. The present results of SASP and its metabolites cannot be explained by their ability of scavenging ROM alone, because compared to 5-ASA and SASP, SP is a weak ROM scavenger [459, 524, 525]. It is possible that the inhibition of peroxidase [522] is involved. The novel drug, BSZ, and its metabolites appear to share the same characteristics as SASP and its metabolites.

In the case of RPMC, it was found in the present study that the inhibitory effect of BSZ on stimulated histamine release was weak, and that of SASP, 5-ASA, 4-ABA, and SP was marked and dose-related, especially the three metabolites with SP being most effective. The discrepancy in the results of RCMC and RPMC could be due to the lower concentrations of HOCl and Nal used in the case of RPMC, or due to some basic difference between the cell preparations. The present results with RPMC are also different from those of the study in which BSZ and SASP (both at 20-500 μM) produced a significant and dose-related inhibition of 48/80-induced histamine release from RPMC, while 5-ASA, 4-ABA, and SP had very little or no inhibitory effect (Chapter 6). In total, these results from stimulated histamine release experiments with RCMC and RPMC indicate that the inhibitory activity of BSZ, SASP, and their respective metabolites differs with the type of cells and stimulant used.

In contrast to its inhibitory effect on RPMC, CsA (10-10000 nM) did not show any inhibitory effect on the HOCl/Nal/HRP system-induced histamine release from guinea pig RCMC, showing the functional heterogeneity of mast cells. CsA has been shown to exhibit anti-allergy and anti-inflammatory effects by inhibition of histamine, leukotriene C4/D4/E4, and prostaglandin D2 release from human basophils, human lung mast cells, human skin mast cells, rat basophilic leukaemia cells (RBL-2H3), and rat peritoneal mast cells [99, 100, 102-104, 514, 526, 527]. The present results also indicate the possible differences between the mechanism of action of CsA and the metabolites of BSZ and
Chapter 5

SASP. The inhibitory effect of CsA also depends on the nature of the inducer of histamine release; for example, 48/80-induced release from RPMC is not inhibited by CsA (see Chapter 6). In general, CsA is a potent inhibitor of calcium-dependent histamine release [100, 514].

Exogenous GSH applied extracellularly was found to be ineffective in preventing histamine release induced by \( \text{H}_2\text{O}_2/\text{NaI/HRP} \) system from RCMC, and was observed to be a relatively poor inhibitor of histamine release from RPMC. This may be due to the capability of HRP to oxidize GSH to harmful thiyi radicals [528]. Ascorbic acid is an aqueous phase compound that appears to be able to act as a primary scavenger of toxic oxidants of physiological importance [420]. However, in doing so, ascorbate radical, which is poorly reactive and may disappear largely by self reaction [496], is formed. It may be that at high dose of ascorbic acid, the ascorbate radical effect exceeds the scavenging effect, hence the absence of inhibitory effect of histamine release at high concentration. Alternately, since ascorbate reduces the inactive redox intermediate of MPO (compound II) back to the active enzyme [522, 529], it is possible that this action becomes apparent at high concentration of ascorbate so that the inhibitory effect of histamine release is reversed. Ascorbate is rapidly oxidized by HOCl [530] and may exerts its inhibitory effect in a manner similar to 5-ASA discussed above (at low concentration).

In conclusion, the present study has demonstrated that the \( \text{H}_2\text{O}_2/\text{halide/peroxidase} \) system induces histamine release from both RCMC and RPMC, indicating a link between the role of ROM and the release of histamine. Both ROM and histamine are known to be involved in inflammatory responses in IBD, and it is possible that the respective metabolites of BSZ and SASP exert their beneficial effects in IBD via inhibition of ROM-induced histamine release. Histamine has been shown to be a potent stimulator of the free radical-generating enzyme xanthine oxidase [195], and it may also interact with neutrophil-derived HOCl via a MPO catalysed oxidation of \( \text{Cl}^- \) by \( \text{H}_2\text{O}_2 \) to generate reactive N-chlorinated derivatives [418] which possess the two oxidizing equivalents of \( \text{H}_2\text{O}_2 \) and HOCl [58]. In other words, as an oxidant, the chlorinated derivatives are more potent than \( \text{H}_2\text{O}_2 \) and HOCl. Hence the inhibition of \( \text{H}_2\text{O}_2/\text{halide/peroxidase} \)-induced histamine release may prevent further formation of ROM and more potent chlorinated derivatives.
Figure 5.1. Some typical experiments showing the effects of various combinations of $\text{H}_2\text{O}_2$, Nal, and horseradish peroxidase (HRP) on histamine release from dispersed guinea pig rectocolonic mucosal mast cells. Samples were incubated for 30 min at 37 °C and pH 7.4. Each column represents the mean±sem of 3-6 samples from 1-2 experiments, and the spontaneous release has not been corrected.

(i) HRP alone; (ii) $\text{H}_2\text{O}_2$ alone; (iii) Nal alone; (iv) $\text{H}_2\text{O}_2$ + HRP (0.25 U/ml); (v) Nal + HRP (0.25 U/ml); (vi) $\text{H}_2\text{O}_2$ + Nal (10 mM); (vii) $\text{H}_2\text{O}_2$ + Nal (10 mM) + HRP (0.25 U/ml); (viii) $\text{H}_2\text{O}_2$ + Nal (10 mM) + HRP (0.5 U/ml).
Figure 5.2. Stimulation of histamine release from dispersed guinea pig rectocolonic mucosal mast cells by horseradish peroxidase (HRP, 0.25 U/ml)/H₂O₂/Nal system with varying concentrations of H₂O₂ or Nal. Results are shown as mean±SEM of 3-24 samples from 1-8 experiments.
Figure 5.3. Some typical experiments showing the effects of various combinations of H$_2$O$_2$, Nal, and horseradish peroxidase (HRP) on histamine release from rat peritoneal mast cells. Samples were incubated for 30 min at 37 °C and pH 7.4. Each column represents the mean±SEM of 3-6 samples from 1-2 experiments, and the spontaneous release has not been corrected. (i) H$_2$O$_2$ alone; (ii) H$_2$O$_2$ + HRP (0.25 U/ml); (iii) H$_2$O$_2$ + Nal (0.5 mM); (iv) H$_2$O$_2$ + Nal (0.5 mM) + HRP (0.25 U/ml); (v) Nal alone; (vi) Nal + HRP (0.25 U/ml); (vii) Nal + H$_2$O$_2$ (0.5 mM); (viii) Nal + H$_2$O$_2$ + HRP (0.5 U/ml).
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Nal (mM)</th>
<th>Nal (mM) + HRP (0.25 U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.1</td>
<td>0.5</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>0.1</td>
<td>43.3 ± 1.8</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>7.6 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>4.5 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>-0.2 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>-2.9 ± 0.5</td>
</tr>
</tbody>
</table>

Table 5.1. Stimulation of histamine release by H₂O₂ + Nal from rat peritoneal mast cells in the presence or absence of horseradish peroxidase (HRP, 0.25 U/ml). Results are shown as mean ± sem of 3-9 samples from 1-3 experiments.
Figure 5.4. Effect of balsalazide (BSZ), sulphasalazine (SASP), and metabolites 5-aminosalicylic acid (5-ASA), 4-aminobenzoyl-β-alanine (4-ABA), and sulphapyridine (SP) (20-500 μM) on horseradish peroxidase (0.25 U/ml)/H₂O₂ (10 mM)/Nal (10 mM) system-induced histamine release from dispersed guinea pig rectocolonic mucosal mast cells. Cell samples were preincubated with drugs for 5 min 37 °C, followed by inducer challenge for 30 min. Each column represents the mean±sem of 3-12 samples from 1-4 experiments. The uninhibited histamine release was 36.7±5.5 %. Student's t - test: *p<0.05, **p<0.01, ***p<0.001.
Figure 5.5. Comparison of the relative effect of balsalazide (BSZ), sulphasalazine (SASP), and their metabolites 5-aminosalicylic acid (5-ASA), 4-aminobenzoyl-ß-alanine (4-ABA), and sulphapyridine (SP) on horseradish peroxidase (HRP)/H\textsubscript{2}O\textsubscript{2}/Nal system-induced histamine release (HR) from dispersed guinea pig rectocolonic mucosal mast cells with various concentrations of H\textsubscript{2}O\textsubscript{2} (1-20 mM) + Nal (10 mM) + HRP (0.25 U/ml). Cell samples were preincubated with drugs at 500 µM for 5 min at 37 °C, then followed by inducer challenge for 30 min. Histamine release controls in the absence of test drugs were expressed as 100%. They were 44.8±0.5, 66.3±1.7, 42.2±6.0, and 72.5±1.4 % for 1, 5, 10, and 20 mM of H\textsubscript{2}O\textsubscript{2} respectively. Results are shown as mean±sem of 3-9 samples from 1-3 experiments. Student's t test: *p<0.05, **p<0.01, ***p<0.001, when compared with the histamine release control.
Chapter 5

Figure 5.6. Effect of drug preincubation time (0-15 min) with balsalazide (BSZ), sulphasalazine (SASP), and metabolites 5-aminosalicylic acid (5-ASA), 4-aminobenzoyl-β-alanine (4-ABA), and sulphapyridine (SP) (all at 500 μM) on histamine release from dispersed guinea pig rectocolic mucosal mast cells induced by horseradish peroxidase (0.25 U/ml)/H₂O₂ (10 mM)/Nal (10 mM) system. Cell samples were preincubated with drugs at 500 μM for the designated length of time at 37 °C, then followed by inducer challenge for 30 min. Results are shown as mean±SEM of 3-12 samples from 1-4 experiments. The uninhibited histamine releases (%) for 0, 5, and 15 min were 57.4±4.4, 36.7±5.5, and 52.0±2.9 respectively. Student's t-test: **p<0.01, ***p<0.001.
Figure 5.7. Effect of balsalazide (BSZ), sulphasalazine (SASP), and metabolites 5-aminosalicylic acid (5-ASA), 4-aminobenzoyl-β-alanine (4-ABA), and sulphapyridine (SP) (20-500 μM) on H$_2$O$_2$ (0.1 mM)/Nal (0.1 mM) system-induced histamine release from rat peritoneal mast cells. Cell samples were preincubated with drugs for 5 min 37 °C, followed by inducer challenge for 30 min. Each column represents the mean±SEM of 6 samples from 2 experiments. The uninhibited histamine release (%) was 43.6±1.9. Student's t-test: *p<0.05, **p<0.01, ***p<0.001.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>0</th>
<th>5</th>
<th>15</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 µM</td>
<td>-1.5 ± 0.3</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>GSH 100 µM</td>
<td>---</td>
<td>-5.6 ± 3.6</td>
<td>-2.1 ± 3.7</td>
</tr>
<tr>
<td>500 µM</td>
<td>-0.2 ± 0.9</td>
<td>-0.4 ± 2.6</td>
<td>-7.0 ± 1.7</td>
</tr>
<tr>
<td>CsA 1 µM</td>
<td>-2.0 ± 0.8</td>
<td>-3.9 ± 3.1</td>
<td>-7.4 ± 2.3</td>
</tr>
</tbody>
</table>

Table 5.2.a. Effect of exogeneous glutathione (reduced form, GSH) and cyclosporin A (CsA) on H$_2$O$_2$ (10 mM)/NaI (10 mM)/horseradish peroxidase (0.25 U/ml)-induced histamine release from enzyme-dispersed guinea pig rectocolonic mucosal mast cells. Inducer challenge was for 30 min at 37 °C. Results are shown as mean±sem of triplicate samples from 3 separate experiments. The mean±sem of uninhibited histamine release (%) for the 3 experiments was 59.9±4.4.

<table>
<thead>
<tr>
<th>Treatment</th>
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<th>100</th>
<th>500</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH</td>
<td>1.6 ± 4.5</td>
<td>1.2 ± 6.0</td>
<td>23.1 ± 3.3*</td>
</tr>
<tr>
<td>Ascorbic Acid</td>
<td>52.9 ± 10.3**</td>
<td>107.1 ± 3.2***</td>
<td>5.1 ± 2.6</td>
</tr>
</tbody>
</table>

Table 5.2.b. Effect of exogeneous glutathione (reduced form, GSH) and ascorbic acid on H$_2$O$_2$ (0.1 mM)/horseradish peroxidase (0.25 U/ml)-induced histamine release from rat peritoneal mast cells. Inducer challenge was for 30 min at 37 °C with drug preincubation time of 5 min. Results are shown as mean±sem of triplicate samples from single experiment. The uninhibited histamine release (%) was 39.0±2.0. Student's t-test: *p<0.05, **p<0.01, ***p<0.001.
Chapter 5

Figure 5.8. Effect of cyclosporin A on histamine release induced by H$_2$O$_2$ (0.1 mM)/Nal (0.1 mM) system from rat peritoneal mast cells (RPMC) or horseradish peroxidase (0.25 U/ml)/H$_2$O$_2$ (10 mM)/Nal (10 mM) system from dispersed guinea pig rectocolonic mucosal mast cells (RCMC). Cell samples were preincubated with drug for 5 or 30 min at 37 °C, then followed by inducer challenge for 30 min. Results are shown as mean±sem of 6-9 samples from 2-3 experiments. The uninhibited histamine releases (%) for RPMC and RCMC with 30 min drug preincubation, and RCMC with 5 min drug preincubation were 41.3±1.9 and 37.9±3.9, and 38.7±4.1, respectively. Student's t-test: *p<0.05, ***p<0.001.
6.1. INTRODUCTION

As mentioned previously (Chapter 1), different mast cell populations respond differently to secretagogues and inhibitory pharmacological agents. There are many stimuli, both immunological and non-immunological, that induce mast cell histamine release. The process of histamine release is regulated by a mechanism which is not yet fully understood and involves many complex and different biochemical pathways [227-230]. Stimulation of mast cells and basophils may result in activation of GTP-binding proteins (G-proteins) which are involved in transducing receptor-mediated signals [223, 238], activation of various membrane-associated enzymes [531-533], mobilization of intracellular calcium [256, 534], and enhancement of the hydrolysis of phosphoinositides [241, 535]. While extracellular calcium is essential for IgE-mediated activation of mast cells [252], it is not required for cell activation by agents typified by compound 48/80 [282, 283]. Using permeabilized mast cells, calcium and guanine nucleotide have been shown to be essential effectors for rat peritoneal mast cells (RPMC) degranulation; and it has been suggested that RPMC activation involves the activation of a G-protein coupled to a polyphosphoinositol phosphodiesterase that renders the formation of inositol 1,4,5-triphosphate (IP$_3$) and 1,2-diacylglycerol (DAG) followed by a G-protein G$_{alpha}$- and calcium-mediated late event that leads to exocytosis [223]. The IP$_3$ has been shown to mobilize calcium from the internal store [272], whereas DAG has been shown to activate protein kinase C (PKC) which in turn leads to protein phosphorylation [302, 305, 324-327] which appears to be a regulatory system used by the cells. The effects of calcium on cell functions have been suggested to be mediated via calmodulin [291], and it has been suggested that calmodulin may be in some way involved in the histamine release from mast cells [265, 299-301].
Platelet activating factor (PAF) is a potent proinflammatory mediator implicated in inflammatory bowel disease [211, 213]. It has been shown to stimulate histamine release from RPMC, most likely by interacting with the cell membrane rather than specific receptors [536]. Histamine release can also be achieved by circumventing the cell surface receptors with agents capable of activating various post-receptor stages of signalling pathways. G-proteins have been reported to be activated by sodium fluoride (NaF), leading to the activation of adenylate cyclase and phospholipase C (PLC) activities, which in turn result in the increase of PKC activity and inositol phosphate formation [349], and eventually histamine release [281, 348]. It has been suggested that compound 48/80 and the neuropeptide substance P (Sub P) may directly activate G-proteins [249]. The elevation of intracellular calcium that triggers histamine release can be stimulated by the application of ionophore A23187 which is capable of transferring calcium ions across the cell membrane [261]. A synthetic 1,2-diglyceride, 1,2-dioctanoyl-sn-glycerol (Dic8), has been reported to activate PKC [336, 337]. Similarly, compounds or drugs which interfere negatively with the signalling pathways are capable of preventing histamine release, hence, restrain the untoward pro-inflammatory effects caused by histamine. In other words, the reactions which control histamine release are potential targets for drug intervention. Using certain secretagogues as a tool, it is possible to find out the mechanisms of how drugs inhibit histamine release.

In this chapter, a further comparison of histamine release from RPMC and enzyme-dispersed guinea pig rectocolonic mucosal mast cells (RCMC) was carried out by using selected secretagogues (PAF, 48/80, Sub P, NaF, PLC, Dic8, and A23187). The effects and mechanisms of action of balsalazide (BSZ), sulphasalazine (SASP), and their metabolites as well as cyclosporin A (CsA) on induced histamine release by selected secretagogues were also investigated and compared. Furthermore, the effects of putative calmodulin antagonists on 48/80-induced histamine release from RPMC were studied.

6.2. MATERIALS AND METHODS

All materials and experimental procedures are outlined in the appropriate sections in chapter 2.
Chapter 6

6.3. RESULTS

6.3.1. Effect of selected secretagogues on histamine release from RPMC and guinea pig RCMC

When the effect of the extracellular calcium was studied, the effect of the selected secretagogues on histamine release in the presence and absence of external calcium was always tested in the same experiment.

6.3.1.1. Response of RPMC to selected secretagogues

RPMC responded in a concentration-related manner to the selected secretagogues which were platelet activating factor (PAF), compound 48/80, substance P (Sub P), sodium fluoride (NaF), phospholipase C (PLC), 1,2-dioctanoyl-sn-glycerol (Dic8), and ionophore A23187 (Fig.6.1-6.4). The response to PAF, compound 48/80, Sub P, and Dic8 was not abolished in the absence of extracellular calcium (Fig.6.1, 6.2, and 6.3.b), while that of NaF and PLC was drastically reduced or abolished, respectively (Fig.6.3.a and Fig.6.4.a). Histamine release induced by Sub P as well as Dic8 was shown to be greater in the absence than in the presence of calcium in the extracellular medium (Fig.6.2.b and 6.3.b).

6.3.1.2. Response of guinea pig RCMC to selected secretagogues

In contrast to RPMC, guinea pig RCMC only responded to PLC and A23187 but not the others, and the response to PLC and A23187 was smaller than that of RPMC (Fig.6.4, Table 6.1 and 6.2). The release induced by PLC was shown to be abolished in the absence of extracellular calcium (Fig.6.4.a).

6.3.2. Effect of BSZ, SASP, and their metabolites on histamine release induced by selected secretagogues from RPMC and guinea pig RCMC

6.3.2.1. Effect on histamine release from RPMC

6.3.2.1.1. Platelet activating factor-induced histamine release

The results are shown in Table 6.3. At 500 μM, both BSZ and SASP showed significant inhibition on PAF (5 μM)-induced histamine release when added simultaneously
with stimulant. SASP was more potent than BSZ. Preincubation (5 min) of the cells with the test compound (20-500 μM) prior to the addition of stimulant did not produce any inhibitory effect by both BSZ and SASP against the stimulated release. The metabolites, 5-ASA, 4-ABA, and SP showed little or no inhibitory effect at the concentrations tested (20-500 μM).

6.3.2.1.2. Compound 48/80-induced histamine release

The results are summarized in Table 6.4. BSZ and SASP (20-500 μM) produced a significant concentration-related inhibition of 48/80 (0.3 μg/ml)-induced release in the presence and absence of calcium in the extracellular medium when the test compound was added simultaneously with the stimulant. However, the inhibition by BSZ at 100 and 500 μM in the absence of extracellular calcium was significantly much lower than that seen in the presence of extracellular calcium; while the inhibition by SASP was only affected slightly by the withdrawal of the extracellular calcium with significant effect observed at drug concentration of 500 μM. Under the same condition, only the BSZ metabolite 4-ABA (500 μM) showed weak inhibition while the common metabolite of BSZ and SASP, 5-ASA and the SASP metabolite SP, were ineffective against the stimulated release at the concentrations tested (20-500 μM).

Preincubation (5 min) of the cells with the test compound (20-500 μM) prior to stimulation appeared to diminish markedly and significantly the inhibitory effect of BSZ and SASP seen when there was no preincubation (i.e. when the drug added simultaneously with the releaser), both in the presence and absence of extracellular calcium. With the drug preincubation (5 min), the withdrawal of extracellular calcium seemed to produce a similar trend on the inhibition of both BSZ and SASP as that observed under the condition with no preincubation mentioned above. The metabolites, 5-ASA, 4-ABA, and SP, showed no inhibition or very slight augmentation on stimulated release at the concentrations tested (20-500 μM).

6.3.2.1.3. Substance P-induced histamine release

The results are summarized in Table 6.5. Simultaneous addition of BSZ produced a significant concentration (20-500 μM)-related inhibition against Sub P (20 μM)-induced release, while SASP only produced significant inhibitory effect at 100 μM (both higher and lower concentrations failed to inhibit release). All the metabolites (5-ASA, 4-ABA,
and SP) at the concentrations tested (20-500 μM) did not show any inhibitory effect on the stimulated release. In the absence of extracellular calcium under the same condition, only the SASP metabolite SP produced a weak inhibitory effect on the induced release, while the parent compounds (BSZ and SASP) and other metabolites (5-ASA and 4-ABA) showed either no effect or a very weak potentiating effect on Sub P-stimulated release.

6.3.2.1.4. Sodium fluoride-induced histamine release

The results are summarized in Table 6.5. At 500 μM, BSZ and SP produced a weak but significant inhibition against NaF (20 mM)-induced histamine release when the test compound was added simultaneously with stimulant, while SASP, 5-ASA, and 4-ABA did not produce any significant effect at the same concentration (500 μM). With 5 min drug (20-500 μM) preincubation with the cells prior to the addition of stimulant, only SASP, 5-ASA, and SP at 500 μM produced significant inhibitory effects on stimulated release; while BSZ and 4-ABA did not produce any effect. The inhibitory effect of SP observed under the condition without drug preincubation was not affected with 5 min drug preincubation; while that of BSZ shown under the condition without drug preincubation was completely abolished with drug preincubation.

6.3.2.1.5. Phospholipase C-induced histamine release

The results are shown in Table 6.6. BSZ, SASP, and their metabolites (20-500 μM) did not produce any inhibitory effect against PLC (20 μg/ml=0.076 U/ml)-induced histamine release under the condition of 5 min drug preincubation with the cells prior to the addition of stimulant. On the contrary, a tendency to augment the stimulated release was shown, especially with SASP.

6.3.2.1.6. Dic8-induced histamine release

The results are shown in Table 6.6. Test compounds (20-500 μM) were preincubated with cells for 5 min prior to the stimulation of histamine release by Dic8 (20 μM). None of the compounds tested produced any inhibitory effect against the release, instead slight potentiation of the release was observed.
6.3.2.1.7. Calcium ionophore A23187-induced histamine release

The results are shown in Table 6.6. Preincubation (5 min) of the cells with test compound (20-500 μM) produced no inhibitory effect against A23187 (10 μM)-induced histamine release by the compounds tested.

6.3.2.2. Effect on histamine release from guinea pig RCMC

6.3.2.2.1. Phospholipase C-induced histamine release

The results are shown in Table 6.7. Test compound (20-500 μM) was preincubated with cells for 5 min prior to the stimulation. BSZ and SASP produced a weak concentration-related inhibition on PLC (20 μg/ml=0.076 U/ml)-induced histamine release, while 5-ASA produced a small augmentation. However, at higher inducing concentration of PLC (40 μg/ml=0.152 U/ml), the inhibitory effect of BSZ and SASP was abolished, while the effect of 5-ASA remained the same as that with PLC at 20 μg/ml, and 4-ABA and SP produced a weak concentration-related augmentation.

6.3.2.2.2. Calcium ionophore A23187-induced histamine release

The results are shown in Table 6.7. Test compound (20-500 μM) was preincubated (5 min) with cells prior to A23187(10 μM) stimulation. Of the compounds tested (BSZ, SASP, 5-ASA, 4-ABA, and SP), only SASP at 500 μM produced a weak inhibition on the induced release, while others produced no effect or slight augmentation.

6.3.3. Effect of cyclosporin A on histamine release from RPMC and guinea pig RCMC induced by selected secretagogues

6.3.3.1. Effect on histamine release from RPMC

6.3.3.1.1. NaF-induced histamine release

Preincubation (5 min) of CsA (10-1000 nM) with the cells prior to NaF (15 mM or 20 mM) stimulation produced a dose-related inhibition of histamine release from RPMC (Fig.6.5). The inhibition was dependent on the concentration of NaF used (Fig.6.5).
Chapter 6

6.3.3.1.2. Phospholipase C-induced histamine release

CsA (10-1000 nM) preincubation (5 min) with the cells prior to stimulation produced a dose-related inhibition of histamine release from RPMC induced by PLC (20 μg/ml=0.076 U/ml) (Fig. 6.6).

6.3.3.1.3 Dic8-induced histamine release

CsA (10-1000 nM) produced a dose-related inhibition of Dic8 (20 μM)-induced histamine release from RPMC in the presence or absence of extracellular calcium (Fig. 6.5). The profiles of inhibition in both cases were not identical in that in the absence of extracellular calcium, the inhibition by CsA reached a plateau at 100 nM of the drug, while that in the presence of extracellular calcium increased beyond 100 nM.

6.3.3.1.4. Calcium ionophore A23187-induced histamine release

Preincubation (30 min) of CsA (10-1000 nM) with the cells prior to the addition of ionophore A23187 (10 μM) produced a dose-related inhibition of histamine release from RPMC (Fig. 6.7). [Result obtained in collaboration with MNK Ho and presented at the 150th meeting of the British Pharmacological Society, 11th September, 1992.]

6.3.3.2. Effect on histamine release from guinea pig RCMC

6.3.3.2.1. Phospholipase C-induced histamine release

Preincubation (5 min) of CsA (10-1000 nM) with the cells prior to stimulation of release only produced a small inhibition of PLC (20 μg/ml=0.076 U/ml)-induced histamine release at 1000 nM of CsA (Fig. 6.6). As shown, the inhibitory effect was smaller than that with RPMC.

6.3.3.2.2. Calcium ionophore A23187-induced histamine release

CsA (10-1000 nM) produced a dose-related inhibition of A23187 (10 μM)-induced histamine release from guinea pig RCMC (Fig. 6.7).
6.3.4. Effect of calmodulin antagonists on compound 48/80-induced histamine release from RPMC

The results are presented in Table 6.8. The calmodulin antagonists (CMA) tested were picumast (PIC), trifluoperazine (TFP), N-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide (W7), and N-(4-aminobutyl)-5-chloro-1-naphthalene-sulphonamide (N-4ABSA). None of the antagonists tested, at 10⁻⁶ to 5x10⁻⁵ M, produced a consistently significant inhibition of histamine release. In some instances there was an increase in release. TFP and W7 themselves were found to induce histamine release at 10⁻⁵ M when tested in experiment 1 and 2. The release (mean±sem) induced by TFP in experiment 1 and 2 was 43.3 ± 1.3 and 68.9 ± 0.8 % respectively, while that of W7 was 69.2 ± 0.4 and 81.7 ± 1.4 % respectively.

6.4. DISCUSSION

Each of the secretagogues selected has a different action on the signalling pathways of mast cell histamine release. To interpret the results of the present study, it is necessary to consider the mode of action of the selected secretagogues.

PAF is a potent proinflammatory mediator implicated in inflammatory bowel disease [211, 213]. It has been shown to have a non-specific mode of action on the mast cell membrane, evoking a dose-dependent, calcium-independent, and non-cytotoxic (up to 5 μM) histamine release from RPMC [536]. The dose-dependency and calcium-independency of histamine release RPMC are confirmed by the present data.

Sodium fluoride has been reported to activate G-proteins in a GTP-independent manner via fluoroaluminate ion AlF₄⁻ which is formed by combination of fluoride ions from NaF with trace quantities of aluminium ions from glassware [243-245]. This action of NaF is not specific; however, it is the only compound available to activate G-protein without the recourse to using the potentially artifactual membrane preparation or cell permeabilization procedures for introducing a nonhydrolyzable GTP analogue [246]. Activation of G-protein by NaF leads to the activation of adenylate cyclase and PLC activities, resulting in the increase of protein kinase C (PKC) activity and inositol phosphate formation [349], and eventually histamine release. The histamine release from isolated rat mast cells induced by NaF has been shown to be dependent on the extracellular calcium [281], and this is supported by the present study. The response
to NaF has also been shown to be accompanied by an elevation of cyclic AMP level within the cell [348] and to be insensitive to pertussis toxin [244].

Compound 48/80 induces histamine release from rat mast cells in the presence as well as in the absence of extracellular calcium [282, 283]. This finding is confirmed by the present results. The mechanism of activation of mast cells by compound 48/80 is not known. It has been suggested that compound 48/80 binds to the proteins on the surface of mast cells [537]. The responsiveness of mast cells from different species and tissues to compound 48/80 appears to be highly restricted, in that 48/80 does not induce histamine release from mucosal mast cells yet it is an effective stimulus (though not universally in all species) of connective tissue mast cells [123, 124] (also see Table 1.2 of chapter 1). This characteristic has been useful in studying mast cell heterogeneity. The neuropeptide Sub P, another secretagogue with unknown mechanism of action on mast cells, has been shown to release histamine from not only RPMC but also the mast cells from some other tissues in various species, though not all, and the release from RPMC can be induced even in the absence of extracellular calcium owing to mobilization of intracellular calcium store [286, 318, 538-540]. The present results agree with the reported effect of extracellular calcium on Sub P-induced histamine release from RPMC. A common target for compound 48/80 and neuropeptide Sub P has been suggested [539]. This notion of sharing the same site, is supported by the similarities in (a) calcium regulation mentioned above; (b) inhibition of histamine release by pertussis toxin, indicating the involvement of pertussis toxin-sensitive G-protein in the biochemical pathway leading to exocytosis; (c) the concomitant increase in inositol phosphates and the sensitivity to pertussis toxin of this generation of second messengers, showing that the activation of a G-protein is followed by that of phospholipase C; and (d) the common inhibitory effect of the hydrolysis of sialic acid residues [241, 289, 314, 317]. The action of 48/80 and Sub P has striking similarities to that of wasp venom peptide mastoparan [317]. Mastoparan has been shown to have a direct effect on G-protein [247, 248]. Hence, it has been suggested that action of compound 48/80 and Sub P may be mediated directly by G-protein [249]. However, Mio et al (1991) [301] have shown the failure of Sub P in inducing histamine release from β-escin permeabilized RPMC, hence the suggestion that Sub P may not be able to activate G-protein directly. The same workers has suggested that the activation of G-protein by Sub P and mastoparan may be ascribable to the indirect action of phospholipid perturbation rather than the direct action on G-protein itself. This suggestion is based on the findings that (a) both Sub P and mastoparan are capable
of binding to acidic phospholipid, resulting in the perturbation of phospholipid conformation in the lipid bilayer [541, 542], and (b) the alterations in phospholipid conformation can modify the protein-lipid interaction [545]. However there were differences in the experimental procedures used by these different groups of workers in that Mousli et al. (1990) [249] and Higashijima et al. (1990) [247] carried out the induction by either mastoparan or Sub P in the presence of phospholipid or detergents which were used in the permeabilization procedures, whereas Mio et al. (1991) [301] carried out the induction in the absence of β-escin (agent for permeabilization) after permeabilization. Moreover, it is also possible that, with normal cells, the primary interaction of these inducers with cell membrane sialic acid residues induces a secondary structure allowing the inducer to cross the membrane to interact with a pertussis toxin-sensitive G-protein and activate PLC, as hypothesized by Mousli et al. (1989) [317]. More recently, using lectins with various sugar-specificities, it has been demonstrated and proposed that one of the action sites for compound 48/80 is glycoproteins having asparagine-linked-sugar residues composed of sialic acid and/or \( N \)-acetylglucosamine, and these glycoproteins possibly play important roles in mast cell activation induced by non-immunological stimuli [543]. Cockcroft et al. (1987) [238] have shown in their system of streptolysin O-permeabilized RPMC that activation of PLC is not involved in triggering of the GTP\(_{\gamma}R\)S (non-hydrolyzable GTP analogue)-dependent histamine release, as the inhibition of PLC by neomycin (hence preventing the production of inositol phosphates) does not affect the histamine release. Thus, a G-protein \( (G_{\gamma}) \), which is distinct from \( G_{\alpha} \) (PLC-associated G-protein), has been proposed to be involved at a late stage in the secretory process [223]. The involvement of \( G_{\alpha} \) in compound 48/80-induced histamine release has been suggested [223, 240].

Dic8, a synthetic 1,2-diglyceride which is an activator of PKC [336, 337], was shown to induce histamine release from RPMC in the presence as well as the absence of extracellular calcium in the present study. DAG level has been shown to increase in a variety of activated mast cells prior to or coincident with the onset of histamine release [332, 544]. DAG together with IP\(_3\) are the products of PLC-catalysed hydrolysis of cell membrane inositol lipid precursor, and they have been regarded as important second messengers in the initiation and/or the regulation of cellular stimulation. IP\(_3\) has been shown to mobilize calcium from internal store, and has also been suggested to induce influx of external calcium [272, 318]. The influx of calcium may also require the presence of inositol-1,3,4,5-tetrakisphosphate [272, 303], a product of IP\(_3\) phosphorylation. DAG has been shown to activate protein kinase C (PKC) which in turn
leads to protein phosphorylation [302, 305, 324-327]. It has been shown that there are more than one species of PKC molecule with multiple discrete subspecies (10 subspecies), and not all of them are calcium- and/or DAG-dependent for their activation [302]. There has been suggestion that activation of PKC is not required for exocytosis to occur [331]. PKC has been proposed as a possible modulator for extracellular calcium-dependent stimuli as the result of the observations of enhanced calcium uptake [325], and the potentiation of the responses to A23187 [323] and NaF [327] with its activation. Moreover, it has been reported that PKC is involved in the transduction process of IgE receptor-mediated mast cell degranulation (an event dependent on extracellular calcium), but not that of compound 48/80 [328].

It was first shown with RPMC that, in the presence of adequate extracellular calcium, A23187 produces marked histamine release accompanied by the uptake of calcium [261]. It has been established that histamine release from various mast cell populations of different animal species can be achieved by A23187 circumventing the cell surface ligand receptors [124, 174, 185, 266, 267]. The finding that A23187 induced histamine release from RPMC was reproduced in the present study. Ionophore A23187 is neither sensitive to pertussis toxin nor neuraminidase-pretreatment, and its calcium requirements are different from those of compound 48/80 or mastoparan and Sub P [317, 546]. The complexity of the mechanisms involved in the activation and regulation of mast cells is exemplified by 48/80, Sub P, and NaF with their common feature of G-protein activation and the incongruity of calcium requirement and sensitivity to pertussis toxin mentioned above. In addition, similar to NaF, IgE-mediated histamine release is insensitive to pertussis toxin [241] and is accompanied by a transient elevation of cAMP, whereas compound 48/80-mediated release is accompanied by a decrease [338]. However, unlike NaF, it has been shown that in the transduction of IgE-mediated triggering signal to PLC in rodent mast cells may not involve a G-protein, though the possibility that a unique G-protein may exist in a special compartment which is not accessible to the experimental procedures used cannot be ruled out [240]. Histamine release in the absence of extracellular calcium is generally attributed to the mobilisation of the intracellular store of calcium [174, 258, 283, 286]. The antagonistic effect of the extracellular calcium on Sub P- and Dic8-induced histamine release from RPMC may reflect competition between the ion and secretagogue for a common binding site (e.g. sialic acid residue) on the RPMC membrane [173, 277, 317]. Alternately, it may be due to the removal of negative feedback role of calcium on cell response, as it has been
shown that calcium is responsible for both activation and inactivation of the cell response [327, 547].

The presented data on selected secretagogues for histamine release show differential effects on enzyme-dispersed guinea pig RCMC and isolated RPMC. PAF, 48/80, Sub P, NaF, Dic8, PLC, and A23187 are potent stimulants of histamine release from RPMC, whereas histamine release from guinea pig RCMC can only be induced by PLC and A23187. As mentioned above, 48/80 does not induce histamine release from mucosal mast cells, and the differential responsiveness of mast cells from various species and tissues to 48/80 has been used as an indication of mast cell heterogeneity. The other basic secretagogue, Sub P, in contrast to its ineffectiveness in inducing histamine release from guinea pig RCMC, has been known to induce histamine release from rat intestinal mucosal mast cells [220]. Apart from attributing the findings to mast cell heterogeneity, it is not yet clear why of the wide range of secretagogues studied, only PLC and A23187 were effective in inducing histamine release from guinea pig RCMC. The failure of guinea pig RCMC to respond to certain selected secretagogues does not necessarily indicate the absence of a certain component from the regulatory pathway of histamine release, as indicated above, it may be attributable to heterogeneity of G-proteins and PKC which may not respond identically.

In general, the present results demonstrate that, in RPMC, the metabolites of BSZ and SASP, 5-ASA, 4-ABA, and SP overall showed little or no inhibitory effect on histamine release induced by the selected secretagogues. In some instances, including the parent compounds BSZ and SASP, augmentation of histamine release was observed, reflecting the possible contribution to the adverse reactions to the said drugs. It is conceivable that, under certain conditions, an ongoing subthreshold stimulation of histamine release present in some patients with inflammatory bowel disease may be augmented by the drugs. Furthermore, in some cases, paradoxical effects of the parent compounds and metabolites were observed. Given the putative mode of actions of the selected secretagogues used, it is possible that BSZ and SASP, as well as, to a much lesser extent in some rare occasions, some of their metabolites, exert their inhibitory effect on RPMC histamine release by a general membrane stabilizing via binding to sialic acid residues on the surface of cells or otherwise, and/or interaction with a regulatory G-protein associated with compound 48/80 and, to a lesser extent as shown, a NaF-associated G-protein which is different from that associated with 48/80. The lesser effect of test drugs on NaF-induced histamine release may also be due to the non-specific action of NaF on G-proteins (as mentioned above) in that NaF activates different
G-proteins [245, 246], and possibly only one (e.g. 48/80-associated G-protein) or only some of the activated G-proteins (but not all) interact with the drugs tested. It is also possible that the inhibitory effect of the drugs may be ascribed to an increase in the intracellular level of cyclic AMP or interfering with the calcium-channels. It has been suggested that the calcium-channels, through which the extracellular calcium enters the mast cells [548], may be closely associated with the sialic acid residues [277]. The calcium-channel has been established as a glycoprotein [275]. The failure of inhibiting histamine release induced by circumventing the cell surface receptors and G-proteins in the present study (i.e. induced by PLC, Dic8, or ionophore A23187) does not indicate whether or not the inhibitory effect of the drugs is associated with a regulatory pathway of histamine release involving PLC and PKC. It is possible that a G-protein which is independent of PLC and/or PKC, or a different PKC (which is not activated by Dic8) may be involved as mentioned above. As to guinea pig RCMC, the effect of the drugs was only tested on A23187-and PLC-induced histamine release, since these two were the only selected secretagogues that guinea pig RCMC responded to. The present data shows that, in guinea pig RCMC, BSZ, SASP, and their metabolites do not inhibit the biochemical events controlling histamine release after the elevation of cytosolic free calcium, as indicated by the inability of inhibiting histamine release induced by A23187.

SASP, and to lesser degree, 5-ASA and SP have been shown to inhibit human granulocyte activation by inhibition of second messenger production at the level of PLC or PLC-associated G-protein (inhibition of the production of IP3 and DAG), as well as at the level of phospholipase D (PLD) or PLD-associated G-protein (inhibition of the production of phosphatidic acid), but independent of cyclic AMP [549, 550]. Sulphidopeptide leukotriene formation induced by A23187 from RPMC has been shown to be inhibited more potently by SASP than 5-ASA, but not SP [161]. Augmentation of IgE-mediated human basophil histamine release by SASP has been reported previously [162, 267]. It has also been reported that SASP inhibits IgE-mediated histamine release from RPMC and mouse bone marrow-derived mast cells [267], and enhances that from human intestinal mast cells [162]. The contrasting results have been attributed to the heterogeneity in different mast cell populations.

The inhibitory effect of BSZ and SASP against compound 48/80- and Sub P-induced histamine release from RPMC was found to be susceptible to whether the extracellular calcium being present or not. In the absence of extracellular calcium, the inhibitory effect was either lessened or abolished. BSZ appeared to be more susceptible to the change in extracellular calcium than SASP in the case of 48/80-induced release,
and the inhibitory effect on Sub P-induced release appeared to be more susceptible to the said change than that on 48/80-induced release. There are several plausible explanations for this observed effect of extracellular calcium. The most obvious explanation is that both drugs require calcium ions in some way to exert their inhibitory effect. Alternately, as referred to above, in the absence of extracellular calcium, the basic secretagogues promote the mobilisation of intracellular sequestered stores of calcium, and the drugs are less effective in modulating the effect of intracellular calcium. As previously suggested, in the presence of extracellular calcium, the mobilisation of internal stores may be prevented [283], and the action of basic secretagogues may be predominantly by promoting the uptake of calcium [285]. It is possible that BSZ and SASP may be more effective in preventing the influx of calcium ions in the presence of extracellular calcium, hence better inhibition. Another possibility is that the removal of calcium from the extracellular medium eliminates the competition presented by the ion against the secretagogue, hence rendering the cells more sensitive to stimulus. Negatively charged sialic acid residues of the cell membrane have been shown to be the common binding site for calcium ions [551, 552] and positively charged secretagogues, compound 48/80 and Sub P [277, 317]. The inhibitory effect of calcium on histamine release induced by 48/80 [546, 553] and Sub P [173, 317] from RPMC has been observed and this inhibitory effect can be counteracted by increasing the concentration of the inducers [553]. Reduction of cell surface sialic acid residues by neuraminidase pretreatment has been shown to decrease histamine release from RPMC induced by 48/80 or Sub P [317]. However, contrary to the finding of Mousli et al (1989) [317], it has also been shown that no decrease of release induced by 48/80 is observed in the presence of external calcium after neuraminidase pretreatment, though there is a neuraminidase dose-dependent decrease of release in the absence of extracellular calcium [277]. Nevertheless, there were differences in the experimental procedures in that the former group [317] used purified RPMC, lower concentrations of 48/80 (0-0.2 μg/ml) and longer neuraminidase preincubation time (60 min); whereas the latter [277] used mixed cells consisting of RPMC and rat pleural mast cells without purification, 48/80 concentration of 1 μg/ml, and shorter neuraminidase preincubation time (10 min). Reduction of cell membrane sialic acid residues has also been shown to increase the sensitivity of RPMC [277] and basophils [276] to extracellular calcium which itself can induce histamine release from RPMC pretreated with neuraminidase [277]. With respect to IgE-mediated histamine release, the change (either increase or reduction) in cell membrane content of sialic acid appears to have no effect on the
release from RPMC [277], whereas the reduction of cell membrane content of sialic acid enhances the release from basophils and vice versa [276]. The reduction in histamine release due to an increase in membrane sialic acid content could be counteracted by raising the concentration of extracellular calcium [554]. The involvement of an interaction between the sialic acid and calcium channel has been suggested [276]. This may explain the effect SASP on IgE-mediated histamine release from human basophils mentioned above, if the drug is to bind to the sialic acid residues, and, hence, to reduce the number of effective sialic acid residues. Lastly, since calcium is responsible for both activation and inactivation of cell response as mentioned above, the effect of inactivation diminishes as a consequence of the absence of extracellular calcium, thus increases the histamine release. It is possible that one or more than one of the above explanations are involved in the observed effect of extracellular calcium on the inhibitory action of the drugs.

It is noteworthy that the possible close association of BSZ and SASP with sialic acid may be relevant to the treatment of inflammatory bowel disease, especially ulcerative colitis, by preventing the degradation of rectocolonic mucus. This is because in the rectocolonic mucosa of patients with ulcerative colitis, there is an increase in representation of sialated mucus and a loss of sulphated mucus [555-557]. Normal colonic mucus is heavily sulphated and this increases its resistance to degradation by bacterial enzymes, and any defect in mucus sulphation could be important in the pathogenesis of ulcerative colitis [557]. It is possible that both drugs may reduce the susceptibility of mucus degradation (due to the lack of sulphated content) by interacting with the sialated components, and thus render the protection.

The present results do not explain the tachyphylaxis exhibited by BSZ and SASP. In view of the suggested effects of 48/80 on cell membrane mentioned above, it is possible that preincubation with either BSZ or SASP predisposes the cells to cell membrane expansion or perturbation as a result of the drug insertion and incorporation into the cell membrane with surface active compounds [448], hence lowers the threshold for inducing histamine release. Therefore, the inhibitory effect of the drugs may be counteracted by the effects of preincubation. Alternately, it is possible that both drugs may act in a similar way to that proposed for disodium cromoglycate (DSCG) which is an inhibitor of histamine release from rat mast cells that exhibits marked tachyphylaxis. It has been proposed that DSCG may transiently increase the phosphorylation of an inhibitory protein of 78,000 Dalton which is thought to regulate
the termination of natural secretion, and the dephosphorylation of the same protein presents tachyphylaxis [558, 559].

In contrast to BSZ and SASP, CsA may exert its inhibition at more distal steps of signal transduction pathways. This view is supported by the findings that CsA significantly inhibited histamine release from RPMC and guinea pig RCMC induced by substances that circumvent receptor stimulation, that is, NaF, PLC, Dic8, and A23187. The inhibitory effect of CsA on PLC- and A23187-induced histamine release from both mast cell populations also shows differential responsiveness. However, in this case, it should be noted that apart from the different drug preincubation time, the difference in responsiveness might be due to the higher number of mixed cells in the RCMC samples than RPMC samples, i.e. the effect of CsA was “diluted” by other cell types. In other words, the actual amount of CsA available to the mast cells is less than that added, since some of it may be taken up by other cell types.

In some studies, CsA has been shown to inhibit 48/80-induced histamine release from RPMC [100, 101]. However, the concentrations of CsA showing inhibitory effect in these studies (800-80,000nM, with different ICso — in one study ICso~8,000nM [100], while in a different study ICso>50,000nM [101]) were higher than those used in the present study (10-1000 nM). They were also higher than those found in the blood of human transplant recipients (125-375 nM) [560] and patients with inflammatory bowel disease (50-500 nM) [84, 87, 561]. In the present study, at lower concentrations (10-1000 nM), CsA showed inhibition against histamine release induced by other selected secretagogues: NaF, PLC, Dic8 and A23187. It should also be noted that purified RPMC were used in the study of Dráberová (1990) [100], and longer CsA preincubation time (30 min) was used in the case of Ezeamuzie & Assem (1990) [101]. Moreover, contrasting results showing CsA to be ineffective in inhibiting 48/80-induced histamine release from RPMC have also been observed consistently in more recent years (unpublished observations from Dr. Assem’s laboratory). Therefore, given the putative modes of action of the selected secretagogues, the present results of inhibition by CsA on the PLC- and A23187-stimulated histamine release together with its effect on 48/80-induced histamine release observed by other investigators may reflect that CsA exerts its inhibitory effects — at least at lower concentrations or partly — by interfering with a signalling pathway different from that activated by 48/80 in RPMC. It may also reflect that 48/80-induced histamine release from RPMC may involve a G-protein different to that activated by NaF, and this 48/80-induced release may not require the activation of PLC or PKC, or the influx of extracellular calcium. This is compatible with the
mechanism of action of 48/80 discussed above. The insensitivity of 48/80 activation of RPMC towards the inhibitory effect of CsA may also be due to a switch of signal transduction pathway from a subpathway involving calcium-binding protein to CsA-insensitive subpathway involving G-protein $G_\varepsilon$ (which mediates the late events of exocytosis — see above). This switch of pathway may arise as a consequence of the enhanced affinity of G-protein $G_\varepsilon$ for its ligands by the effect of 48/80 in conjunction with the inhibition of the intracellular rise of calcium by the inhibitory effect of CsA on calcium uptake (see below). It has been proposed in RPMC that there is a communication between calcium-binding protein and G-protein $G_\varepsilon$, in that in the permeabilised cells the elevation of one of the two essential effectors for exocytotic reaction — calcium and guanine nucleotide — reduces the requirement for the other [223]. CsA has also been shown to be ineffective on Sub P-induced histamine release from human skin mast cells [102], a mast cell population which shows responses to various secretagogues similar to those of RPMC [124].

It has been shown that CsA does not affect the binding of Con A to phosphatidylserine-treated RPMC [100], or interfere with binding of formyl-methionyl-leucyl-phenylalanine to differentiated HL-60 cells [106]. The activity of CsA thus appears to be mediated by intracellular rather than membrane receptors. It has been suggested that CsA interferes with calcium signalling either by directly affecting the signal transduction pathway downstream of this second messenger involving cyclophilin, calmodulin, and calcineurin [298] as mentioned in Chapter 1; or by blocking the calcium channels and inhibiting the rise in intracellular calcium which is associated with cell activation [562, 563]. As alluded to above, PKC may have a modulatory role on extracellular calcium-depedent stimuli and it has also been suggested that CsA interferes with PKC [564]. It has been demonstrated in RPMC that CsA-mediated inhibition of histamine release is related to external calcium uptake, not activation-related early increase in the intracellular free calcium [100]. CsA has also been shown to be a potent anti-inflammatory agent acting on human skin mast cells, presumably by interacting with cyclophilin [102]. The present results of inhibitory effect by CsA on the actions of selected secretagogues agree with the above-mentioned observations. Except Dic8, all the selected secretagogues whose actions were inhibited by CsA induce extracellular calcium-depedent histamine release. Dic8 was shown to induce RPMC histamine release which was extracellular calcium-independent in the present system (see above), and CsA was shown to inhibit Dic8-induced histamine release in the presence or absence of extracellular calcium. The difference in inhibitory profile between the two
cases may reflect the possibility that CsA acts via different mechanisms depending on the presence or absence of extracellular calcium. Alternately, it may be possible that the inhibitory action of CsA requires the presence of calcium ions at a concentration to achieve a certain minimal concentration ratio of calcium to CsA; and in the absence of extracellular calcium, the intracellular free calcium mobilized from the internal stores is not sufficient to achieve the said ratio with high concentration of CsA. It has been demonstrated that binding of the cyclophilin-cyclosporin complex to calcineurin is calcium-dependent [95, 566]. Apart from the direct interference with PKC by CsA mentioned above, the inhibitory effect of CsA on Dic8-induced histamine release in the present study may also be due to an indirect effect on PKC secondary to an inhibition of the rise in cytosolic free calcium. This is because the translocation of PKC into cell membrane, a process that may be crucial for histamine release, is dependent on intracellular calcium concentration [326]. Based on the data that CsA was without inhibitory effect on superoxide formation induced by NaF and by stable guanine nucleotides (e.g. GTPrS) in a cell-free system from differentiated HL-60 cells, it has been suggested that CsA does not directly interfere with G-protein [106]. This finding may also be applied to the present system of mast cell histamine release. The relatively smaller inhibitory effect of CsA on histamine release induced by exogenous PLC suggests that CsA may interfere with part of the signals or processes generated by PLC. PLC is capable not only of generating a mixture of messengers, which are primarily inositol phosphates and 1,2-diacylglycerol, but also other signals such as the cascade of mediators arising from phospholipase A₃ [272, 302]. Admittedly, the PLC (lecithinase C) used in the present study is not phosphatidylinositol specific. Nevertheless, phosphatidylcholine (PC)-reactive PLC has been proposed to occur in several tissues, and PC may contribute to the formation of DAG [310, 567, 568]. It has been proposed that DAG could itself promote membrane fusion [569] and also the membrane phospholipids, which are the substrates of PLC, might themselves be inhibitory to fusion so that their depletion could render the membrane more susceptible to fusogenic influence [570, 571].

Compound 48/80-induced histamine release from RPMC and mixed serosal cells from peritoneal and thoracic cavities has been found to be inhibited by calmodulin antagonists, including PIC, TFP, W7, and others [265, 300]. However, disparate findings have also been obtained by other authors with calmodulin antagonists (CMA), causing no effect, weak inhibition or augmentation of histamine release by inducers, or even induction of histamine release [572]. The present results showed that over the
Chapter 6

ccentration range used by Gigi et al (1987) [300] CMA did not consistently inhibit histamine release from RPMC, and higher concentrations increased both spontaneous and 48/80-induced release. The disparity of findings and conclusions may be partly due to the unsuitability of certain CMA in the study of cellular responses such as 48/80-induced histamine release from RPMC, since there is the possibility of inter-switching between signal transduction subpathways as mentioned above. It may be also due to the differences in experimental methods. The histamine assay method probably played a minor role, and the present results took into account the interference by certain CMA with the fluorometric assay of histamine and were further confirmed by radioimmunoassay. The disparate effects shown by CMA on 48/80-induced histamine release from RPMC appear to be comparable with those shown by CsA, whose inhibitory action is thought to be associated with calmodulin inhibition.

To summarize, the present results obtained in the experimental conditions employed have demonstrated that selected secretagogues for mast cell histamine release showed differential effects on dispersed guinea pig RCMC and RPMC. BSZ and SASP inhibited RPMC histamine release induced by PAF, 48/80, Sub P, and NaF (much smaller inhibition by BSZ), but not that induced by PLC, A23187, and Dic8. The metabolites of BSZ and SASP (5-ASA, 4-ABA, and SP) in general showed little or no inhibitory effect. Given the putative modes of action of the selected secretagogues, it is possible that, in RPMC, the target(s) of BSZ and SASP intervention could be a G-protein(s) and/or the sialic acid residues on cell membrane. With guinea pig RCMC, the histamine release induced by PLC and A23187, the only two selected secretagogues to which RCMC responded, was not inhibited by BSZ, SASP, and their metabolites. In contrast, CsA attenuated histamine release by NaF, PLC, Dic8, and A23187 from RPMC, as well as that induced by PLC and A23187 from guinea pig RCMC. It is possible that the intervention by CsA occurs at a more distal step (relative to that of BSZ and SASP) of signalling pathways controlling histamine release; possibly at a step downstream of second messenger calcium or at calcium channels. In RPMC, the pathway(s) involved in the CsA intervention may be different from that activated by 48/80.
Figure 6.1. Rat peritoneal mast cell histamine release induced by Platelet Activating Factor (PAF, 10 min incubation) in full or calcium-free HEPES-buffered Tyrode solution (HBTS). Results are shown as mean±SEM of 2-14 samples from 1-4 experiments.
Figure 6.2. Rat peritoneal mast cell histamine release induced by (a) Compound 48/80 (10 min incubation) and (b) Substance P (10 min incubation) in full or calcium-free HEPES-buffered Tyrode solution (HBTS). Results are shown as mean of 3-6 samples from 1-2 experiments.
Figure 6.3. Rat peritoneal mast cell histamine release induced by (a) Sodium Fluoride (NaF; 15, 30, or 60 min incubation) and (b) 1,2-Dioctanoyl-sn-glycerol (Dic8, 60 min incubation) in full or calcium-free HEPES-buffered Tyrode solution (HBTS). Results are shown as mean of 3-19 samples from 1-6 experiments.
Figure 6.4. Histamine release from rat peritoneal cells (RPMC) and enzyme dispersed guinea pig rectocolonic mucosal mast cells (RCMC) induced by (a) Phospholipase C (PLC, 60 min incubation) in full or calcium-free HEPES-buffered Tyrode solution (HBTS) and (b) Ionophore A23187 (10 min incubation) in full HBTS. Results are shown as mean of 3-9 samples from 1-3 experiments.
Table 6.1. Histamine release induced by (a) Platelet Activating Factor (PAF), (b) Compound 48/80, and (c) Substance P, from enzyme-dispersed guinea pig rectocolonic mucosal mast cells. Results are shown as mean±sem of 3-12 samples from 1 to 4 experiments.

Concn.: concentration; HBTS: HEPES-buffered tyrode solution; Ca\(^{2+}\)F: calcium-free HBTS.
# Table 6.2. Histamine release induced by (a) Sodium Fluoride (NaF) and (b) 1,2-Dioctanoyl-sn-Glycerol (Dic8) from enzyme-dispersed guinea pig rectocolonic mucosal mast cells. Results are shown as mean ± sem of 3-12 samples from 1 to 4 experiments.

Concn.: concentration; HBTS: HEPES-buffered tyrode solution.
### Table 6.3. Effect of balsalazide (BSZ), sulphasalazine (SASP), and their metabolites 5-aminosalicylic acid (5-ASA), sulphapyridine (SP), and 4-aminobenzoyl-S-alanine (4-ABA) on platelet activating factor (PAF, 5.0 μM, 10 min incubation)-induced histamine release from rat peritoneal mast cells. Results are presented as mean±sem of 6 samples from two experiments. Negative value indicates potentiation. The induced histamine releases (%) in the absence of test drugs are in parentheses. Student’s t-test: * p<0.05, ** p<0.01, *** p<0.001 — significant difference from the induced histamine release in the absence of test drug; † p<0.05, ‡ p<0.001 — significant difference from 0 min drug preincubation.

<table>
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<tr>
<th>Drug</th>
<th>Conc. (μM)</th>
<th>0 min Drug Preincubation</th>
<th>5 min Drug Preincubation</th>
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</thead>
<tbody>
<tr>
<td>BSZ</td>
<td>20</td>
<td>10.2 ±2.9</td>
<td>- 1.8 ±3.0^</td>
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<tr>
<td></td>
<td>100</td>
<td>7.2 ±2.6</td>
<td>- 5.7 ±4.7†</td>
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<tr>
<td></td>
<td>500</td>
<td>26.7 ±3.8*</td>
<td>0.1 ±3.2‡‡‡</td>
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<tr>
<td>SASP</td>
<td>20</td>
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<td>- 0.5 ±2.4</td>
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<tr>
<td></td>
<td>100</td>
<td>- 1.1 ±3.2</td>
<td>- 1.1 ±6.4</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>49.2 ±3.6***</td>
<td>6.0 ±4.5‡‡‡</td>
</tr>
<tr>
<td>5-ASA</td>
<td>20</td>
<td>- 5.4 ±2.1</td>
<td>2.0 ±2.7</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>- 3.9 ±8.4</td>
<td>- 1.5 ±2.2</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>- 3.1 ±10.9</td>
<td>- 0.4 ±2.1</td>
</tr>
<tr>
<td>4-ABA</td>
<td>20</td>
<td>0.9 ±3.6</td>
<td>9.0 ±2.0**</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>3.9 ±8.4</td>
<td>3.7 ±4.5</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>- 2.4 ±9.0</td>
<td>- 2.3 ±4.3</td>
</tr>
<tr>
<td>SP</td>
<td>20</td>
<td>2.7 ±3.6</td>
<td>0.5 ±7.2</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>2.7 ±3.2</td>
<td>- 0.3 ±2.9</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>3.0 ±4.7</td>
<td>- 4.3 ±1.5*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(34.8 ±3.9)</td>
<td>(36.2 ±2.3)</td>
</tr>
</tbody>
</table>

Concn.: concentration.
## Table 6.4. Effect of balsalazide (BSZ), sulphasalazine (SASP), and their metabolites 5-aminosalicylic acid (5-ASA), sulphapyridine (SP), and 4-aminobenzoyl-B-alanine (4-ABA) on compound 48/80 (0.3 µg/ml, 10 min incubation)-induced histamine release from rat peritoneal mast cells. Results are shown as mean ± sem of 6 samples from 2 experiments except † (5 min Drug Preincubation) where results are mean ± sem of triplicate samples from single experiments carried out simultaneously with one of the experiments of 0 min drug preincubation. Negative value indicates potentiation. The induced histamine releases (%) in the absence of test drugs are in parentheses. Student’s t-test: *p<0.05, **p<0.01, ***p<0.001—significant difference from the induced histamine release in the absence of test drug; x p<0.05, xx p<0.01, xxx p<0.001—significant difference from HBTS; † p<0.05, †† p<0.01, ††† p<0.001—significant difference from 0 min drug preincubation.

Concn.: concentration; HBTS: HEPES-buffered tyrode solution.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concn. (µM)</th>
<th>0 min Drug Preincubation</th>
<th>5 min Drug Preincubation †</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>HBTS (µM)</td>
<td>Ca²⁺-Free HBTS (µM)</td>
</tr>
<tr>
<td>BSZ</td>
<td>20</td>
<td>1.6 ± 0.8</td>
<td>3.4 ± 0.5*</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>8.0 ± 0.7***</td>
<td>4.2 ± 0.7‡‡</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>60.1 ± 3.4***</td>
<td>26.1 ± 4.4‡‡‡</td>
</tr>
<tr>
<td>SASP</td>
<td>20</td>
<td>2.5 ± 0.6**</td>
<td>4.0 ± 0.7‡‡</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>22.6 ± 5.5**</td>
<td>12.0 ± 2.1***</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>111.1 ± 2.5***</td>
<td>99.7 ± 0.9‡‡‡</td>
</tr>
<tr>
<td>5-ASA</td>
<td>20</td>
<td>- 0.6 ± 1.5</td>
<td>- 2.2 ± 1.7</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>- 2.8 ± 1.0*</td>
<td>- 1.9 ± 1.7</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>- 0.3 ± 1.2</td>
<td>- 0.1 ± 2.7</td>
</tr>
<tr>
<td>4-ABA</td>
<td>20</td>
<td>- 0.8 ± 1.8</td>
<td>- 0.8 ± 2.5</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>- 0.3 ± 0.5</td>
<td>3.8 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>7.7 ± 1.5***</td>
<td>9.2 ± 0.7***</td>
</tr>
<tr>
<td>SP</td>
<td>20</td>
<td>- 0.9 ± 0.9</td>
<td>3.6 ± 3.3</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>- 1.9 ± 2.1</td>
<td>5.7 ± 3.2</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>0.6 ± 2.0</td>
<td>5.6 ± 3.5</td>
</tr>
</tbody>
</table>

(63.4 ± 2.5) (65.9 ± 5.4) (62.3 ± 0.5) (60.7 ± 0.7)
Chapter 6

Table 6.5. Effect of balsalazide (BSZ), sulphasalazine (SASP), and their metabolites 5-aminosalicylic acid (5-ASA), sulphapyridine (SP), and 4-aminobenzoyl-B-alanine (4-ABA) on histamine release from rat peritoneal mast cells induced by substance P (20 μM, 10 min incubation) and NaF (20 mM, 15 min incubation). Results are shown as mean ±sem of 6-12 samples from 2 to 4 experiments. Negative value indicates potentiation. The induced histamine releases (%) in the absence of test drugs are in parentheses. Student’s t-test: * p < 0.05, ** p < 0.01, *** p < 0.001 — significant difference from the induced histamine release in the absence of test drug; * p < 0.05, ** p < 0.01, *** p < 0.001 — significant difference from HBTS; * p < 0.05 — significant difference from 0 min drug preincubation.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Conc. (μM)</th>
<th>HBTS</th>
<th>Ca²⁺-Free HBTS</th>
<th>5 min Drug Preincubation</th>
<th>0 min Drug Preincubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSZ</td>
<td>20</td>
<td>25.9 ± 0.8**</td>
<td>- 1.3 ± 0.7‡x</td>
<td>- 1.7 ± 4.9</td>
<td>- - - -</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>34.6 ± 4.1***</td>
<td>- 4.2 ± 1.1‡xx</td>
<td>- 0.3 ± 4.7</td>
<td>- - - -</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>43.0 ± 4.3***</td>
<td>- 4.1 ± 0.8‡‡x</td>
<td>1.4 ± 2.4‡+</td>
<td>11.5 ± 2.6**</td>
</tr>
<tr>
<td>SASP</td>
<td>20</td>
<td>- 9.8 ± 12.5</td>
<td>- 2.4 ± 0.8*</td>
<td>3.8 ± 2.3</td>
<td>- - - -</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>28.5 ± 2.9***</td>
<td>- 2.9 ± 0.6‡‡x</td>
<td>5.9 ± 3.4</td>
<td>- - - -</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>9.1 ± 4.5</td>
<td>- 6.5 ± 1.1‡‡x</td>
<td>23.8 ± 2.3‡‡**</td>
<td>11.2 ± 6.0</td>
</tr>
<tr>
<td>5-ASA</td>
<td>20</td>
<td>- 9.0 ± 5.8</td>
<td>- 0.7 ± 1.5</td>
<td>- 3.0 ± 2.4</td>
<td>- - - -</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>- 8.0 ± 15.1</td>
<td>0.1 ± 1.9</td>
<td>7.2 ± 5.7</td>
<td>- - - -</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>- 18.3 ± 34.4</td>
<td>- 4.3 ± 2.2</td>
<td>13.7 ± 2.7‡**</td>
<td>0.7 ± 4.0</td>
</tr>
<tr>
<td>4-ABA</td>
<td>20</td>
<td>- 33.7 ± 30.0</td>
<td>0.6 ± 2.2</td>
<td>- 7.2 ± 3.3</td>
<td>- - - -</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>- 29.3 ± 10.6*</td>
<td>- 2.3 ± 0.9‡</td>
<td>- 0.3 ± 4.6</td>
<td>- - - -</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>- 11.0 ± 2.6</td>
<td>0.2 ± 1.0xx</td>
<td>- 1.0 ± 4.3</td>
<td>- 8.0 ± 5.1</td>
</tr>
<tr>
<td>SP</td>
<td>20</td>
<td>- 25.3 ± 5.1**</td>
<td>0.4 ± 1.2xxx</td>
<td>3.2 ± 3.2</td>
<td>- - - -</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>- 9.5 ± 6.1</td>
<td>0.8 ± 1.0</td>
<td>6.2 ± 2.8</td>
<td>- - - -</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>- 7.0 ± 12.4</td>
<td>9.8 ± 4.1*</td>
<td>15.1 ± 3.9*</td>
<td>11.8 ± 2.6**</td>
</tr>
<tr>
<td></td>
<td>(22.0 ± 1.6)</td>
<td>(56.1 ± 2.7)</td>
<td>(32.2 ± 2.4)</td>
<td>(40.0 ± 3.2)</td>
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</tr>
</tbody>
</table>

Concn.: concentration; HBTS: HEPES-buffered tyrode solution.
### Inhibition of Histamine Release (%)

<table>
<thead>
<tr>
<th>Drug</th>
<th>Conc. (µM)</th>
<th>Dic8 (20 µM)</th>
<th>A23187 (10 µM)</th>
<th>PLC (20 µg/ml = 0.076 U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSZ</td>
<td>20</td>
<td>- 4.2 ±3.1†</td>
<td>- 2.2 ±1.3</td>
<td>- 5.1 ±2.4†</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>- 6.8 ±1.7**</td>
<td>- 6.7 ±1.8**</td>
<td>- 5.1 ±4.2</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>- 10.5 ±1.1***</td>
<td>- 3.7 ±1.6</td>
<td>- 33.8 ±4.2**†</td>
</tr>
<tr>
<td>SAP</td>
<td>20</td>
<td>- 5.4 ±0.9**†</td>
<td>- 9.9 ±4.3*</td>
<td>- 17.6 ±4.0†</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>- 16.4 ±3.9**</td>
<td>- 10.5 ±3.6*</td>
<td>- 26.3 ±3.1***</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>- 9.2 ±2.6**</td>
<td>- 1.4 ±2.9</td>
<td>- 60.9 ±3.0***†</td>
</tr>
<tr>
<td>5-ASA</td>
<td>20</td>
<td>- 4.7 ±0.9**†</td>
<td>- 1.3 ±1.6</td>
<td>- 5.7 ±7.4†</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>- 6.0 ±2.6*</td>
<td>- 5.5 ±8.3</td>
<td>- 7.0 ±4.7</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>- 8.4 ±3.2*</td>
<td>- 2.0 ±1.5</td>
<td>- 31.0 ±6.5*†</td>
</tr>
<tr>
<td>4-ABA</td>
<td>20</td>
<td>- 10.9 ±1.7**†</td>
<td>- 2.8 ±3.4</td>
<td>- 14.5 ±2.4†</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>- 5.3 ±4.1</td>
<td>2.3 ±3.6</td>
<td>- 6.5 ±4.6</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>- 3.4 ±2.6</td>
<td>15.8 ±8.9</td>
<td>- 23.2 ±3.9**†</td>
</tr>
<tr>
<td>SP</td>
<td>20</td>
<td>- 18.1 ±4.2**†</td>
<td>- 1.9 ±2.2</td>
<td>- 15.2 ±0.8**†</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>- 7.3 ±2.3**</td>
<td>- 2.2 ±2.7</td>
<td>- 5.4 ±3.4</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>- 10.5 ±2.7**</td>
<td>2.9 ±5.3</td>
<td>- 22.3 ±1.0**†</td>
</tr>
</tbody>
</table>

(52.7 ±0.3) (66.0 ±3.0) (41.7 ±3.5)

**Table 6.6.** Effect of balsalazide (BSZ), sulphasalazine (SASP), and their metabolites 5-aminosalicylic acid (5-ASA), sulphapyridine (SP), and 4-aminobenzoyl-B-alanine (4-ABA) on histamine release from rat peritoneal mast cells induced by 1,2-dioctanoyl-sn-glycerol (Dic8, 20 µM, 60 min incubation), ionophore A23187 (10 µM, 10 min incubation), or phospholipase C (PLC, 20 µg/ml = 0.076 U/ml, 60 min incubation). Test drugs were preincubated with cells for 5 min prior to the addition of inducer. Results are shown as mean±sem of 6-9 samples from 2 to 3 experiments except † where results are mean±sem of triplicate samples from single experiment. Negative value indicates potentiation. The induced histamine releases (%) in the absence of test drugs are in parentheses. Student’s t-test: * p<0.05, ** p<0.01, *** p<0.001.

Concn.: concentration.
# Chapter 6

## Inhibition of Histamine Release (%)

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration (µM)</th>
<th>A23187 (10 µM)</th>
<th>PLC (20 µg/ml) 0.076 U/ml</th>
<th>PLC (40 µg/ml) 0.152 U/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSZ</td>
<td>20</td>
<td>- 4.2 ± 3.3</td>
<td>2.1 ± 1.8</td>
<td>2.3 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>3.7 ± 3.1</td>
<td>5.7 ± 1.7*</td>
<td>0.2 ± 2.5</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>1.9 ± 2.0</td>
<td>8.3 ± 2.7*</td>
<td>3.0 ± 2.5</td>
</tr>
<tr>
<td>SASP</td>
<td>20</td>
<td>0.0 ± 2.4</td>
<td>7.9 ± 2.0**</td>
<td>- 5.1 ± 1.3‡‡‡</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0.7 ± 3.2</td>
<td>10.7 ± 2.0***</td>
<td>- 0.7 ± 2.1‡‡</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>9.2 ± 2.7**</td>
<td>11.1 ± 2.9**</td>
<td>2.3 ± 0.8*</td>
</tr>
<tr>
<td>5-ASA</td>
<td>20</td>
<td>- 5.3 ± 3.2</td>
<td>- 2.7 ± 1.7</td>
<td>- 9.1 ± 0.6‡‡‡</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>- 7.5 ± 2.1*</td>
<td>- 10.4 ± 3.1**</td>
<td>- 13.5 ± 2.6***</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>- 3.8 ± 3.2</td>
<td>- 24.2 ± 2.1***</td>
<td>- 26.0 ± 2.7***</td>
</tr>
<tr>
<td>4-ABA</td>
<td>20</td>
<td>- 13.4 ± 1.9***</td>
<td>2.2 ± 1.5</td>
<td>- 5.6 ± 1.6‡‡‡</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>- 7.2 ± 1.3*</td>
<td>- 2.8 ± 1.1</td>
<td>- 7.0 ± 2.6*</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>- 0.4 ± 3.7</td>
<td>- 4.5 ± 2.7</td>
<td>- 15.6 ± 4.2‡‡‡</td>
</tr>
<tr>
<td>SP</td>
<td>20</td>
<td>- 4.1 ± 4.9</td>
<td>- 0.5 ± 3.7</td>
<td>- 8.7 ± 1.1***</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>- 7.9 ± 3.0*</td>
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<td>- 10.2 ± 2.4‡*</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>- 7.2 ± 5.4**</td>
<td>- 0.9 ± 6.6</td>
<td>- 10.1 ± 1.3***</td>
</tr>
<tr>
<td></td>
<td>(24.4 ± 2.2)</td>
<td>(35.2 ± 3.9)</td>
<td>(45.8 ± 3.2)</td>
<td></td>
</tr>
</tbody>
</table>

Table 6.7. Effect of balsalazide (BSZ), sulphasalazine (SASP), and their metabolites 5-aminosalicylic acid (5-ASA), sulphapyridine (SP), and 4-aminobenzoyl-S-alanine (4-ABA) on histamine release from enzyme-dispersed guinea pig rectocolonic mucosal mast cells induced by ionophore A23187 (10 µM, 30 min incubation) and phospholipase C (PLC, 20 µg/ml = 0.076 U/ml and 40 µg/ml = 0.152 U/ml, 60 min incubation). The test drugs were preincubated with cells for 5 min prior to the addition of inducers. Results are shown as mean ± sem of 6-9 samples from 2 to 3 experiments. Negative value indicates potentiation. The induced histamine releases (%) in the absence of test drugs are in parentheses. Student's t-test: * p<0.05, ** p<0.01, *** p<0.001 — significant difference from the induced histamine release in the absence of test drug; † p<0.05, ‡‡ p<0.01, \‡‡‡ p<0.001 — significant difference from PLC (20 µg/ml).

Concn.: concentration.
Figure 6.5. Effect of cyclosporin A (CsA) on histamine release from rat peritoneal mast cells induced by sodium fluoride (NaF, 15 or 20 mM, 15 min incubation) or 1,2-dioctanoylsn-glycerol (Dic8, 20 μM, 60 min incubation). Results are shown as mean±SEM of 6-9 samples from 2-3 experiments except results of the calcium-free experiment with Dic8, which are mean±SEM of triplicate samples from a single experiment. The uninhibited histamine release (%) induced by NaF (15 mM) = 33.8±1.8, NaF (20 mM) = 46.3±1.3, Dic8 (20 μM) in the presence of Ca²⁺ = 50.1±3.1, and Dic8 (20 μM) in the absence of Ca²⁺ = 15.2±0.2. Student's t-test: *p<0.05, ***p<0.001.
Figure 6.6. Effect of cyclosporin A (CsA) on histamine release from rat peritoneal mast cells (RPMC) and enzyme-dispersed guinea pig rectocolonic mucosal mast cells (RCMC) induced by phospholipase C (PLC, 20 μg/ml = 0.076 U/ml, 60 min incubation). CsA was preincubated with cells for 5 min prior to the addition of inducer. Results are shown as mean±sem of 6-9 samples from 2-3 experiments. Uninhibited histamine release (%) induced by PLC from RPMC = 64.2±1.0, that from RCMC = 32.5±7.1. Student's t-test: **p < 0.01, ***p < 0.001.
Figure 6.7. Effect of cyclosporin A (CsA) on histamine release from rat peritoneal mast cells (RPMC) and enzyme-dispersed guinea pig mucosal mast cells (RCMC) induced by ionophore A23187 (10 μM, 30 min incubation). CsA was preincubated with cells for 30 min (RPMC) or 5 min (RCMC) prior to addition of inducer. Results are shown as mean±sem of 6-9 samples from 2-3 experiments. Uninhibited histamine release (%) induced by A23187 for RPMC = 65.6±2.3 and that for RCMC = 27.6±4.6. Student's t-test: **p<0.01, ***p<0.001.
## Table 6.8

Effect of calmodulin antagonists on compound 48/80 (0.2 or 0.3 μg/ml, 10 min incubation)-induced histamine release from rat peritoneal mast cells. Results are presented as mean ± sem of 3 observations except \(^b\) (n = 9). Drug preincubation time prior to the addition and incubation of 48/40 (0.3 μg/ml for experiment 1, and 0.2 μg/ml for experiments 2 and 3) was 30 min (expt 1 & 2) or 10 min (expt.3). Student’s *t*-test: * \(p<0.05\), ** \(p<0.01\), *** \(p<0.001\) — significant difference from *a* 48/80 control.

<table>
<thead>
<tr>
<th>Expt</th>
<th>Compd</th>
<th>0(^a)</th>
<th>10(^{-8})</th>
<th>5x10(^{-8})</th>
<th>10(^{-5})</th>
<th>5x10(^{-5})</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PIC</td>
<td>68.0 ±0.4</td>
<td>69.2 ±0.6</td>
<td>66.1 ±1.0</td>
<td>61.3 ±1.5(^*)</td>
<td>-----</td>
</tr>
<tr>
<td></td>
<td>TFP</td>
<td>68.0 ±0.4</td>
<td>68.3 ±0.0</td>
<td>68.6 ±0.3</td>
<td>51.8 ±0.2(***)</td>
<td>-----</td>
</tr>
<tr>
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<td>W7</td>
<td>69.6 ±0.1</td>
<td>69.7 ±0.3</td>
<td>69.7 ±0.6</td>
<td>72.3 ±0.5(^**)</td>
<td>-----</td>
</tr>
<tr>
<td></td>
<td>N-4ABSA</td>
<td>69.6 ±0.1</td>
<td>68.9 ±0.7</td>
<td>66.8 ±0.3</td>
<td>67.9 ±1.0</td>
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</tr>
<tr>
<td>2</td>
<td>PIC</td>
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<td>51.3 ±0.2(^*)</td>
<td>48.8 ±0.7</td>
<td>44.6 ±1.6</td>
<td>-----</td>
</tr>
<tr>
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<td>TFP</td>
<td>45.5 ±1.3</td>
<td>57.1 ±1.1(^**)</td>
<td>43.6 ±0.9</td>
<td>66.9 ±2.0(***)</td>
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</tr>
<tr>
<td></td>
<td>W7</td>
<td>52.6 ±1.6</td>
<td>52.8 ±2.9</td>
<td>39.5 ±1.6(^**)</td>
<td>81.8 ±1.4(***)</td>
<td>-----</td>
</tr>
<tr>
<td></td>
<td>N-4ABSA</td>
<td>52.6 ±1.6</td>
<td>42.8 ±1.1(^**)</td>
<td>46.6 ±3.6</td>
<td>51.5 ±2.7</td>
<td>-----</td>
</tr>
<tr>
<td>3</td>
<td>PIC</td>
<td>42.8 ±1.3(^b)</td>
<td>-----</td>
<td>-----</td>
<td>46.9 ±2.3</td>
<td>49.8 ±2.6(^*)</td>
</tr>
<tr>
<td></td>
<td>TFP</td>
<td>44.6 ±1.0</td>
<td>-----</td>
<td>-----</td>
<td>49.7 ±0.5(^*)</td>
<td>53.0 ±1.4(**)</td>
</tr>
</tbody>
</table>

Expt: experiment; Compd: compound; PIC: picumast; TFP: trifluoperazine; W7: N-(6-aminohexyl)-5-chloro-1-naphthalene-sulphonamide; N-4ABSA: N-(4-aminobutyl)-5-chloro-1-naphthalene-sulphonamide.
CHAPTER 7

EFFECT OF BALSALAZIDE AND SULPHASALAZINE ON MEMBRANE STABILIZATION

7.1. INTRODUCTION

The most fundamental task of the biological membrane is to separate physical properties and chemical composition of a living organism from its environment. Membranes also form barriers between various intracellular compartments with their specialized chemical contents. While membranes have also been bestowed with many functions, such as carrier-mediated transport, enzyme activity, and ligand binding to surface receptors and their functional coupling to effector systems, their performance in this isolatory respect remains crucial for the living cell. There are occasions, such as during the inflammatory process, when the efficiency of membrane’s isolatory capacity may be overwhelmed, resulting in eventual cell death. Drug compounds that stabilize the cell membrane could enhance cellular resistance to membrane perturbation and preserve cell membrane integrity, hence cell survival, as well as modulate membrane functions.

In the preceding chapters, it has been shown that sulphasalazine (SASP) and balsalazide (BSZ) inhibit histamine release from both rat peritoneal mast cells and \textit{in situ} ethanol-perfused rat rectocolon; and in the study of \textit{in situ} perfused rat rectocolon, both drugs show cytoprotection and anti-inflammatory activity. Moreover, both drugs also reduce ethanol-induced gastric damage in rats, suggesting a potential for peptic ulcer treatment [76, 573] apart from being potent drugs for ulcerative colitis therapy.

Due to their ready availability and the ease of handling, erythrocytes are widely used in the studies of membrane structure and function, including the pharmacological property of membrane-stabilizing activity by different drugs. Moreover, the mature mammalian erythrocyte is devoid of nucleus, mitochondria, and all other subcellular structures, hence, any surface active effects of a drug will mainly be due to its action of the plasma membrane.

The anti-allergic drug — disodium cromoglycate [443], many anti-inflammatory drugs [444-446], or some tranquilizers, antihistamines, and anaesthetics [447-449] have
been shown to stabilize the membrane of erythrocytes. In particular, the inhibitory effect of non-steroid anti-inflammatory drugs on heat-induced haemolysis is potent, and a relationship between their membrane stabilizing action and anti-inflammatory activity has been discussed [445, 446, 450].

In this chapter, the hypothesis that SASP and BSZ might exert their inhibitory and protective action through enhancing cellular resistance to inducing and damaging agents by stabilizing cell membrane and maintaining its integrity was tested. The effects of both drugs and their respective metabolites on haemolysis of human and rat erythrocytes induced by membrane labilizers such as heat, the bee-venom peptide melittin, bile acid (deoxycholic acid, DA), and the enzyme (phospholipase C, PLC) was studied. The relative role of plasma membrane lipids and proteins in the erythrocyte membrane stabilizing action of these drugs was assessed by modifying the membrane with 2,4,6-trinitrobenzene sulphonic acid (TNBS), an impermeable reagent which denatures protein; with PLC which interacts directly with the lipid components of the membrane; and with DA which alters membrane spatial organization. The metabolites of BSZ and SASP, as discussed in chapter 1, are 5-aminosalicylic acid (5-ASA, active moiety common to SASP and BSZ), sulphapyridine (SP, carrier moiety for SASP), and 4-aminobenzoyl-L-alanine (4-ABA, carrier moiety for BSZ). Disodium cromoglycate (DSCG), a known "mast cell stabilizer" and a non-steroid anti-inflammatory drug phenylbutazone (PBZ), both with the erythrocyte membrane stabilizing effect, were used as a reference [443, 446].

The effect of cyclosporin A, an immunosuppressant which has also been used in treating inflammatory bowel disease [83, 84] and two selected calmodulin antagonists, picumast (PIC) and trifluoperazine (TFP), were also assessed for comparison. Glutathione, which may rearrange disulphide bonds in membrane protein via its participation in a disulphide interchange reaction, was also tested for its effect.

7.2. MATERIALS AND METHODS

The methods used were based on that of Mizushima et al (1970) [446], and all materials and experimental procedures are outlined in the appropriate sections in chapter 2.
Chapter 7

7.3. RESULTS

7.3.1. Heat-induced haemolysis

7.3.1.1. Effects of drugs on heat-induced lysis of unmodified rat erythrocytes

Both references, DSCG and PBZ, showed a dose-dependent inhibitory effect on heat-induced haemolysis of rat erythrocytes within the concentration range of 20-500 µM in the case of DSCG, and 100-500 µM in the case of PBZ (Fig. 7.1). The magnitudes of the effect were comparable to those of the published literature [443, 446]. The reduced form of glutathione (GSH) did not show a significant effect on this heat induced haemolysis (Fig. 7.1).

In general, BSZ and SASP also showed a dose-dependent inhibitory effect, with the metabolites showing very much smaller effect at the higher dose (500 µM), except SP which showed a small inhibitory effect which was inversely proportional to its concentration (Fig. 7.2). At 500 µM, the inhibitory effect of BSZ and SASP was superior to that of DSCG and PBZ (Fig. 7.1 and Fig. 7.2).

As cyclosporin A (CsA) was initially dissolved in ethanol and then diluted with saline to the required concentrations, the effect of vehicle at corresponding concentrations on heat-induced haemolysis of rat erythrocytes was tested and compared to saline (Fig. 7.3.a). In order to eliminate the vehicle effect, the result for each concentration of CsA was expressed as the % of the corresponding drug-free control containing the same amount of ethanol. CsA showed dose-dependent potentiation of heat-induced haemolysis of rat erythrocytes within the concentration range of 0.01-100 µM (Fig. 7.3.b).

Based on the same rationale as in the case of CsA, the effect of PIC on heat-induced haemolysis was assessed in a similar manner, since it was initially dissolved in dimethyl sulphoxide (DMSO). The effect of DMSO is shown in Table 7.1.a. Both calmodulin antagonists (5-50 µM), PIC and TFP, showed potentiation of heat-induced haemolysis of rat erythrocytes (Table 7.1.b).

Deoxycholic acid (DA, 100 µM) augmented heat-induced rat erythrocyte lysis (Table 7.2.a). BSZ, SASP, and their metabolites were tested for their effects on this augmentation. The drug and DA (100 µM) were added concurrently to the washed erythrocyte suspension followed immediately by heating. The inhibitory effects of BSZ at 100 µM and 4-ABA at 500 µM were reduced by the inclusion of DA, while the rest was not affected (Fig. 7.4).
7.3.1.2. Heat-induced haemolysis of modified rat erythrocytes

Three different agents were used for the modification of the rat erythrocyte membrane. They were TNBS (4 mM), phospholipase C (PLC, 2 µg/ml), and deoxycholic acid (DA, 100 µM).

7.3.1.2.1. Effect of modification procedure on erythrocytes

A small degree of haemolysis was caused during the incubation with each of the modifying agents. There was about 9-10% of haemolysis being observed in the case of TNBS and PLC, and 3% in the case of DA, while negligible haemolysis was observed in the control tubes without the agent. Figure 7.5 shows how the procedure of modifying erythrocytes with DA (100 µM) affects heat-induced haemolysis. Two sets of washed erythrocyte suspension were incubated with DA at 37 °C for 30 min. At the end of incubation, one set was heated immediately for 20 min, while the other set was cooled, spun, resuspended in the original supernatant, and then heated. It was apparent that the process of resuspension caused the erythrocytes to be more thermolabile. Low concentrations of DA seemed to protect the erythrocytes, a phenomenon which is shared by many lipid-soluble and surface-active substances at very low concentrations (e.g. alcohol, some tranquilizers, and anaesthetics). This could be due to the ability of surface active substances to expand biological membranes, as reviewed in detail by Seeman (1972) [448]. Overexpansion of the cell membrane by high concentrations of surface active substance brings about cell disruption, whereas at sublytic concentrations, the expansion may give rise to thermodynamically more favourable configurations of membrane components.

7.3.1.2.2. Effect of drugs on heat-induced haemolysis of modified rat erythrocytes

The stabilizing effect of BSZ, SASP, 5-ASA, 4-ABA, and SP against heat-induced haemolysis of modified rat erythrocytes is summarized in Figure 7.6. The stabilizing effect of BSZ and SASP was reduced more by TNBS than by DA or PLC pretreatment, especially in the case of BSZ where the stabilizing effect was almost completely abolished by TNBS pretreatment. In general, except in the case of 4-ABA where the stabilizing effect against heat was reversed by DA pretreatment, the much smaller stabilizing effect of metabolites 5-ASA, 4-ABA, and SP was not significantly affected by the modification of erythrocytes, though there was a trend of slightly greater effect by
TNBS modification than other modifications on reducing the stabilizing effect of metabolites.

The stabilizing effect of DSCG on heat-induced haemolysis was not affected by the modification with PLC and DA (Fig. 7.6).

7.3.2. Haemolysis induced by other membrane labilizers

7.3.2.1. Pilot tests for determining experimental conditions using other labilizers

The experimental conditions that produced about 150±10% haemolysis were chosen for the experiments on the effect of test compounds. The results for PLC, melittin, and DA are summarized in Fig. 7.7.a and 7.7.b, and Table 7.2.b respectively. PLC at 2 μg/ml with 30 min incubation at 37 °C, melittin at 7.5 μg/ml with 30 min incubation at 37 °C, and DA at 500 μM with 1 hour incubation at 37 °C were chosen for testing the effect of drugs.

7.3.2.2. Effect of BSZ and SASP on melittin-induced human erythrocyte lysis

As shown in Fig. 7.8, within the concentration range of 20-500 μM, both BSZ and SASP showed dose-dependent inhibitory effects on melittin-induced haemolysis of human erythrocytes. At 20 μM, SASP showed greater inhibition than BSZ, but at the two higher concentrations, the two drugs were equi-potent.

7.3.2.3. Effect of drugs on PLC-induced rat erythrocyte lysis

The results are summarized in Fig. 7.1 and 7.9. BSZ, 5-ASA, 4-ABA, and SP were equi-active in general, whereas SASP showed a smaller effect which was comparable with DSCG. 4-ABA showed an inhibitory effect which was inversely proportional to its dose within the dose range tested. GSH caused a dose-dependent augmentation of PLC-induced haemolysis.

7.3.2.4. Effect of drugs on DA-induced rat erythrocyte lysis

The results are summarized in Fig. 7.1 and 7.10. All the compounds tested showed dose-dependent inhibitory effects against DA-induced haemolysis of rat erythrocytes within the dose range of 20-500 μM, except 5-ASA which showed an
inhibitory effect which was inversely proportional to its dose. SASP produced a greater inhibitory effect than the others which were generally equi-active, though among the others, GSH was slightly more active, and 5-ASA was slightly less active.

7.3.3. Effect of SASP and BSZ on heat-induced albumin denaturation

Bovine serum albumin solution (1% w/v) was added to BSZ or SASP (both at 500 μM) and left standing at room temperature for different time periods (0 to 60 min), then followed by heating at 80 °C for 3 min (induction of denaturation). The turbidity of the solution was measured spectrophotometrically at 660 nM. Fig. 7.11 showed that overall SASP produced better protection against heat-induced denaturation than BSZ. In both cases, the protective effect diminished rapidly from a maximum effect which occurred when the drug was added at the commencement of heating.

7.4. DISCUSSION

The present results demonstrated the membrane stabilizing effect of BSZ and SASP with all the labilizers tested, while their metabolites showed stabilizing effects on PLC- and DA-induced haemolysis only. The modification of rat erythrocyte membrane by protein denaturation with TNBS decreased the protective effect of BSZ and SASP on heat-induced haemolysis, indicating that the cell membrane stabilizing action of the drugs (especially that of BSZ) may be affected by protein modification, hence the site of action could be protein components of the membrane. Since membrane stabilization may reduce inflammatory manifestations [446] and may also render cytoprotection, it may account partly for the beneficial actions of SASP and BSZ in inflammatory bowel disease, implicating that both drugs, as parent compounds, are not merely pro-drugs for delivering their active components in the colon.

Apart from demonstrating some similarities in the mechanism of action between CsA and the calmodulin antagonists, PIC and TFP, the potentiation of heat-induced haemolysis shown by CsA, PIC, and TFP also shows that the mechanism of action of these three compounds are fundamentally different from that of both BSZ and SASP. CsA is a lipophilic, cyclic undecapeptide which penetrates membrane and appears to exert its immunosuppressive activity via intracellular rather than cell membrane receptors [82]. PIC and TFP also seem to exert their activities via intracellular components, as
calmodulin is an intracellular calcium receptor which has been implicated in histamine release from mast cells [291, 299, 300]. It has been shown that drugs exerting strong haemolytic action tend to penetrate the membrane much more effectively (i.e. more lipophilic, such as CsA) than those with less activity [574].

As mentioned in chapter 1, GSH is biologically active and serves several independent functions, such as, among others, stabilizing protein structure by thiol/disulphide exchange; regulating enzyme activities as cofactor, and playing a critical role in detoxification and antioxidation processes of the cell. The augmentation by GSH in PLC-induced haemolysis of rat erythrocytes could be due to GSH being a cofactor for the enzyme. The inhibitory effect of GSH on DA-induced haemolysis, could be due to it being an antioxidant that prevents lipid peroxidation and/or being a stabilizer that prevents the alteration of membrane spatial organization by detergents.

A drug molecule may exert its action by targeting several distinct membrane properties. These include fluidity which could be affected in either direction; permeability of membrane; spatial organization of membrane which may be altered drastically by agents that are exemplified by detergents; fusion of membrane; and charge distribution and density of membrane. Cell membrane integrity is essential for cellular functions such as carrier-mediated transport, enzyme activity, ligand binding to surface receptors and their functional coupling to effector system. For example, it has been shown that membrane fluidity altered by lipid-modification affected the efficiency of β-adrenergic action in the isolated prostatic epithelial cells [575].

Apart from being a general membrane model for studying pharmacological activity, stabilizing erythrocyte membrane and maintaining its integrity in its own right may have relevance to the pathophysiology of oxidative injury. Erythrocytes are rich in endogenous anti-oxidant enzymes, and they may metabolize reactive oxygen metabolites such as superoxide radical and hydrogen peroxide which can cross the erythrocyte membrane rapidly by diffusion via membrane anion channels in the case of former and by free diffusion, the latter [451]. It has been shown that erythrocytes scavenge extracellular hydrogen peroxide and inhibit the formation of hypochlorous acid and hydroxyl radical [452]. Moreover, it has been demonstrated that erythrocytes, either in intact or lysed form, can protect isolated lungs and pulmonary artery endothelial cells [453], and isolated heart [432] against endogenous or exogenous hydrogen peroxide-mediated injury. However, intact cells are preferred, as haemolysis releases haemoprotein-associated oxidant or haemoglobin which will interact with hydrogen peroxide or lipid hydroperoxides to yield further haemoprotein-associated oxidant that
Chapter 7

is capable of peroxidizing a variety of polyunsaturated fatty acids, phospholipid, and cellular membrane [454-457]. As active episodes of intestinal mucosal inflammation and injury coincide with interstitial haemorrhage [458] and lipid peroxidation [459], it has been suggested that haemoglobin-catalysed reactions may play an important role in mediating and/or exacerbating inflammatory tissue injury such as that of inflammatory bowel disease [58]. The presence of haemoglobin indeed has been proposed to mediate and/or exacerbate oxidative injury in tissues such as central nervous system [460], retina [461], kidney [462], and synovial cavity [463]. It is that erythrocytes are protective, but when lysed, the released haemoglobin may exacerbate oxidative injury.
Figure 7.1. Effect of disodium cromoglycate (DSCG), phenylbutazone (PBZ), and reduced glutathione (GSH) on heat (20 min incubation)-, phospholipase C (PLC, 2 μg/ml, 60 min incubation)-, and deoxycholic acid (DA, 500 μM, 60 min incubation)-induced rat erythrocyte lysis. Results are shown as mean±sem of 3-9 observations. Student’s t-test: *p<0.05, **p<0.01, ***p<0.001.
Figure 7.2. Effect of balsalazide (BSZ), sulphasalazine (SASP), and metabolites 5-aminosalicylic acid (5-ASA), 4-aminobenzoyl-β-alanine (4-ABA), and sulphapyridine (SP) (all at 20-500 μM) on heat-induced rat erythrocyte haemolysis. Results are shown as mean±sem of 3-9 observations. Student’s t-test: *p<0.05, **p<0.01, ***p<0.001.
Figure 7.3. Effect of (a) Ethanol and (b) Cyclosporin A (CsA) on heat-induced rat erythrocyte lysis. Results are shown as mean±sem of 3 observations. Ethanol-free heated control and CsA-free heated control were expressed as 100%. Student's t -test: *p<0.05, **p<0.01, ***p<0.001.
Table 7.1. Effect of (a) dimethyl sulphoxide (DMSO) and (b) calmodulin antagonists on heat-induced haemolysis of rat erythrocytes. Results are shown as mean±sem of 3 observations except * (n=9). The mean extracellular haemoglobin content of the heated control (drug free) sample was expressed as 100 as a relative amount of haemolysis. Student’s t-test: * p<0.05, ** p<0.01, *** p<0.001 — significant difference from haemolysis of 0% DMSO or 0 M drug.

PIC: picumast; TFP: trifluoperazine.
Table 7.2. (a) Heat-induced rat erythrocyte lysis (%) in the absence/presence of deoxycholic acid (DA, 100 μM, 20 min incubation at 54-55 °C). (b) DA-induced rat erythrocyte lysis (%) at 37 °C.
Results are shown as mean±SEM of 3-9 observations. Student's t-test: ***p<0.001—Absence vs Presence.

(a)

<table>
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<th>DA (100 μM)</th>
<th>Absence</th>
<th>Presence</th>
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<tr>
<td>Haemolysis (%)</td>
<td>35.4 ± 2.7</td>
<td>49.3 ± 3.2***</td>
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</table>

(b)

<table>
<thead>
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<th>DA Concentration (μM)</th>
<th>Incubation Time (min)</th>
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<th>500</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>30</td>
<td>2.3 ± 0.5</td>
<td>15.2 ± 1.7</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>-- ---</td>
<td>42.3 ± 1.6</td>
</tr>
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</table>
Figure 7.4. Inhibitory effect of balsalazide (BSZ), sulphasalazine (SASP), and metabolites 5-aminosalicylic acid (5-ASA), 4-aminobenzoyl-β-alanine (4-ABA), and sulphapyridine (SP) (all at 100-500 µM) on heat-induced rat erythrocyte haemolysis in the absence/presence of deoxycholic acid (DA, 100 µM). Results are shown as means±SEM of 3-9 observations. Student’s t-test: **p<0.01-- significant difference between the inhibition in the absence and presence of DA.
Figure 7.5. Effect of resuspension and preincubation with deoxycholic acid (DA, 30 min preincubation at 37 °C) on heat-induced rat erythrocyte haemolysis. Results are expressed as % change calculated with respect to corresponding DA-free control and mean±sem of 3 observations.
Figure 7.6. Effect of balsalazide (BSZ), sulphasalazine (SASP), metabolites 5-aminosalicylic acid (5-ASA), 4-aminobenzoyl-ß-alanine (4-ABA) and sulphapyridine (SP), and disodium cromoglycate (DSCG) (all at 500 μM) on heat-induced haemolysis using rat erythrocytes unmodified or modified by trinitrobenzene sulphonic acid (TNBS, 4 mM), phospholipase C (PLC, 2 μg/ml), and deoxycholic acid (DA, 100 μM). Results are shown as means ± SEM of 3-6 observations. Student’s t-test: *p<0.05, **p<0.01 ***p<0.001 -- significant difference from unmodified erythrocytes.
Figure 7.7. Dose-response of (a) phospholipase C (PLC)-induced rat erythrocyte lysis (30 or 60 min incubation) and (b) melittin-induced human erythrocyte lysis (30 min incubation). Results are shown as mean of 3 observations. Values of sem (0.1-2.9) are too small to be represented graphically.
Figure 7.8. Effect of balsalazide (BSZ) and sulphasalazine (SASP) on melittin (7.5 µg/ml)-induced (30 min incubation) human erythrocyte lysis. Results are shown as mean±sem of 3 observations. Student's t-test: *p<0.05, **p<0.01, ***p<0.001.
Figure 7.9. Effect of balsalazide (BSZ), sulphasalazine (SASP), and metabolites 5-aminosalicylic acid (5-ASA), 4-aminobenzoyl-β-alanine (4-ABA), and sulphapyridine (SP) (all at 20-500 µM) on phospholipase C (2 µg/ml, 30 min incubation)-induced rat erythrocyte lysis. Results are shown as mean±sem of 3-9 observations. Student's t-test: *p<0.05, **p<0.01, ***p<0.001.
Figure 7.10. Effect of balsalazide (BSZ), sulphasalazine (SASP), and metabolites 5-aminosalicylic acid (5-ASA), 4-aminobenzoyl-β-alanine (4-ABA), and sulphapyridine (SP) (all at 20-500 μM) on deoxycholic acid (100 μM, 30 min incubation)-induced rat erythrocyte lysis. Results are shown as mean±sem of 3 observations. Student's t-test: *p<0.05, **p<0.01, ***p<0.001.
Figure 7.11. Effect of balsalazide (BSZ) and sulphasalazine (SASP) (both at 500 μM) on heat-induced albumin denaturation with varying duration of drug preincubation. Results are shown as mean±SEM of 3 observations. Student's t-test: *p<0.05, **p<0.01, ***p<0.001.
CHAPTER 8

GENERAL DISCUSSION

Inflammatory bowel disease (IBD), which includes ulcerative colitis and Crohn’s disease, remains one of the major unsolved and complex clinical problems in gastroenterology today. The aetiology and aetiological agents of the disease are unknown, the pathophysiological mechanism of the attack is in essence unclear, and the chemical mediators of inflammation are largely undefined. It is most probable that IBD is caused by an array of aetiological and potentiating factors which act independently or in concert, resulting in immune responses of an inflammatory nature.

The treatment of IBD remains largely empirical. The primary mode of action of sulphasalazine (SASP), the most widely prescribed agent for IBD therapy for about 50 years, is unknown. The therapeutic efficacy of SASP in IBD has been attributed to the 5-aminosalicylic acid (5-ASA) moiety [66], although the possible therapeutic effect of SASP itself and one of its metabolites, sulphapyridine (SP) cannot be completely ruled out [71, 74]. The adverse effects of SASP have been ascribed mainly to SP, and this has prompted the development of a series of sulphfa-free 5-ASA preparations, thus a new generation of SASP-like drugs without the sulphfa-moiety is emerging [64, 576]. Balsalazide (BSZ) is one of such new compounds. It consists of 5-ASA linked to 4-aminobenzoyl-S-alanine (4-ABA, an inert carrier) [75], and has been shown clinically to have the same efficacy as SASP, but with fewer side effects [79-81]. Cyclosporin A (CsA), an effective immunosuppressant which is a lipophilic, cyclic fungal undecapeptide used widely in organ transplantation to prevent graft rejection, has more recently received increasing attention in the treatment of IBD [85-87]. The exact mechanism of action of CsA remains unclear [91, 93, 109, 110, 298].

There is ultrastructural [149, 153, 154] and biochemical evidence [156-158] for local mast cell activation in IBD specimens, suggesting the involvement of mast cells in the disease. The mechanism responsible for mast cell activation in IBD is unknown. It appears that IgE-mediated degranulation may not play a major role in IBD, since the number of local IgE-containing immunocytes generally remains normal in ulcerative colitis or slightly decreased in Crohn’s disease patients [145, 165]. It is most likely that other mast cell secretagogues which are readily present locally in the
Chapter 8

diseased tissue, such as reactive oxygen metabolites (ROM) [168], epithelial cell-associated components [157], interleukin 1 [577], macrophage-derived histamine releasing factor [133], subclass IgG\textsubscript{4} antibody [155], neuropeptides (particularly substance P and neurotensin) [138, 578], and bile acid, may play a significant role. Recently, it has become apparent that the control of gastrointestinal functions, which may be relevant to IBD, involves a large number of complex neuroimmune regulatory mechanisms [139]. Increased substance P distribution has been demonstrated immunocytochemically in the colon of patients with IBD [579]. Mast cells have been increasingly implicated in this neuroimmune control system of gastrointestinal functions [580, 581]. Histamine, the best known mast cell mediator, exerts its widespread biological effects through H\textsubscript{1}, H\textsubscript{2}, and H\textsubscript{3} histamine receptors [134, 181]. Acting through both the H\textsubscript{1} and H\textsubscript{2} receptors, it causes smooth muscle contraction, increased mucus production, and oedema through vasodilation and capillary leakage and could contribute significantly to the symptoms of IBD. Recent studies suggest that the biological relevance of histamine extends beyond its well characterized role in mediating allergic reactions, and it may have a possible function in cytokine-governed regulation of inflammatory and immune cascades [191]. Therefore, as histamine is involved so widely in physiological as well as pathophysiological processes, it is more desirable to control its aberrant release, which is generally associated with pathological conditions, rather than inhibit its actions after the aberrant release. This is because the inhibition of its actions via its specific receptors may also interfere with its normal physiological actions.

In the present study, SASP treatment of experimental chronic and active rat colitis unexpectedly showed no ameliorating activity but slight aggravation of inflammation, while BSZ reduced both rectocolonic lesions and myeloperoxidase (MPO) activity (index of inflammation) in the lesions. In contrast, in an acute inflammatory model represented by the \textit{in situ} ethanol-perfused rat rectocolon, both BSZ and SASP showed marked prophylactic effects of cytoprotection (as shown by the inhibition of lactate dehydrogenase release) and anti-inflammation (as shown by the inhibition of MPO activity, tissue oedema, and histamine release). These findings are compatible with the fact that both drugs are more successful in the maintenance treatment in remission rather than severe active disease. Reduced glutathione (GSH) which is important in the detoxification of reactive oxygen metabolites (ROM) and inhibition of lipid peroxidation, presented a complex profile and unclear role in the experimental rat colitis models studied. This may be because, as mentioned and discussed in the preceding chapters, GSH may act as a peroxidation inhibitor/antioxidant as well as a pro-oxidant, it may
Chapter 8

prevent or reverse the inhibitory effect on neutrophil motility mediated by peroxidase/H$_2$O$_2$/halide system, and it has an important role in the activation of lymphocytes [426]. Its effect may reflect the balance or the net result of its several contrary actions. In the experimental rat colitis studied, GSH appeared to be more susceptible to insult by acute ethanol than by ROM generated by leucocyte activities (i.e. GSH is depleted more readily by acute ethanol insult than by leucocyte activities which were indicated by MPO activity), and the tissue GSH pool could be restored rather rapidly.

Secretagogues for mast cell histamine release and test compounds for modulating stimulated histamine release showed differential effects on dispersed guinea pig rectocolonic mucosal mast cells (RCMC) and isolated rat peritoneal mast cells (RPMC). The functional heterogeneity of mast cells is well known. There are variations in response by mast cells from different species and tissues to histamine release inducers as well as to the modulatory drugs of histamine release. These variations reflect the complexity and the different regulatory processes of controlling histamine release from mast cells.

Deoxycholic acid (DA) evoked dose-dependent histamine release which is at least partly cytotoxic from isolated RPMC and guinea pig rectocolonic mucosal scraping. This is compatible with the ability of DA to act as a biological detergent and to decrease cell membrane stability, and may be of relevance in inflammatory bowel conditions, at least as an enhancer. DA is also a calcium chelating agent that may mobilize intracellular calcium store [503, 504].

The peroxidase/H$_2$O$_2$/halide system (free radical generating system) induced histamine release from both RCMC and RPMC, indicating a link between the role of free radicals and histamine release. Metabolites of BSZ and SASP, namely 5-ASA, 4-ABA, and SP, produced marked and dose-related inhibition of this stimulated histamine release from both RCMC and RPMC, while the parent molecules, BSZ and SASP, were less effective, with only SASP showing considerable inhibition of release from RPMC at a high dose. Both CsA and exogenous GSH were found to be effective with RPMC, but not effective with RCMC. However, exogenous GSH, when showed inhibitory effect, was in general less effective than all the other drugs mentioned. The present results of inhibition cannot be explained by the ability of scavenging reactive oxygen metabolites alone. It is possible that the ability of inhibiting peroxidase may be involved. The inhibition by metabolites of BSZ and SASP on release from RCMC showed tachyphylaxis.
Using selected secretagogues for mast cell histamine release, the inhibitory effects of BSZ, SASP, and their respective metabolites on stimulated histamine release were studied. With RPMC, the histamine release induced by PAF, which has been suggested to be mediated by non-specific action on mast cell membrane, was inhibited by BSZ and SASP. Also inhibited by BSZ and SASP was the histamine release induced by 48/80 and Sub P, both of which have been suggested to activate G-protein directly, and their actions are closely associated with the sialic acid residues on the cell surface. The BSZ metabolite 4-ABA, at high dose, also showed a very small inhibition against 48/80-induced release. The NaF-induced histamine release, which is also mediated by direct activation of a G-protein(s), was inhibited by SASP, and, to a lesser extent, by BSZ, 5-ASA, and SP. The G-protein(s) activated by 48/80 and Sub P is thought to be different from those activated by NaF, according to their sensitivity toward pertussis toxin. The different profiles of inhibition by BSZ and SASP on histamine release induced by the mentioned putative G-protein activators in the present study may also reflect the differences in the G-proteins activated. The histamine release induced by PLC, A23187, and Dic8 was not inhibited by BSZ, SASP, and their metabolites. Therefore, with RPMC, given the putative modes of action of the selected secretagogues, it is possible that BSZ and SASP exert their inhibitory effects by interfering with the early stages of mast cell activation prior to the elevation of cytosolic calcium, and/or by general stabilizing action on the mast cell membrane. The target(s) of intervention could be a G-protein(s) and/or the sialic acid residues on cell surface. This inhibition by BSZ and SASP on histamine release showed tachyphylaxis. With guinea pig RCMC, the histamine release induced by PLC and A23187, the only two secretagogues to which RCMC responded, was not inhibited by BSZ, SASP, and their metabolites. In contrast, CsA attenuated histamine release induced by NaF, PLC, Dic8, and A23187 from RPMC as well as that induced by PLC and A23187 from guinea pig RCMC. CsA has also been shown to be ineffective in inhibiting 48/80-induced histamine release from RPMC and Sub P-induced release from human skin mast cells (as discussed in Chapter 6). Hence, it is possible that CsA exerts its inhibitory effects at a more distal step (relative to that of BSZ and SASP) of signalling pathways controlling histamine release; possibly by interfering with a step downstream of second messenger calcium, or by blocking calcium channels, hence inhibiting the rise in intracellular calcium. In RPMC, the pathways involved may be different from that activated by 48/80.

BSZ, SASP, and in some cases their metabolites were shown to stabilize erythrocyte membrane against the membrane labilizers tested. This may account partly
for the beneficial actions of both drugs in rendering cytoprotection and preventing inflammation. CsA showed an opposite effect to that of BSZ and SASP, indicating the fundamental differences in the mechanism of action between the compounds.

Apart from the role in detoxication of ROM and inhibition of lipid peroxidation, GSH also participates in the activation of human lymphocytes [426] and the formation of sulphidopeptide leukotrienes [425] which are mediators of mast cells. Its sulphhydryl group may also play a role in the regulation of enzyme activities [404, 421] and ligand binding to receptors [134, 582]. It is a substrate for enzymic peroxidation (to produce sulphidopeptide leukotrienes as mentioned above) and glutathione redox cycle (to reduce peroxides to water by glutathione peroxidase — see Section 1.6.4.1 and Figure 1.4 of Chapter 1), and both of these reactions have been shown to be involved in the processes of histamine release from RPMC and rat pleural mast cells [583]. In view of all these effects, it is not difficult to envisage the significant role that GSH plays in mast cell activation and IBD. Further studies of GSH with respect to mast cell histamine release, including the modulatory effect of drugs are warranted.

It is conceivable that one would expect some discrepancies between the effects of the same drug on mast cell histamine release and erythrocyte lysis, as they involve two different processes: the former exocytotic and the latter, cytolytic. The two processes may express different sensitivities towards the same inducer. Moreover, histamine is a much smaller molecule than haemoglobin, hence requiring smaller perturbation of cell membrane for its release. In other words, histamine is more readily released from mast cells than haemoglobin from erythrocytes.

Since mixed cell preparations which contained a large number of other cell types, apart from mast cells, were used in the study, the possibility that the observed drug effects on histamine release from mast cells might be modulated by an effect on/of contaminating cells cannot be ruled out. For example, it has been shown in rat peritoneal cells, that phorbol ester-activated neutrophils and eosinophils initiate mast cell degranulation and histamine release [584], and antigen-induced histamine release is potentiated through direct interaction between mast cells and non-mast cells [585]. Moreover, compared with other peritoneal cells, mast cells exhibit a relatively small PKC activity [323] compared with other peritoneal cells, and as discussed in Chapter 5, superoxide produced by activated phagocytic leucocytes can influence the effect of a drug on myeloperoxidase. Nevertheless, the present study has demonstrated, at least within the limit of the tests carried out, that BSZ shares the same actions and is as effective as SASP; and like SASP, the parent molecule may not merely be a pro-drug
but also as a drug. Besides the suggested therapeutic moiety, the parent molecule and carrier moiety may contribute to the beneficial effect of both compounds. It has also been demonstrated that the inhibitory activity of BSZ, SASP, 5-ASA, 4-ABA, SP, and CsA differs with the type of mast cells and stimulant used. This differential inhibition of mast cell histamine release by the drugs reflects the complexity of regulatory mechanism of mast cell activation, the selectivity of inhibitory effect of agents depending on the nature of stimuli, and the heterogeneity of mast cell. Therefore, it is essential to identify and define stimuli for mast cell activation in IBD in order to establish the action of therapeutic agents, and to be cautious in extrapolating results obtained from one mast cell population to the other. The stimuli may be mediators from other inflammatory cells, such as ROM, cytokines, prostaglandins, and leukotrienes. Hence, with the identification of new mediators and agents present in IBD, the investigations of the effects of these mediators and agents on mast cell activation as well as the modulatory effects of drugs on activation are essential. Human mast cell populations, in this case intestinal mucosal mast cells, are most ideal for studying human disease. However, very often functional studies of human mast cells have not been performed because of the difficulty in handling and assaying histamine in biological specimens which are not readily available and usually small in size. Apart from improving the experimental methodology of using human specimens, the alternative is to search for an easily obtainable substitute mast cell population which resembles closely the human counterpart by performing the comparative study of different mast cell populations.

The mast cell is only one of the cell types involved in inflammatory reactions in the intestinal tract and in order to establish the overall action of therapeutic agents, the effect of the drugs on other inflammatory cells, such as macrophages, neutrophils, and eosinophils must also be investigated.
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202
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