THE PHARMACOLOGY OF CALCIUM AND ATP-DEPENDENT POTASSIUM CHANNELS IN ERYTHROCYTES AND IN SMOOTH AND SKELETAL MUSCLE

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A thesis submitted for the degree of Doctor of Philosophy

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Abstract

Potassium channels are a diverse class of membrane proteins found in virtually all cells. The first part of this study concerns the pharmacology of the calcium activated potassium channel ($K_{Ca}$) found in mammalian erythrocytes. This channel is blocked by cetiedil, an anti-sickling agent, and the initial section of the thesis explores the structure activity relationship (SAR) of compounds related to cetiedil, investigates their mechanism of action and compares them with other blocking agents. This was studied by measuring the effects of the test compounds on the $K_{Ca}$-mediated net $K^+$ loss which results from the application of A23187 to a suspension of rabbit erythrocytes. Qualitative differences between the action of the cetiedil analogues and another potent $K_{Ca}$ blocker, clotrimazole, were observed, suggesting a different mechanism of channel block. The relative activity of the optical enantiomers of cetiedil, UCL 1348 and UCL 1349, as anti-muscarinic and calcium entry blocking agents were also examined. Finally, the ability of cetiedil and two of its analogues, UCL 1495 and UCL 1285, to inhibit levcromakalim stimulated $^{86}\text{Rb}$ efflux from rat aorta was compared. The results indicate that the SAR for cetiedil at this site differs from that for $K_{Ca}$ in erythrocytes.

In the second part the effects of $K^+$ channel openers (KCO's) and metabolic inhibition on $K^+$ channels in skeletal muscle were investigated. In the frog, cromakalim and levcromakalim increased $^{86}\text{Rb}$ efflux and caused membrane hyperpolarisation associated with a decrease in input resistance. The effects of cromakalim were blocked by glibenclamide and tolbutamide. These findings are consistent with an action of cromakalim on ATP-sensitive potassium channels ($K_{ATP}$). Comparisons are made between this channel and the KCO sensitive $K^+$ channel of smooth muscle. Levcromakalim was far less effective in mammalian skeletal muscle and possible reasons for the difference between frog and mammalian muscle are discussed. An incidental observation was that metabolic inhibition caused a large $\text{Ba}^{2+}$ sensitive increase in $^{86}\text{Rb}$ efflux from mouse soleus. However, only a small component was blocked by glibenclamide suggesting involvement of channels other than $K_{ATP}$. 


Acknowledgments

I am indebted to Professor D.H. Jenkinson for acting as my supervisor and for his encouragement and unfailing patience. I am also grateful to Dr. D.G. Haylett and Dr. P. M. Dunn who have given me much advice over the years. I would also like to thank Professor C. R. Ganellin for his help on medicinal chemistry and to his team of chemists, Dr. C. Roxburgh, Dr. S. Athmani, Dr. W. Quaglia and Ms Z. Miscony who synthesized the novel compounds used in this study and who have also given me valuable advice.

The other members of the K⁺ channel group have provided constant support as well as productive and lively discussion on all aspects of my work. In particular I would like to thank Mr. M. Shiner and Ms M. Malik with whom I have worked closely on the erythrocyte project. Ms K.S. Burke and Mr. A.G. Jones have also been generous in allowing me access to their results.

This thesis is dedicated to Georgina and to Eleanor.
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Section I

Introduction
1 INTRODUCTION

Ion channels are protein molecules found in the lipid bilayer of cell membranes which serve to regulate the passive movement of ions across the membrane. Most ion channels discriminate between cations and anions. Of the cation channels, some are non-selective, though channels exist that are selective for one or other of the most physiologically important cations (Na\(^+\), K\(^+\) and Ca\(^{2+}\)). Potassium selective channels are found in the membranes of most if not all cells and have a wide range of functions including the regulation of excitability, secretion and cell volume. K\(^+\) channels are not only the most ubiquitous but also the most diverse class of ion channels; over 15 different types have been described and many cells possess several kinds of K\(^+\) channel.

The discovery of highly selective ligands for K\(^+\) channel proteins, the introduction of the patch clamp technique and more recently the cloning of K\(^+\) channel genes has led to a rapid growth in interest in K\(^+\) channels. Many K\(^+\) channels are, however, still poorly understood in terms of both their structure and function and there is still a need for selective pharmacological probes. A number of drugs which act by modulating K\(^+\) channels are in clinical use and many recently discovered compounds show therapeutic potential. It can be hoped, therefore, that further developments in K\(^+\) channel pharmacology will be useful not only in the study of the structure and function of K\(^+\) channels but also in the search for useful therapeutic agents.

1.1 Diversity of K\(^+\) channels

K\(^+\) channels have been classified according to their pharmacology, biophysical properties and physiological modulation. The cloning and expression of K\(^+\) channels has allowed them to be grouped into families according to structure, though, this process is by no means complete. Table 1 shows the major K\(^+\) channels with some information on their properties.

As can be seen, no single method of classification is wholly satisfactory.
<table>
<thead>
<tr>
<th>Name</th>
<th>Gene</th>
<th>Physiological Modulator</th>
<th>Properties</th>
<th>Pharmacological Modulator</th>
<th>Unitary conductance (pS)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Voltage dependent</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$K_v$</td>
<td>Shaker</td>
<td>Activated by depolarisation</td>
<td>Activate with short delay after depolarisation. Marked heterogeneity in terms of threshold and inactivation.</td>
<td>TEA, Cs$^+$, Ba$^{2+}$, Zn$^{2+}$, forskolin, 4AP, 9-aminoacridine, phencyclidine, imperator toxin, phallolidin.</td>
<td>5-60</td>
</tr>
<tr>
<td>$K_{Va}$ Delayed rectifier, fast component</td>
<td>Shab, Shal, Shaw</td>
<td>Activated by depolarisation</td>
<td></td>
<td>Dofetilide, sotalol, E4031, tedisamil</td>
<td></td>
</tr>
<tr>
<td>$K_{Vs}$ Delayed rectifier, slow component</td>
<td>minK</td>
<td>Activated by depolarisation</td>
<td>Very slow activation and inactivation.</td>
<td>LY97241, NE10118</td>
<td></td>
</tr>
<tr>
<td>$K_A$ A-channel</td>
<td>Shaker</td>
<td>Activated by depolarisation</td>
<td>Rapid activation and inactivation. Inactivation complete at potentials positive to -50mV.</td>
<td>4AP, phencyclidine, dendrotoxin, toxin I, MCDP</td>
<td></td>
</tr>
<tr>
<td>$K_r$ Inward (anomalous) rectifier</td>
<td>Irk RomK</td>
<td>Current reduced during depolarisation</td>
<td>Conductance greatest at negative potentials. Inward rectification due to block by Mg$^{2+}$, and some polyamines e.g. spermine. Gating depends on $[K^+]_o$.</td>
<td>Gaboon viper venom, TEA, Cs$^+$, Ba$^{2+}$, Sr$^{2+}$</td>
<td>5-18</td>
</tr>
<tr>
<td>$K_M$ M-channel</td>
<td></td>
<td>Activated by depolarisation. Inhibited by activation of muscarinic and other receptors. Nature of transduction mechanism unknown.</td>
<td>Time- and voltage dependent current. Activated at potentials positive to -65 mV.</td>
<td>Ba$^{2+}$</td>
<td></td>
</tr>
<tr>
<td><strong>Ca$^{2+}$-activated</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>100-250</td>
</tr>
<tr>
<td>BKCa Large conductance Ca$^{2+}$-activated K$^+$ channel</td>
<td>Slo</td>
<td>Activated by depolarisation and $\uparrow[Ca^{2+}]$.</td>
<td>Synergistic activation by Ca$^{2+}$ and depolarisation.</td>
<td>TEA, charybdotoxin Activated by NS 1619</td>
<td>18-50</td>
</tr>
<tr>
<td>IKCa Intermediate conductance Ca$^{2+}$-</td>
<td></td>
<td>Activated by $\uparrow[Ca^{2+}]$.</td>
<td>May be voltage sensitive (molluscan neurones) or</td>
<td>charybdotoxin, nitrendipine, clotrimazole,</td>
<td>6-14</td>
</tr>
<tr>
<td>Channel Type</td>
<td>Action</td>
<td>Sensitivity</td>
<td>Blockers</td>
<td></td>
<td></td>
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<tr>
<td>--------------</td>
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<tr>
<td>SK&lt;sub&gt;Ca&lt;/sub&gt;, Small conductance Ca&lt;sup&gt;2+&lt;/sup&gt;-activated K&lt;sup&gt;+&lt;/sup&gt; channel</td>
<td>Activated by [Ca&lt;sup&gt;2+&lt;/sup&gt;]&lt;sup&gt;+&lt;/sup&gt;. Voltage insensitive. Apamin insensitive low conductance channels have been described.</td>
<td>Apamin, tubocurarine, dequalinium&lt;sup&gt;2&lt;/sup&gt;, leiurotoxin&lt;sup&gt;1&lt;/sup&gt;, quinine&lt;sup&gt;1&lt;/sup&gt;</td>
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<td></td>
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<td>Receptor operated channels</td>
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<td></td>
<td>7-50</td>
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<tr>
<td>G-protein linked eg K&lt;sub&gt;ACH&lt;/sub&gt;</td>
<td>Directly activated by G&lt;sub&gt;αi&lt;/sub&gt;.</td>
<td>Inwardly rectifying. May also be regulated by βγ sub-unit via stimulation of PL&lt;sub&gt;α&lt;/sub&gt;.</td>
<td>Propafenone&lt;sup&gt;3&lt;/sup&gt;, clotrimazole&lt;sup&gt;4&lt;/sup&gt;, cetiedil&lt;sup&gt;4&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K&lt;sub&gt;S-HT&lt;/sub&gt;</td>
<td>Inactivated by stimulation of 5-HT receptors via cAMP-dependent phosphorylation.</td>
<td>Weakly voltage dependent but open at resting potential.</td>
<td>Ba&lt;sup&gt;2+&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other K&lt;sup&gt;+&lt;/sup&gt; channels</td>
<td></td>
<td></td>
<td>5-90</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K&lt;sub&gt;ATP&lt;/sub&gt;, ATP-sensitive K&lt;sup&gt;+&lt;/sup&gt; channel</td>
<td>Inhibited by ATP&lt;sub&gt;i&lt;/sub&gt; [ATP]/[ADP] ratio and pH, may also be physiologically important</td>
<td>Weak inward rectification in some tissues.</td>
<td>Glibenclamide, phentolamine, quinine Ba&lt;sup&gt;2+&lt;/sup&gt;, 8-hydroxy decanoate. Activated by potassium channels openers eg levocromakalim.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K&lt;sub&gt;vol&lt;/sub&gt;, Cell-volume-sensitive K&lt;sup&gt;+&lt;/sup&gt; channel</td>
<td>Activated by increase in cell volume.</td>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;-insensitive in hepatocytes. May contribute to regulatory volume decrease in some cells.</td>
<td>Cetiedil</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K&lt;sub&gt;Na&lt;/sub&gt;, Sodium-activated K&lt;sup&gt;+&lt;/sup&gt; channel</td>
<td>Activated by increase in [Na&lt;sup&gt;+&lt;/sup&gt;]&lt;sub&gt;i&lt;/sub&gt;</td>
<td>Believed to be activated by transient increase in [Na&lt;sup&gt;+&lt;/sup&gt;]&lt;sub&gt;i&lt;/sub&gt; during action potential or during hypoxia</td>
<td>TEA, 4AP</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Notes 1 Most of the information in this table is taken from the Trends in Pharmacological Sciences Receptor & Ion Channel Nomenclature Supplement, 1994. Other information was taken from the references shown below:
1. Ficker et al. 1994, Lopatin et al. 1994
2. Castle et al. 1993
3. Inomata et al. 1993
4. Jones et al. 1994
5. Ashford et al. 1994
6. Sandford et al. 1992
Functionally, K⁺ channels have been classified by gating mechanism. Thus channels may be
gated by changes in membrane potential or by interaction with intracellular messengers. This
method of classification is not ideal since many channels are modulated both by membrane
potential and by interaction with ligand molecules (e.g. BK, Kₐ). Although still in its early
stages of development, classification according to molecular structure is useful and indeed
potentially decisive. In particular it has become apparent that the fast delayed rectifier (Kᵥᵣ)
resembles the A-current channel (Kᵥₐ) far more closely than it does the slow delayed rectifier
(Kᵥₛ). Classification according to pharmacological criteria is also fraught with problems. For
example, charybdotoxin has been found to block at least three groups of K⁺ channel (BK, IK
and Kᵥₐ) which differ markedly by functional and structural criteria.

Recent growth in the field makes a comprehensive review of K⁺ channels impractical in the
space available. This introduction will concentrate instead on those aspects of K⁺ channel
physiology and pharmacology most relevant to the present work, which concerns, the
intermediate conductance calcium activated K⁺ channel (IK) of erythrocytes, the ATP-
sensitive K⁺ channel of skeletal muscle (Kₐₜₚ), and the associated pharmacology of the K⁺
channel openers (KCO).

1.1.1 K⁺ channel nomenclature
At present there is no universally accepted nomenclature for K⁺ channels. The nomenclature
adopted in this thesis is that suggested by Edwards and Weston (1993b) using the
abbreviations shown in table 1. Thus the names of K⁺ channels are abbreviated as K
followed by a subscript which indicates the channel type (e.g. Kₐₜₚ for the ATP-sensitive K⁺
channel). K⁺ current is abbreviated as I𝐾 and an additional subscript in brackets is used to
indicate the type of channel involved (e.g. I𝐾(ᵣₜₚ) for the K⁺ current carried by Kₐₜₚ).
Similarly, K⁺ permeability has been abbreviated as P𝐾 and the K⁺ permeability associated
with a particular channel is indicated by a further subscript (e.g. P𝐾(ᵣₚ₉ₚ) for calcium-activated
K⁺ permeability).

Ca²⁺-activated K⁺ channels (Kᵥₙₚ) are also classified according to unitary conductance using
the terms BK, IK and SK for large, intermediate and small conductance channels
respectively. Although not consistent with the system described above, these terms are now
so widespread that they have been used in this thesis. However, the IK channel found in mammalian erythrocytes has been referred to as the ‘erythrocyte K\textsubscript{ca}’ in order to emphasize the fact that its properties differ considerably from other IK channels.

1.2 Calcium-activated K\textsuperscript{+} channels in erythrocytes

Mammalian erythrocytes contain an intermediate conductance calcium activated K\textsuperscript{+} channel (IK). Although a calcium-stimulated K\textsuperscript{+} permeability was first described in the erythrocyte (Gardos 1958) it was not until data were published from patch clamp experiments (Hamill 1981) that it became widely accepted that this process involves ion channels rather than a carrier-mediated transport system.

This section will discuss previously published work on the properties of these channels, their pharmacology and their possible role in sickle cell anaemia.

1.2.1 The Gardos effect

In 1935 Orskov reported that exposure of red blood cells to lead ions caused cell shrinkage resulting from a net loss of K\textsuperscript{+} ions. Subsequently, it became apparent that a number of procedures, including depletion of ATP by incubation with inhibitors of glycolysis such as iodoacetate, also caused a selective increase in the potassium permeability (P\textsubscript{K}) of the red cell membrane. Gardos (1958) showed that the increase in P\textsubscript{K} caused by ATP depletion was dependent on extracellular calcium ions. This led to the suggestion that the increase in P\textsubscript{K} was caused by a rise in intracellular calcium resulting secondary to ATP depletion. This phenomenon came to be known as the ‘Gardos effect’. The relationship between ATP depletion, intracellular [Ca\textsuperscript{2+}] and P\textsubscript{K} was further studied by Lew (1971) who showed that P\textsubscript{K} increased in parallel with the decline in ATP content of the cell. The discovery of the Gardos effect was of considerable importance since it was the first time a link was made between intracellular calcium ions and potassium permeability.

1.2.2 Effects of Ca\textsuperscript{2+} and other divalent metal ions on the K\textsuperscript{+} permeability of erythrocytes

The sensitivity of the erythrocyte K\textsubscript{ca} to Ca\textsuperscript{2+} and other divalent cations has been studied
using intact red cells, resealed membrane ghosts, inside-out vesicles and by the patch clamp technique.

Simons (1976a, b) investigated the sensitivity of the $P_{K(Ca)}$ by using buffered calcium solution sealed into red cell ghosts. In the absence of Mg$^{2+}$, calcium caused half-maximal activation at 0.4μM. At concentrations above 3 μM potassium efflux was depressed. Inclusion of Mg$^{2+}$ at mM concentrations caused a rightward shift in the curve. Similar results were obtained in experiments on inside-out vesicles in which the inner surface of the red cell membrane was in contact with the bathing solution (Lew et al. 1982) although the sensitivity to calcium was somewhat greater. In experiments using inside out membrane patches Grygorczyk and co-workers (Grygorczyk and Schwarz 1983, 1985, Grygorczyk et al. 1984) found a similar sensitivity to Ca$^{2+}$ and confirmed the inhibitory effect of Mg$^{2+}$. Kinetic analysis of the records showed that increasing [Ca$^{2+}$] increased $P_{\text{open}}$ by reducing closed times with no apparent effect on open times (Leinders et al. 1992b).

Most workers have reported that the concentration activation curve for calcium is rather steep, having a Hill slope close to two (Simons 1976a, Grygorczyk and Schwarz 1983) suggesting that 2 Ca$^{2+}$ ions must bind in order to activate the channel. More recently, however, Leinders (1992b) reported a Hill slope of 1. This discrepancy has yet to be explained.

The erythrocyte $K_{Ca}$ is also activated by a number of other cations. The most thoroughly studied is Pb$^{2+}$ (Orskov 1935, Shields et al. 1985, Simons 1985). Lead ions are thought to enter the erythrocyte via an anion exchanger and to be extruded by the calcium ATPase (Simons 1984, 1988). It is believed that the action of Pb$^{2+}$ on $K_{Ca}$ is direct and presumably similar to that of Ca$^{2+}$ since channel activation occurs in conditions where the application of calcium ionophore is ineffective (Shields et al. 1985). In a study of the ability of several divalent cations to activate $K_{Ca}$ in isolated patches Leinders et al. (1992a) reported a rank order of potency of Pb$^{2+}$, Cd$^{2+}$>Ca$^{2+}$ = Co$^{2+}$>>Fe$^{2+}$, Mg$^{2+}$. This is in contrast to results obtained with BK channels where the order was Ca$^{2+}$>Cd$^{2+}$>Fe$^{2+}$>>Pb$^{2+}$ (Oberhauser et al. 1988). Presumably these findings reflect differences in the Ca$^{2+}$ binding sites of different classes of Ca$^{2+}$-activated K$^+$ channels.
The sensitivity of $K_{Ca}$ to activation by both $Ca^{2+}$ and $Pb^{2+}$ is modified by propranolol. For example, Porzig (1975) found that at concentrations below 1 mM propranolol stimulated $K^+$ efflux whereas greater concentrations caused inhibition. More detailed experiments have shown that the effect of propranolol depends on the concentration of free calcium in the cells, the inhibitory effect becoming more pronounced as $[Ca^{2+}]_i$ increases (Schwarz et al. 1989). In patch clamp experiments it was found that propranolol had a combined effect of increasing the sensitivity to $Ca^{2+}$ and reducing the maximum open probability which could be attained. In addition propranolol caused a reduction in the single channel conductance (Schwarz et al. 1989). These results indicate that the effect of propranolol results from a direct effect on the channel and not from the release of membrane bound calcium as was originally suggested by Porzig (1975).

In experiments on cell suspensions and on inside-out vesicles several workers noted that the responses to $Ca^{2+}$ and $Pb^{2+}$ had an all-or-none character in that as the concentration of activating agent increases it appears that the pool of available $K^+$ increased rather than the rate at which equilibrium was reached (Lew et al. 1983, Alvarez et al. 1986). Thus each cell or vesicle must have a threshold concentration at which it switches from a low to a high permeability state. Since individual cells contain more than one channel such a mechanism would require that a) the gating of individual channels was of a threshold nature and b) that the thresholds for channels in a given cell should be the same. Since experiments on isolated patches showed that a graded increase in $P_{\text{open}}$ with ligand concentration was observed, these results are difficult to explain at the level of single channels. Alvarez et al. (1988) have suggested that the apparent all or none nature of the responses arises not from co-ordination of thresholds for the $K$ channels but from a heterogeneity of ligand concentrations achieved in different cells.

1.2.3 Calpromotin

Plishker and co-workers have suggested that activation of erythrocyte $KCa$ requires a cytoplasmic protein called calpromotin (Moore et al. 1990). The first indication of the involvement of this protein came from experiments on the effect of iodoacetate (IAA) on $K^+$ efflux from erythrocytes. IAA has long been known to activate $K_{Ca}$ by causing an increase in cytoplasmic $[Ca^{2+}]$ secondary to depletion of ATP. However, it was found that prolonged exposure to IAA caused inhibition of $K_{Ca}$ and that tritiated IAA labelled a 23 kD protein
(Pliskher 1985). Subsequently this protein was purified and it was shown that inclusion of a polyclonal antibody against it into erythrocyte ghosts caused inhibition of KCa. (Pliskher et al. 1986, Moore et al. 1990)

Most of the calpromotin appears to be located in the cytoplasm. However, in experiments on reconstituted membrane vesicles it was found that the membrane bound levels of the protein increased with cytoplasmic Ca\(^{2+}\) concentration (Moore et al. 1991). This suggested that Ca\(^{2+}\) combines with cytoplasmic calpromotin and that this complex binds to the membrane. Membrane bound calpromotin appears to consists mostly of a high molecular weight multimeric form.

As yet the role of calpromotin in the regulation of K\(_{Ca}\) is unclear. Since Ca\(^{2+}\) alone can activate channels in cell-free patches it is unlikely that it is a Ca-calpromotin complex formed in the cytoplasm which activates the channel. It is possible that the protein is simply needed for the maintenance of channel activity or that Ca\(^{2+}\) can complex with membrane bound calpromotin in order to activate the channel.

1.2.4 Biophysical properties of erythrocyte K\(_{Ca}\)

Estimates of the single channel conductance of K\(_{Ca}\) in erythrocytes vary from 25 pS in symmetrical high K\(^+\) (Grygorczyk and Schwarz 1983) to 15 pS in a physiological K\(^+\) gradient (Leinders et al. 1992b). As would be expected the single channel conductance increases with external potassium concentration (Leinders et al. 1992b). In experiments on cell suspensions (Knauf et al. 1975) found that prolonged incubation of red cells in K\(^+\) free medium caused a prolonged inhibition of Ca\(^{2+}\)-activated K\(^+\) fluxes and it has been reported that similar treatment of cells caused a reduction in the number of active channels detected in membrane patches (Grygorczyk and Schwarz 1983). The mechanism of this inactivation is unclear.

In symmetrical K\(^+\) both the single channel conductance and channel activity exhibit inward rectification (Grygorczyk et al. 1984, Grygorczyk and Schwarz 1985). The inward rectification of single channel current is less marked in low [K\(^-\)]\(_o\) solutions (Christophersen 1991) and absent in a physiological K\(^+\) gradient over the range from -25 to +25 mV (Leinders
Since rectification is apparent in symmetrical solutions and in the absence of potential blocking ions it would appear to arise from an asymmetry in the channel.

The rectification of the Ca$^{2+}$-activated K$^+$ conductance has been studied more indirectly in cell suspension by estimating net K$^+$ fluxes and membrane potential using the proton gradient method (Vestergaard-Bogind et al. 1987, Stampe and Vestergaard-Bogind 1988). These studies demonstrated that $g_{K(Ca)}$ varied with [K$^+$]$_o$ in a similar manner to the single channel conductance. Similarly, the estimated K$^+$ current showed inward rectification which was more marked at higher [K$^+$]$_o$. Measurements of flux ratios have shown the channel exhibits single file diffusion with a flux ratio exponent of 2.7 (Vestergaard-Bogind et al. 1985) suggesting that it behaves as a multi-ion pore with at least 3 K$^+$ binding sites.

Single erythrocyte K$_{Ca}$ channels are blocked by a number of small ions including Cs$^+$, Ba$^{2+}$, and Na$^+$ (Grygorczyk and Schwarz 1985, Shields et al. 1985). Na$^+$ causes a voltage dependent flickery block (Christophersen 1991) which is alleviated when [K$^+$] is increased on the opposite side of the membrane (Grygorczyk and Schwarz 1985, Shields et al. 1985). In experiments on suspensions of intact cells and resealed ghosts, it has been observed that internal Na$^+$ depresses calcium activated K$^+$ efflux and that this effect is reduced by increasing external [K$^+$] (Knauf et al. 1975, Stampe and Vestergaard-Bogind 1989). Although this effect was first explained by suggesting an allosteric interaction between separate K$^+$ and Na$^+$ binding sites on opposite sides of the membrane (Knauf et al. 1975), it is also explicable in terms of the predicted behaviour of a multi-ion pore (Yellen 1984, Hille 1992).

The ionic selectivity of the channel was first investigated by Simons (1976a) who used red cell ghosts and reported that monovalent cations were transported in the ratio K$^+>$Rb$^+$>Cs$^+>$Na$^+$. By measuring changes in reversal potential in isolated patches (Grygorczyk and Schwarz 1983) reported a ratio for K$^+$:Na$^+$ of 1:15. More recently a detailed study by Christophersen (1991) has been published. In a series of experiments with symmetrical cation solutions, the conductance series was K$^+>$NH$_4^+$>Rb$^+$>Na$^+$=Cs$^+$=Li$^+$ whereas the permeability series based on bi-ionic reversal potentials was K$^+>$Rb$^+$>Cs$^+$>NH$_4^+$>Li$^+$>$Na^+$. 

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This result shows that the principle of independence is not obeyed (Hodgkin and Huxley 1952) and is consistent with the behaviour of a multi-ion pore.

1.2.5 Channel density
Several estimates have been made of the number of $K_{Ca}$ channels per cell. Lew and co-workers (1982) produced an estimate of 100-200 based on the proportion of inside-out vesicles responding to the application of $Ca^{2+}$. Calculations based on measurements of ion fluxes and single channel properties suggested that the channel density was heterogeneous with 25% of cells containing 1-5 channels and 75% containing 11-55 channels (Grygorczyk et al. 1984). A similarly low estimate of 1-3 channels per cell was obtained by Wolff et al. (1988) using comparable methods. More recently binding studies with $^{125}$I labelled charybdotoxin yielded an estimate of 120 binding sites per cell (Brugnara et al. 1993a).

1.2.6 Pharmacology of erythrocyte $K_{Ca}$
Several agents have been reported to have the ability to block erythrocyte $K_{Ca}$. The structures of some of these agents are shown in Fig. 1.1.

1.2.6.1 Charybdotoxin
The most potent inhibitor of erythrocyte $K_{Ca}$ is charybdotoxin, a 37 amino acid peptide purified from the venom of Leiurus hebraeus quinquestriatus. In functional studies this toxin blocks calcium activated $K^+$ efflux from metabolically inhibited red cells with an $IC_{50}$ of 0.8 nM (Wolff et al. 1988) and blocks A23187 stimulated $^{86}$Rb uptake into red cells with an $IC_{50}$ of 4 nM (Brugnara et al. 1993a). $^{125}$I labelled CTX has been prepared and has been shown to retain biological activity. In low ionic strength solutions at pH 8 labelled CTX binds with a $K_d$ of 94 pM. Under these conditions the $IC_{50}$ for inhibition of A23187 stimulated $^{86}$Rb influx is 21 pM (Brugnara et al. 1993a).

1.2.6.2 Dihydropyridines
The dihydropyridine calcium channel blockers nitrendipine (Fig. 1.1A) and nifedipine have been shown to block A23187 stimulated $^{86}$Rb influx in human erythrocytes. Nitrendipine is the most potent with an $IC_{50}$ of 130 nM (Ellory et al. 1992) compared with 4 μM for nifedipine (Kaji 1990).
Fig. 1.1 Structures of some blockers of erythrocyte $K_{Ca}$. A nitrendipine. B clotrimazole. C quinine. D 3,3'-diethylthiadicarbocyanine (diS-C2(5)). E bepridil. F cetiedil.
A number of dihydropyridines, including the calcium channel opener BAY K 8644, were active whereas the non-dihydropyridine calcium entry blockers verapamil and diltiazem were not.

1.2.6.3 Clotrimazole and other imidazole anti-mycotic agents

Clotrimazole (Fig. 1.1B), an anti-mycotic agent, has been shown to be a potent inhibitor of \( ^{86}\)Rb influx in red cells (Alvarez et al. 1992). The IC\(_{50}\) for inhibition of A23187-induced shrinkage (measured by changes in light scattering) was 0.05 \( \mu \)M and A23187-stimulated \( ^{86}\)Rb uptake was inhibited with an IC\(_{50}\) of 0.1 \( \mu \)M. Other agents related to clotrimazole including tisoconazole, econazole and miconazole were also found to be active. Clotrimazole has also been shown to inhibit binding of \(^{125}\)I-CTX and there is a good correlation between the ability of clotrimazole and related compounds to inhibit \(^{86}\)Rb influx and to displace \(^{125}\)I-CTX (Alvarez et al. 1992).

1.2.6.4 Quinine

Quinine (Fig. 1.1C) and quinidine block several types of K\(^+\) channel. The activity of quinine as an inhibitor of the erythrocyte K\(_{Ca}\) was first demonstrated by Armando-Hardy \textit{et al.} (1975)

The effectiveness of quinine is strongly dependent on extracellular [K\(^+\)]. Thus the IC\(_{50}\) for inhibition of A23187-stimulated tracer efflux ranged from 5\( \mu \)M in a nominally K\(^+\) free bathing solution to 98\( \mu \)M in 5 mM K\(^+\). In contrast the effect of quinine was enhanced when the intracellular Na\(^+\) concentration was elevated (Reichstein and Rothstein 1981)

In isolated membrane patches, quinine inhibits single channel currents when applied to the inner surface of the membrane (Grygorczyk and Schwarz 1985). In keeping with experiments on intact cells the effect of quinine was enhanced when the K\(^+\) concentration at the outer surface of the membrane was decreased. Blockade by quinine was also increased at positive membrane potentials.

1.2.6.5 Carbocyanine dyes

Carbocyanine dyes were introduced for use as fluorescent indicators of membrane potential.
Sims et al. (1974) demonstrated that 3.3'-diethylthiadicarbocyanine iodide (diS-C2(5)) (Fig. 1.1D) was a potent inhibitor of calcium activated K\(^+\) fluxes in red cell ghosts. As with quinine, the effectiveness of diS-C2(5) was reduced in potassium rich solutions. The degree of inhibition caused by diS-C2(5) decreases as haematocrit increases, presumably because a large proportion of the compound partitions into the plasma membrane. At a 0.3% haematocrit complete inhibition of K\(^+\) efflux was caused by 0.3 \(\mu\)M diS-C(5).

The anionic and membrane impermeable dye diS-C3SO\(_3\)\(^-\) was active when incorporated into the ghosts but not when applied to the exterior, suggesting that these compounds bind to an intracellular site.

1.2.6.6 Bepridil

Bepridil (Fig. 1.1E) is a smooth muscle relaxant with calcium entry blocking actions. It has been found to cause abolition of A23187 stimulated K\(^+\) efflux at 100 \(\mu\)M (Johnston et al. 1989). In experiments where the Gardos effect was activated by cyclical oxygenation-deoxygenation, K\(^+\) efflux was inhibited with an IC\(_{50}\) of 25 \(\mu\)M.

1.2.6.7 Cetiedil

Cetiedil (Fig. 1.1F) was first introduced as a peripheral vasodilator and has a wide range of actions including antagonism of bradykinin and serotonin, inhibition of phosphodiesterase and in vivo has an analgesic action (Simaan and Aviado 1976). It was first shown to inhibit the Gardos effect by Berkowitz and Orringer (1981). In experiments using the protonophore equilibration method cetiedil inhibited A23187 stimulated hyperpolarisations with an IC\(_{50}\) of 25 \(\mu\)M (Christophersen and Vestergaard-Bogind 1985).

Cetiedil is now known to block a number of K\(^+\) channels including those concerned with volume regulation in lymphocytes and hepatocytes (Sarkadi et al. 1985, Sandford et al. 1992), G-protein linked K\(^+\) channels in atrial myocytes and sub-mucosal plexus neurones (Jones, A. G. et al. 1994) and the K\(^+\) channels activated by levcromakalim in smooth and skeletal muscle (Benton et al. 1994) (see also Chapters 9 and 12 of this thesis). One of the principle aims of the present work was to study the structure activity relationship of cetiedil and a series of novel related compounds (synthesised in the Department of Chemistry, UCL) with the intention of developing more potent blockers of the erythrocyte K\(_{Ca}\). This work is described
in Chapter 7. Given the broad spectrum of activity of cetiedil it is also important to obtain information concerning the selectivity the novel compounds as blockers of other K⁺ channels. Chapter 9. describes work in which the activity of cetiedil and two novel analogues were compared as blockers of the levromakalim activated K⁺ channel in rat aorta. Little is known of the mechanism whereby cetiedil blocks \( K_{Ca} \); experiments designed to study this problem are described in Chapter 8.

1.2.7 Sickle cell anaemia

Sickle cell anaemia was the first genetic disorder to be understood at the molecular level; the substitution of glu by val at position 6 of the B chain of haemoglobin was described by Ingram (1956). The pathology of the disorder is, however, extremely complex and there is a marked heterogeneity of in the clinical manifestations of the disease (see e.g. Joiner (1993) for review). The major pathological event in a sickle cell crisis is vaso-occlusion caused by the formation of dense 'sickle' cells. Cell sickling results from the polymerisation of HbS following a reduction in the water content of the cell. A number of pathways may contribute to red cell dehydration, however, it seems likely that the transient increase in intracellular calcium following de-oxygenation may stimulate the activity of \( K_{Ca} \) resulting in loss of K⁺ and water. Agents that prevent red cell dehydration may, therefore, be useful in the treatment of this condition. (see Brugnara (1993) for review).

The first agent for which this mechanism was proposed was cetiedil which was shown to possess anti-sickling properties \textit{in vivo} (Cabannes and Maron 1977). \textit{In vitro} experiments (Asakura et al. 1980, Benjamin et al. 1980) showed that cetiedil improved the cation and water content of sickle cells at similar concentrations to those used in the treatment of peripheral vascular disease. Subsequently, inhibition of the Gardos effect was suggested as a possible mechanism (Berkowitz and Orringer 1982). A number of agents described in the preceding section have also been shown to have \textit{in vitro} anti-sickling actions including charybdotoxin (Ohnishi et al. 1989), bepridil (Johnston et al. 1989), the dihydropyridines (Ohnishi et al. 1986) and clotrimazole (Brugnara et al. 1993b). As yet little \textit{in vivo} evidence concerning the efficacy of agents other than cetiedil has been published. Nifedipine has been found to have a beneficial action (Rodgers et al. 1988) but nitrendipine, the most potent dihydropyridine blocker of \( K_{Ca} \) was found to have no beneficial effect on blood parameters in a small scale study (Nash et al. 1991). In a recent study on transgenic SAD mice, which have
been transfected with the human HbS gene, it was found that clotrimazole had a beneficial effect \textit{in vivo} as well as protecting cells from sickling \textit{in vitro} (De-Franceschi et al. 1994).

It seems likely, therefore, that although inhibition of erythrocyte K\textsubscript{Ca} has an \textit{in vitro} protective effect on sickle cells it is not yet clear whether or not useful therapies are likely to emerge. It is to be hoped, however, that development of more potent and selective agents targeted at erythrocyte K\textsubscript{Ca} will allow this issue to be resolved. The novel compounds described in Chapter\textit{7} have a wide range of potencies as blockers of K\textsubscript{Ca} and so may be of use in studying this problem.

1.3 ATP-Sensitive K\textsuperscript{+} channels
K\textsuperscript{+} channels inhibited by intracellular ATP were first described by Noma and co-workers in ventricular myocytes (Noma 1983). Similar channels were then described in the pancreatic β-cell (Ashcroft, F. M. et al. 1984) and in vesicles formed from frog skeletal muscle (Spruce et al. 1985, 1987)

The main feature of these channels was that they were inhibited by the application of ATP to the cytoplasmic face of the membrane. The effectiveness of non-hydrolysable analogues of ATP such AMP-PNP ruled out the role of phosphorylation by ATP and instead suggested that ATP interacts with an inhibitory site at the intracellular part of the channel.

After K\textsubscript{ATP} was characterised in these tissues similar channels were described in neurones (Ashford et al. 1988, 1990) and in smooth muscle (Kajioka et al. 1991, Beech et al. 1993\textit{a}). However, it appears that there are quite large differences between K\textsubscript{ATP} in different tissues. This section will concentrate on the properties of K\textsubscript{ATP} in smooth and skeletal muscle.

1.3.1 K\textsubscript{ATP} in skeletal muscle
Depletion of intracellular ATP by metabolic exhaustion has long been known to cause a selective increase in membrane K\textsuperscript{+} (Fink and Luttgau 1976). Initially it was supposed that this was secondary to an increase in [Ca\textsuperscript{2+}], and it was not until the patch clamp technique was applied that a direct modulation of K\textsuperscript{+} channels by ATP was demonstrated (Spruce et al. 1985).
Further experiments on metabolically exhausted frog skeletal muscle confirmed that the increase in $P_K$ seen under these conditions was largely due to the activation of $K_{ATP}$ (Castle and Haylett 1987). $K_{ATP}$ has now been identified in mouse (Weik and Neumcke 1989) and human skeletal muscle (Burton et al. 1988).

1.3.1.1 Biophysical properties of $K_{ATP}$ in frog skeletal muscle

The biophysical properties of single $K_{ATP}$ channels in patches excised from vesicles of frog skeletal muscle have been studied in some detail by Stanfield and co-workers (Spruce et al. 1985, 1987, Standen et al. 1985, Quayle et al. 1988). When $[K]_i=140$ mM and $[K]_o=60$ mM the unitary conductance of the channels was 42 pS. This was reduced to 15 pS when $[K]_o=2.5$ mM. Unitary conductance was found to saturate as $[K]_o$ was increased.

Measurement of reversal potential in solutions containing different proportions of Na$^+$ and K$^+$ showed that $P_K/P_{Na} = 0.015$. The channels are much less permeable to Rb$^+$ than K$^+$ and blocked by Cs$^+$ and Ba$^{2+}$. The voltage dependence of the block by external Cs$^+$ and Ba$^{2+}$, together with the saturation of unitary conductance at high $[K^+]_o$, suggests that the channels is a multi-ion channel with single-file diffusion properties. The channels have complex kinetics, with openings occurring in bursts, suggesting at least three closed states and two open states.

1.3.1.2 Effect of ATP and other nucleotides on $K_{ATP}$

The principal characteristic of $K_{ATP}$ is that channel activity is inhibited by in the presence of intracellular ATP. In the frog half-maximal inhibition occurs with 135 μM ATP (Spruce et al. 1987) and the concentration inhibition curve has a Hill slope of unity. This is in contrast to $K_{ATP}$ in ventricular myocytes where a Hill slope of 3-4 has been reported (Kakei et al. 1985).

In the frog, channel opening is inhibited by ADP and AMP, albeit at higher concentrations than ATP and there appeared to be no interaction between ATP and ADP (Spruce et al. 1987). This is in contrast to findings with ventricular myocytes and β-cells where in the presence of inhibitory concentrations of ATP, ADP causes channel activation (Dunne and Petersen 1986, Kakei et al. 1986). However, subsequent workers (Vivaudou et al. 1991, Forestier and Vivaudou 1993) found that, in the presence of Mg, ADP does reduce the effectiveness of inhibition by ATP. In mouse skeletal muscle, Lazdunski and co-workers
(Allard and Lazdunski 1992) reported that both ADP and GDP enhanced channel activity in the absence of ATP.

1.3.1.3 Regulation of $K_{\text{ATP}}$ by pH

In addition to modulation by nucleotide phosphates, it appears that $K_{\text{ATP}}$ is also sensitive to intracellular pH. In frog skeletal muscle alterations of pH$_i$ have little effect on the activity of channels in the absence of ATP, however, in the presence of inhibitory concentrations of ATP reducing pH$_i$ causes a marked increase in $P_{\text{open}}$ (Davies 1990, Davies et al. 1992). From these experiments it would appear that protons may actually inhibit the binding of ATP to its inhibitory site. The relationship between pH$_i$ and $P_{\text{open}}$ suggests that two protons bind to inhibit the action of ATP with an equilibrium constant of 0.11 μM. This is close to the normal value of [H$^+$]$_i$ of 0.085 μM. Thus the activity of $K_{\text{ATP}}$ would be expected to be very sensitive to changes in pH$_i$. Indeed, it has been found that pH$_i$ may change from 7.3 to 6.1 during fatigue (Renaud 1989). In intact muscle it has been shown that intracellular acidosis induces a glibenclamide sensitive current (Standen et al. 1992) suggesting that pH$_i$ may indeed regulate $K_{\text{ATP}}$ in physiological circumstances.

1.3.1.4 Blockers of skeletal muscle $K_{\text{ATP}}$

$K_{\text{ATP}}$ can be inhibited by a number of agents. In a comprehensive study of K$^+$ blockers on $^{86}$Rb efflux from frog semitendinosus, (Castle and Haylett 1987) found that $K_{\text{ATP}}$ was sensitive to a wide range of known K$^+$ channel blockers including quinine, TEA, Ba$^{2+}$ and also local anaesthetic agents such as lignocaine. The most potent blockers of $K_{\text{ATP}}$ are the sulphonylurea oral hypoglycaemic agents. Block of $K_{\text{ATP}}$ by these agents was first described in the pancreatic β cell (Sturgess et al. 1985) and this finding was extended to $K_{\text{ATP}}$ in heart and skeletal muscle. Sensitivity to glibenclamide has come to be considered as diagnostic of $K_{\text{ATP}}$ although other actions of glibenclamide have been described, for example, antagonism of thromboxane A$_2$ (Cocks et al. 1990) and block of certain chloride channels (Sheppard and Welsh 1992). In excised patches the onset of action of glibenclamide is slow and appears to reduce $P_{\text{open}}$ by inducing a long closed state.

Glibenclamide has been shown in ligand binding studies to bind with high affinity to membrane preparations from heart, pancreas and CNS tissue. However, no accounts of binding to skeletal muscle have been published (see Ashcroft & Ashcroft (1992) for review).
1.3.2 $K_{ATP}$ in smooth muscle

The first report of $K_{ATP}$ in vascular smooth muscle was published by Standen and co-workers (Standen et al. 1989). These channels had a very high unitary conductance (150 pS in symmetrical 140 mM $K^+$). Subsequently Nelson (Kovacs and Nelson 1991) reported that similar channels could be detected in artificial lipid bilayers containing material from cell membranes from canine aorta. However, many other workers have been unable to detect these channels.

Van Breemen and co-workers (Gelband et al. 1990, Groschner et al. 1991) described large conductance channels sensitive to both Ca$^{2+}$ and ATP in tracheal smooth muscle. However, it is now accepted that the apparent sensitivity of these channels to ATP was an artefact arising from the ability of ATP to chelate Ca$^{2+}$ (Klockner and Isenberg 1992) and that they were in fact BK channels. It seems possible that the ATP-sensitive channels described by Standen et al. may also in fact have been BK channels.

Attempts to identify $K_{ATP}$ in smooth muscle by the use of metabolic inhibitors to deplete ATP have met with mixed success. Using pulmonary arterial myocytes, Clapp & Gurney (1992) found that depletion of ATP caused the activation of a glibenclamide-sensitive current which could be blocked by flash photolysis of caged ATP. Similarly, treatment of cells isolated from rat portal vein with metabolic inhibitors also caused the activation of glibenclamide-sensitive $K^+$ currents (Noack et al. 1992c).

Kajioka et al. (1991) described the activation by pinacidil of a small conductance channel which was sensitive to ATP but ran down almost immediately in the absence of GDP. In keeping with this, Beech et al. (1993a) have described a 24 pS channel in rabbit portal vein which could be activated by intracellular dialysis of nucleoside diphosphates in the presence of Mg$^{2+}$. The channel was also inhibited by intracellular ATP, though a reduction in ATP alone was not sufficient to activate the channels. Thus, although these channels have some similarities to $K_{ATP}$, there are clearly major differences. Accordingly, Beech et al. referred to them as $K_{NDP}$ in order to highlight the role of GDP in channel modulation. Further experiments showed that levcromakalim activated the same current. The dependence of these channels on nucleoside diphosphates may explain why these channels have not previously been detected.
1.4 The Pharmacology of K⁺ Channel Openers

K⁺ channel openers are a structurally diverse group of compounds which are characterised by an ability to cause the opening of plasmalemmal K⁺ channels (Hamilton and Weston 1989). The therapeutic potential of these agents in the treatment of hypertension and bronchial asthma has stimulated extensive synthetic programs aimed at discovering new KCO's as well as many pharmacological studies of the mechanism of action of these compounds.

The first KCO to be described was BRL34915 (cromakalim) (Hamilton et al. 1986, Weir and Weston 1986a, b). The observations which suggested that cromakalim may act by causing the opening of K⁺ channels were that the vasorelaxant action was accompanied by cessation of spontaneous electrical activity, membrane hyperpolarisation and an increase in ⁸⁶Rb efflux. Further evidence of K⁺ channel opening was supplied by the observation that cromakalim was unable to cause relaxation in preparations contracted with high KCl (>30 mM) (Cook et al. 1988). This finding is consistent with a mechanism involving membrane hyperpolarisation and clearly sets cromakalim apart from other vasodilators such as calcium entry blockers and activators of guanylate cyclase. After the activity of cromakalim as a K⁺ channel opener was described it was established that a number of other compounds previously classified as directly acting vasodilators such as diazoxide, nicorandil and minoxidil sulphate were also capable of opening K⁺ channels. Completely novel compounds such as RP49356 were also shown to have similar actions.

The KCO's fall into six main groups: benzopyrans (e.g. levocromakalim), thioformamides(RP49356), benzothiadiazines(diazoxide), guanidine (pinacidil) pyridines (nicorandil) and pyrimidines ( minoxidil sulphate). The structures of the prototype members of these groups are shown in Fig. 1.1

After the discovery of the actions of cromakalim in vascular and gastrointestinal muscle, similar actions were described in a wide variety of other tissues including heart (Osterreider 1988, Escande et al. 1989), skeletal muscle (Spuler et al. 1989) and hippocampal neurones (Alzheimer and ten-Bruggencate 1988). This section will focus on the activity of KCO's in smooth and skeletal muscle, which were the tissues used in the present work.
Fig. 1.2 Structures of prototype members of the 6 classes of KCO. 
A levcromakalim (benzopyran). B RP 52891 (aprikalim) (thioformamide) 
C Pinacidil (guanidine). D Diazoxide (benzothiadiazine). E Minoxidil sulphate 
(pyrimidine). F Nicorandil (pyridine). Note that nicorandil contains a nitro group which 
confers the ability to cause relaxation via activation of guanylate cyclase. Accordingly 
these compounds are sometimes referred to as being 'mixed action' vasodilators.
1.4.1 Effects of KCO's in skeletal muscle

An effect of cromakalim on skeletal muscle was first reported by Spuler and colleagues (Quasthoff et al. 1989, Spuler et al. 1989). Working with biopsy samples of human skeletal muscle, these authors demonstrated a hyperpolarisation and decrease in input resistance in response to cromakalim. The effect of cromakalim was inhibited by tolbutamide. Pinacidil and RP 49356 were shown to have a similar effect. Cromakalim also repolarised fibres taken from patients suffering from periodic hyperkalaemic paralysis, indicating a possible therapeutic application. Further, Neumcke et al (1990) have shown that cromakalim, RP49356 and pinacidil can activate identified ATP-sensitive K channels in excised patches of membrane from mouse skeletal muscle. In the frog SR44866 (a member of the benzopyran family of KCO's) activated ATP-sensitive channels in excised patches and stimulated an outward current in fibres held at 0 mV in a vaseline gap voltage clamp (Sauviat et al. 1991). This current reversed close to $E_K$ and had a linear I-V relationship. SR 44866 caused a reduction in action potential duration and at high concentration caused a reduction in action potential height. This latter effect was accompanied by a reduction in twitch size and was indistinguishable from the effects of inhibition of sodium currents. However this effect of SR44866 was also partially reversed by glibenclamide.

In addition to activating $K_{ATP}^{\text{inside out patches from human sarcolemmal vesicles,}}$, cromakalim and the KCO's RP49356 and EMD 52692 activated a second, outwardly rectifying channel which was not blocked by ATP but which was only seen in its presence (Quasthoff et al. 1990).

In mouse diaphragm, Hong and Chang (1991) reported that levocromakalim (100 μM) did not hyperpolarise innervated muscle unless the fibre was depolarised by current injection to less than 60 mV. At this potential levocromakalim caused a decrease in input resistance and hyperpolarisation. In contrast, denervated muscle, which has a lower resting potential responded to levocromakalim without the need for depolarisation.

1.4.2 Smooth Muscle

Although the properties of KCO's were first described in smooth muscle preparations it is in
this tissue that there has been most doubt as to the identity and indeed the number of types of channels involved.

1.4.2.1 Mechanism by which KCO's cause relaxation

There is a good correlation between the ability of different KCO's to cause smooth muscle relaxation and to increase \( P_k \) (as measured by studies of tracer efflux) (Cook and Quast 1990). However many authors have noted that there is a discrepancy between the concentrations required to cause tracer efflux and relaxation. Furthermore, these compounds are able to inhibit electrically silent contractions (e.g. contraction of rabbit aorta by noradrenaline).

Under these circumstances it is not immediately obvious why an increase in \( P_k \) should lead to relaxation and many authors have been led to suggest mechanisms other than \( K^+ \) channel opening. There is, however, some evidence to suggest that KCO's can inhibit IP\(_3\) turnover, albeit in a way that is as yet unclear, and that this is linked to membrane hyperpolarisation since the effect is blocked in \( K^+ \) rich solution and by \( K^+ \) channel blockers (see Quast (1993) for a recent review). The question of whether or not the relaxant action of KCO's is exerted solely through the activation of \( K^+ \) channels is still, therefore, unresolved.

1.4.2.2 Blockers of the effects of KCO's in smooth muscle

A number of agents have now been shown to inhibit the effects of KCO's, presumably by inhibiting the opening of \( K^+ \) channels, and the use of previously characterised \( K^+ \) channel blockers has yielded some evidence about the identity of the channels involved.

1.4.2.2.1 Glibenclamide and sulphonylureas

The finding that the effects of cromakalim could be reversed by glibenclamide (Quast and Cook 1988b) rapidly led to the suggestion that KCO's might act at an ATP-sensitive channel although at the time there was no direct evidence that such channels existed in smooth muscle. Although the concentrations required to inhibit KCO activity in smooth muscle are higher than required for inhibition of \( \beta \)-cell ATP-sensitive \( K^+ \) channels there is a good correlation between the ability of a wide range of these compounds to displace binding of \(^3\text{H}\)-glibenclamide from RIN 5mf cells and to inhibit cromakalim stimulated Rb efflux (Cook and Quast 1990). This suggests that a similar site may be involved.
Fig. 1.3 Structure of some compounds which inhibit the actions of KCO's in smooth muscle. A glibenclamide. B tedisamil. C guanethidine. D ciclazindol. E phentolamine.
In tension recording studies, glibenclamide appears to inhibit the action of cromakalim in a competitive manner (Eltze 1989) and pA₂ values in the range of 7-6 have been reported. It has, therefore been suggested the sulphonylureas and cromakalim may act at the same or a closely related site. The limited solubility of cromakalim, however, means that the range of concentrations which can be tested is limited and these experiments have not been performed using more direct measurements of K⁺ channel opening such as changes in tracer efflux or input resistance. These data should, therefore be regarded with caution.

1.4.2.2.2 Tedisamil
Tedisamil is a novel bradycardic agent which is thought to act by blocking K channels. It has been shown to inhibit $^{86}$Rb efflux stimulated by levcromakalim with an IC₅₀ of 50 nM (Bray and Quast 1991, 1992, Kreye et al. 1992).

1.4.2.2.3 Ciclazindol
Ciclazindol has been found to inhibit the effects of levcromakalim in rat portal vein (Noack et al. 1992b). In experiments on intact portal vein it caused an non-parallel shift in the dose response curve for levcromakalim and at 3 μM caused complete inhibition of levcromakalim-stimulated $^{42}$K efflux. In experiments using whole cell recording ciclazindol blocked both levcromakalim-stimulated current ($I_{K(KCO)}$) and the delayed rectifier current ($I_{K(V)}$). The IC₅₀ for inhibition of $I_{K(KCO)}$ was 2.5 μM whereas the IC₅₀ for inhibition of $I_{K(V)}$ was 20 μM.

1.4.2.2.4 Guanethidine and bretylium
The adrenergic neurone blocking agents have been found to block the actions of KCO's in vascular, gastrointestinal, vascular and tracheal smooth muscle at high micromolar concentrations (Berry et al. 1992a, b). Guanethidine and bretylium caused almost complete inhibition of levcromakalim-stimulated $^{86}$Rb efflux at 50 μM.

1.4.2.2.5 Phentolamine
The non-selective α-adrenoceptor antagonist phentolamine has been shown to inhibit the relaxation caused by cromakalim in rat aorta with an pD₂ of 5.2 (McPherson and Angus...
1989). The rank order of potency for a series of related \( \alpha \)-adrenoceptor blockers was different from that for \( \alpha \)-blockade suggesting that the two actions are unrelated.

1.4.2.6 Toxins

Crude venom of the scorpion *Leirus quinquestriatus* was found to block the action of cromakalim in rat portal vein (Quast and Cook 1988a) leading to the suggestion that the K\(^+\) channel acted on by cromakalim was sensitive to charybdotoxin. Subsequent purification of the toxin showed, however, that the active component of the venom was not charybdotoxin but a minor component which may in fact be leiurotoxin I (Strong et al. 1989).

1.4.2.3 Target channel of KCO's in vascular smooth muscle

As already noted, the precise identity of the channel or channels at which KCO's act is as yet unclear. Though the effectiveness of the sulphonylureas suggested a role for ATP-sensitive channels, the existence of these channels in smooth muscle is controversial and evidence has been presented to suggest the involvement of other channel types. This will now be considered.

1.4.2.3.1 Calcium-activated channels

A number of authors have reported that KCO's can activate BK channels in excised patches (e.g. Klockner et al. (1989)). However, in experiments on intact smooth muscle and in intact cells charybdotoxin has been found to have no effect of the actions of KCO's (Strong et al. 1989, Winquist et al. 1989). In addition, noise analysis of whole cell currents has indicated that the major channel acted on by the KCO's has a relatively small conductance (15-25 pS) (Noack et al. 1992a). It seems likely, therefore, that even if KCO's do activate BK under some conditions this is not their major effect.

1.4.2.3.2 \( K_{\text{ATP}} \)

The finding that the actions of KCO's in smooth muscle are antagonised by glibenclamide and other sulphonylurea drugs (Quast and Cook 1988b) led to the suggestion that these compounds act on \( K_{\text{ATP}} \) as they have been shown to do in other tissues. However, as has been discussed above the precise nature of \( K_{\text{ATP}} \) in smooth muscle has been the subject of some controversy. Experiments by Weston and co-workers (Noack et al. 1992a, c) showed that levocromakalim and metabolic inhibition induced time-independent K\(^+\) currents which, by
noise analysis, appeared to have similar unitary conductances. Bolton and co-workers (Beech et al. 1993b) reported that levcromakalim and intracellularly applied GDP induced whole cell currents with similar characteristics. They also showed in excised patches the activation by these agents of small conductance, ATP-inhibited channels.

It seems likely, therefore, that the main target of KCO's in smooth muscle is a small conductance channel, $K_{NDP}$, which is similar to $K_{ATP}$ but which nevertheless differs in some important respects.

1.4.2.3.3 The 'delayed rectifier hypothesis'

A number of reports using the whole cell patch recording technique have reported that the activation of $K^+$ current by KCO's is accompanied by inhibition of the delayed rectifier current, $I_{Kv}$ (Beech and Bolton 1989). In addition it was noted that the sensitivities of the two currents to blocking agents, with the exception of glibenclamide, were remarkably similar. These findings lead to the suggestion that in smooth muscle cromakalim acted to alter the gating properties of $K_v$.

Weston and co-workers (Edwards et al. 1993) reported that activators of protein kinase A inhibited the action of levcromakalim whereas the 'de-phosphorylating agent' butanedione monoxime mimicked it. This led them to suggest that the KCO's act by de-phosphorylating $K_v$ which causes it to lose its voltage dependence and acquire the characteristics of $K_{ATP}$ (i.e. sensitivity to ATP, and sulphonylureas.) This group went on to show that a similar phenomenon could be demonstrated in the insulin secreting cell line RIN5MF (Edwards and Weston 1993a) and more recently it has been found that both levcromakalim and metabolic inhibition cause a reduction in $I_{Kv}$ in ventricular myocytes (Heath and Terrar 1994).

The proposition that $K_{ATP}$ is really a modified form of $K_v$ is controversial (see Ashcroft et al. (1994) and Edwards and Weston (1994)) and the recent cloning of a rat heart $K_{ATP}$, RCK$_{ATP}$-1 (Ashford et al. 1994), which appears to have little resemblance to any of the cloned voltage dependent channels, makes it seem unlikely.

Further evidence against the delayed rectifier hypothesis comes from work by McHugh and Beech (1995). These workers have reported that in jejunal smooth muscle cells
levcromakalim does indeed appear to inhibit \( K_V \) at the same time as inducing a time and voltage independent \( K^+ \) current. However, \( Cs^+ \) and \( Rb^+ \) discriminated between \( I_{K\text{KCO}} \) and \( I_{K(V)} \). Furthermore, it was found that only outward current carried by \( K_V \) was inhibited; in experiments in which inward \( K_V \) currents were studied it was found that levcromakalim caused no attenuation, despite the fact that \( K_{\text{KCO}} \) was still activated. This led the authors to suggest that the apparent inhibition of \( K_V \) by levcromakalim arises secondary to depletion of \( K^+ \) from a sub-membrane pool. If such depletion did arise from an increase in \( P_K \) induced by KCO's then attenuation of \( K_V \) would be expected.

### 1.4.3 Site and Mechanism of action of KCO's

Little is known of the mechanism of action of \( K^+ \) channel openers. Early experiments showed that cromakalim did not increase cGMP or cAMP or interact with protein kinase C (Coldwell and Howlett 1987, Coldwell 1988) or depend on pertussis toxin sensitive G-proteins (Quast et al. 1988). The fact that they are active in cell free patches seem to indicate that they act at a site which is closely linked to the channel even if it is not the channel itself. However, since the compounds are active when applied to either the extra-cellular or the intracellular surface of the membrane the location of the binding site remains uncertain.

Early attempts to demonstrate binding of \(^3\text{H}\)-cromakalim proved unsuccessful more recently however, high affinity binding of \(^3\text{H}\)-P1075 to rat aorta has been demonstrated (Quast et al. 1993). These results showed that binding of P1075 was competitively inhibited by members of all families of KCO's and there was a good correlation between pKi for binding and ability to increase \(^{86}\text{Rb} \) efflux. Studies of the dissociation of P1075 from its receptor showed first order kinetics with a half-life of 19 min. This result was obtained irrespective of which KCO was used as the displacing ligand. This data strongly suggests that, at least in this tissue, KCO's have a common site of action and that the high affinity binding site for \(^3\text{H}\)-P1075 is indeed the binding site for channel activation. The oral hypoglycaemic sulphonylureas glibenclamide, glipizide and AZ-DF 265 were also able to displace binding of P1075, however, studies of the dissociation kinetics of P1075 binding showed that glibenclamide caused a dose-dependent decrease in the half-life of dissociation. This finding suggests an allosteric interaction between the binding site for KCO's and for sulphonylureas. It is to be hoped that the availability of a radio-labelled ligand will allow further biochemical
characterisation of the KCO binding site and help to establish how closely linked it is to the channel.

The discovery of novel blockers of KCO-activated channels should prove useful in the further characterisation of the actions of KCO’s in different tissues. In Chapters 9 and 11 attempts to characterise KCO activated channels in smooth and skeletal muscle by using K⁺ channel blockers are described.
Section II

Methods
2 MEASUREMENT OF K⁺ LOSS FROM ERYTHROCYTES

2.1 Preparation of red blood cells
Blood (2-5 ml) was withdrawn from the ear vein of adult New Zealand White rabbits and mixed with heparin (5 units/ml whole blood). It was stored at 4°C for up to 2 hours until used. Human blood was withdrawn from the median cubital vein and treated in a similar manner.

The blood was centrifuged (3 min at 1600 g) and the supernatant and buffy coat aspirated. The packed cells were then resuspended in 5 volumes of a solution containing (in mM): NaCl 145, KCl 0.1, EGTA 1, TRIS 10, inosine 10, pH adjusted to 7.4 by adding 1 M NaOH. This suspension was centrifuged and the pellet re-suspended twice. The cells were stored as a pellet in this solution at 4°C for up to 3 days.

2.2 Preparation of K⁺ sensitive electrodes
Potassium loss from a suspension of erythrocytes at 37°C was followed by measuring the resulting changes in extracellular [K⁺] using valinomycin-based K⁺ sensitive electrodes. The potassium selective membranes were prepared using the method described by Hill et al. (1978) and Castle (1987). The membrane mixture was prepared by dissolving 4 mg valinomycin, 1 mg sodium tetraphenylborate and 25 mg high molecular weight PVC in 1 ml tetrahydrofuran and 75 μl dibutyl sebacate. This mixture was stored at -20 °C.

Membranes were formed by applying 5 μl of mixture to filter paper discs inserted into the end of a short length (∼ 2 cm) of PVC tubing (2.0 mm i.d.). After the solvent had evaporated a further 5 μl was applied and the membranes were allowed to dry for at least a further 2 hours. The dry membranes could be stored in a dessicator at 4 °C for several weeks. When required for use the PVC tube carrying the membrane was filled with electrolyte (145 mM NaCl, 5.4 mM KCl) and connected to a second tube containing an Ag/AgCl bead. The reference electrode was formed from a tube containing a similar bead and filled with a salt solution identical to the one in which the cells were suspended.
The P.D. across the electrodes was measured using a high impedance amplifier (WPI F-223 A) and displayed on a pen recorder (Kipp & Zonen BD 100).

The recording chamber comprised a water jacketed glass bath containing 2 ml of solution which was stirred by a small magnetic flea driven by a hydraulic magnetic stirrer placed below the chamber.

2.2.1 Calibration of K⁺ sensitive electrodes

Electrodes were calibrated by cumulative addition of aliquots of a solution containing 145 mM NaCl and either 10 mM or 100 mM KCl. For experiments in 0.1 mM K⁺ the calibration range was from 0.1 to 2.7 mM and for experiments in 5.4 mM K⁺ a range from 5.4 to 22 mM was used.

For a perfectly selective electrode the P.D. developed (E) should be determined by the Nernst equation:

\[ E = \frac{RT}{F} \log_e \frac{a_{K^+ \text{bath}}}{a_{K^+ \text{electrode}}} \]

where,  
\( R = \) universal gas constant 
\( T = \) absolute temperature 
\( F = \) Faraday's constant 

and \( a_{K^+ \text{bath}} \) and \( a_{K^+ \text{electrode}} \) are the K⁺ activities on either side of the membrane.

Thus, assuming activity can be replaced by concentration for the present purposes, a ten-fold change in potassium ion concentration at 37 °C should produce a change in potential of 61.5 mV. Since the potassium concentration at the inner face of electrode was constant at 5.4 mM, calibration data were in practice fitted using a least squares method to the equation:-
\[
E = k \log [K^+]_{bath} + C
\]

where, \( k = \text{slope, formally equivalent to } \frac{RT}{F} \)
and \( C = \text{the intercept on the abscissa} \)

Observed values of \( k \) and \( C \) were used to calculate potassium concentrations in the experiment proper. Figure 2.1 shows typical a calibration curve for an electrode to be used in 0.1 mM K\(^+\) solution.

A complicating factor was that the presence in the bathing solution of compounds containing a quaternary ammonium group was found to cause a shift in potential and to reduce the potassium sensitivity of the electrode, presumably because the electrode responded to such cations. This was allowed for by re-calibrating the electrode in the presence of the appropriate concentration of the agent being tested.

### 2.3 Measurement of K\(^+\) loss initiated by the calcium ionophore A23187

In these experiments the bathing solution contained (in mM) NaCl 145, MgSO\(_4\) 1, CaCl\(_2\) 1, inosine 10, TRIS 10. pH was adjusted to 7.4 by addition of 1 M NaOH. The concentration of KCl was usually 0.1 mM, though sometimes 5.4 mM (see later). Cells were suspended in 2 ml of solution to give a final haematocrit of 1%. When the K\(^+\) sensitive electrode gave a stable reading K\(^+\) loss was stimulated by the addition of 2 \( \mu \)M A23187. This concentration caused a rapid response and was probably supra-maximal.

Preliminary experiments showed that, even after washing the bath twice with saline solution, sufficient ionophore remained to cause a slow loss of K\(^+\) from the next batch of cells. This problem was overcome by washing the bath with methanol between responses. 3 min after the application of A23187, digitonin (100 \( \mu \)M) was added to cause cell lysis and so allow the total available potassium to be estimated (Cook 1983).

The size of the response was calculated as the increase in extracellular potassium concentration three minutes after addition of A23187 expressed as a percentage of the total increase after addition of digitonin. This is equivalent to the quantity of K\(^+\) released by A23187 as a percentage of total K\(^+\) content of the cells (see Fig. 2.2 for further explanation).
Fig. 2.1 Typical calibration curve for a $K^+$-sensitive electrode. Linear regression shows that a ten-fold increase in $[K^+]$ causes a 59 mV change in P.D. as compared with 61.5 mV predicted by the Nernst equation.
Fig. 2.2 Schematic representation of potential changes during ionophore stimulated K release from erythrocytes. Addition of A23187 causes loss of K from the cells in suspension. After three minutes addition of digitonin causes lysis of the cells and total release of K. Bath [K] is measured at points a, b and c. The size of the response is expressed as:

\[
\%K^+\text{loss} = 100 \times \frac{(b-a)}{(c-a)}
\]
2.4 Activation of $K_{Ca}$ by Lead ions

The cells were treated exactly as for ionophore experiments. Lead II nitrate was added to the bath to give a nominal $Pb^{2+}$ concentration of 20 μM. Lead ions are extensively bound by red blood cell proteins (see e.g. Simons, 1993) so the free concentration was unknown. Since the effect of lead was slower than ionophore and had a greater latency the $K^+$ loss was measured after 5 min. It was found that prolonged exposure to lead had a deleterious effect on the potassium sensitive electrodes which could be prevented by washing the bath with a solution containing $10^{-5}$ M EDTA.

2.5 Preparation of buffered calcium solutions

Buffered calcium solutions were prepared in a solution containing (in mM) NaCl 145, KCl 0.1, MgSO$_4$ 1, inosine 10, HEPES 10, EGTA 5, pH 7.4. The desired Ca$^{2+}$ concentration was attained by adding the appropriate volume of 1 M CaCl$_2$ and re-adjusting pH to 7.4 with 1 M NaOH. Free Ca$^{2+}$ concentrations were calculated using the program REACT (by G.L. Smith) based on affinity constants published by Smith and Miller (1985).

2.6 Effect of blocking agents on the $K^+$ release caused by the calcium ionophore A23187

Test substances were added to the bath directly for an equilibration period (usually 3 min) before the addition of either ionophore or lead to initiate $K^+$ release via $K_{Ca}$. In experiments requiring longer equilibration periods the cells and test substance were mixed in a glass vial and placed in a shaking water bath at 37 °C for the appropriate time before transfer to the recording chamber.
3. **RUBIDIUM EFFLUX STUDIES WITH SKELETAL AND SMOOTH MUSCLE**

The effects of potassium channel openers and of metabolic inhibition were studied in smooth and skeletal muscle preparations by following changes in efflux of $^{86}$Rb, serving as a marker for potassium. The method used was similar for all the series of experiments and so they can be described together.

### 3.1 Muscle preparations

#### 3.1.1 Frog Skeletal Muscle

Most experiments were carried out on the sartorius muscle, though the semitendinosus was used in some preliminary work.

Adult *Rana temporaria* were killed by a blow to the head followed by destruction of the brain and spinal cord. Sartorius muscles were dissected out with the aid of a microscope since the fibres are particularly vulnerable to mechanical damage, especially along the edge and at the insertion into the pubis. It was usually possible to obtain both sartorii from a single animal.

The semitendinosus is a two headed muscle; in these experiments only the anterior head was used.

#### 3.1.2 Mouse Skeletal Muscle

Adult male mice were used in all experiments. The mice were killed by a lethal injection of sodium pentobarbitone. In the studies on potassium channel openers and in the initial work on metabolically poisoned muscle, soleus from BALB/c mice were used. In a few experiments strips of mouse diaphragm were used. Four strips of tissue $\approx 5$ mm wide were cut from the medial portion.

#### 3.1.3 Rat Aorta and Annococcygeus

Male Sprague Dawley rats (200-300g) were filled by a blow to the head followed by exsanguination. The thoracic aorta was removed and fat dissected off. The aorta was
then cut into 4-6 rings which were cut longitudinally to form strips. The endothelium was removed by gently rubbing the luminal surface with a piece of moistened cotton wool.

In experiments using annococcygeus, both muscles were removed and each was cut into two pieces.

3.2 Solutions and experimental conditions

Experiments on frog skeletal muscle were conducted at room temperature (20-25 °C). Preliminary experiments examining the effects of potassium channel openers on mammalian muscle were carried out at room temperature but later experiments were performed at 37 °C (see discussion). All experiments on metabolically inhibited muscle and on rat aorta were carried out at 25 °C and 37 °C respectively.

The frog Ringer had the following composition (in mM): NaCl 116, KCl 2.5, CaCl₂ 1.8, NaH₂PO₄ 0.25, Na₂HPO₄ 1.75. The pH was 7.2.

Mammalian muscle was bathed in a modified Krebs' solution containing (in mM): NaCl 152, KCl 4.62, MgSO₄ 1.2, NaH₂PO₄ 1.2, CaCl₂ 2.5, glucose 11, HEPES 8. The pH was adjusted to 7.4 by the addition of approximately 4 ml of 1 M NaOH per litre of solution. In experiments studying the effects of metabolic inhibition on $^{86}$Rb efflux from mammalian muscle 1 mM iodoacetic acid and 2 mM NaCN were added to the solution.

Preliminary experiments on rat aorta used the same solution as was used for mammalian skeletal muscle. Later experiments were carried out using a Krebs' solution composed of (in mM): NaCl 118, KCl 4.62, MgSO₄ 1.2, NaH₂PO₄ 1.2, CaCl₂ 2.5, NaHCO₃ 25, glucose 11. This solution was gassed with 95% O₂/CO₂ to maintain pH at 7.4

3.3 'Loading' of tissue with $^{86}$Rb and preparation of samples

Freshly dissected tissues were tied to frames made from stainless steel tubing, through which gas was passed to provide stirring.
The tissue was loaded with tracer by incubating it in a solution containing $^{86}$Rb at a specific activity of 3 μCi/ml for a period of 90 minutes. The concentration of Rb$^+$ in the load solution did not exceed 100 μM. At the end of the loading period the muscles were washed in a large volume of non-radioactive solution to remove excess tracer from the extracellular space. Thirty minutes after removal from the load the muscles were transferred through a series of test-tubes each containing 5 ml of solution. When a bicarbonate buffered solution was used the tubes were capped immediately after preparation and pre-gassed for 4 minutes before the tissue was placed in them. Finally the tubes were emptied into scintillation vials and 5 ml of 5 mM KCl was used to wash any activity remaining in the tube into the vial.

At the end of the experiment the tissue was blotted dry and dissolved in 0.2 ml of concentrated nitric acid. The extract was then diluted into 10 ml of bathing solution. The difference in quenching between the tissue extract and the other samples was taken into account by internal standardisation ('spiking'). Two 2 ml aliquots of tissue extract were diluted to 10 ml with 5 ml of 5 mM KCl and 3 ml of bathing solution. A known quantity of tracer (20 μl of load solution - the 'spike') was added to tissue extract and to the solution used for the other samples. The observed count was compared to the unspiked tissue sample and a correction factor calculated by the equation:-

$$Q = \frac{m_s - m}{s}$$

where, $m$= cpm in tissue extract  
$m_s$= cpm in tissue extract + spike 
$s$ = cpm in saline + spike

The activity in the samples was determined by counting Čerenkov radiation in a scintillation spectrophotometer (Beckman LS801).

The rate of efflux of expressed as the fractional loss of tracer from the tissue per minute:-
where, \( k \) = the rate constant for tracer efflux

\[
\Delta c = \text{cpm in sample}
\]

\[
c_m = \text{mean cpm remaining in tissue during collection period}
\]

\[
\Delta t = \text{duration of collection period in minutes}
\]

In some instances in which the response to drugs was small relative to variations in resting efflux between preparations the data were normalised by dividing each point by the mean of the 4 rate constants prior to exposure to the drug.

3.4 Design of experiments

3.4.1 Studies on the action of potassium channel openers on \(^{86}\text{Rb efflux from skeletal muscle}\)

Fifteen two-minute collection periods were used in these experiments. The tissue was exposed to the potassium channel opener for a ten minute period beginning 40 minutes after removal from the load solution. In experiments which examined the effect of blocking agents, the blocker was present 20 minutes prior to, and during, exposure to channel opener. One muscle from each pair served as a control while the other was exposed to blocker.

The effect of cromakalim was calculated from the difference between average of the 2nd, 3rd and 4th drug application periods and the average of the three periods immediately prior to drug application. This difference was expressed as a percentage of the pre-drug average. The inhibition by blocking agents was calculated from the percentage difference between control and test preparations. In the case of mouse skeletal muscle it was found that the response peaked during the first exposure period and tended to decline more rapidly than with frog muscle and so the increase was calculated from the mean rate constants for the first and second periods.
In some preliminary experiments the muscle was exposed to cromakalim twice. This approach was quickly abandoned since a marked tachyphylaxis was apparent (see Chapter 12).

### 3.4.2 Rubidium efflux from metabolically inhibited muscle

Twenty two-minute collection periods were used. For the first ten minutes of the experimental period the muscle was exposed to normal solution which was then replaced by 'poisoning' solution containing cyanide and iodoacetate for the remaining 30 minutes. Glibenclamide was added 20 minutes prior to poisoning; all other blockers were present 4 minutes before poisoning. As with the potassium channel opener experiments pairs of muscles were used, one acting as a control, the being exposed to blocker.

Percentage inhibition was calculated from the formula:

\[
\%\text{Inhibition} = \left[1 - \frac{T_e - T_n}{C_e - C_n}\right] \times 100
\]

Here \(T_n\) and \(C_n\) are the means of the two rate constants before poisoning for the test and control muscles respectively, \(C_e\) is the mean of the three peak rate constants for the control during poisoning and \(T_e\) is the mean of the three corresponding points for the test muscle.

### 3.4.3 Effects of potassium channel openers on \(^{86}\text{Rb} \) efflux from smooth muscle

Eleven four-minute collection periods were used. Potassium channel openers were present from between 16 and 28 minutes after the tissue was removed from the load solution. Blockers were present 8 minutes prior to exposure to the potassium channel opener.

The increase in \(^{86}\text{Rb} \) efflux stimulated by potassium channel openers was calculated from the difference between the peak rate during exposure and the mean rate for the two collection periods immediately before exposure. This was expressed as a percentage of
the pre-drug average. In each experiment at least two pieces of tissue were used as controls. Inhibition by test compounds was calculated from the difference between the response in the presence of test compound and the average of the control responses. This difference was expressed as a percentage of the average control response.
4 ELECTROPHYSIOLOGICAL RECORDINGS

The effects of potassium channel openers on skeletal muscle were further investigated using conventional micro-electrode recording techniques which will now be briefly summarised.

4.1 Design of experiments

As has been discussed in the introduction, the opening of potassium channels in a cell membrane causes the membrane potential to shift towards the equilibrium potential for potassium \( E_K \). At the same time the electrical resistance of the membrane would be expected to decrease (though the possible complication of changes in resistance secondary to the alteration of membrane potential has to be kept in mind). It is possible to estimate the input resistance of a large spherical cell by measuring the electrotonic potential produced by the injection of a known current. Because of the cable-like properties of muscle fibres, however, it is not possible to calculate absolute values unless the space constant of the fibre and the separation of the micro-electrodes are known. Nevertheless, monitoring electrotonic potentials can give a useful indication of relative changes in input resistance and this was considered satisfactory for the present work. A more difficult problem is that the chloride conductance of skeletal muscle is high relative to potassium and so may mask changes in membrane resistance secondary to alterations in potassium conductance. Also, chloride ion redistribution can be expected to follow maintained changes in membrane potential (see e.g. Jenkinson & Terrar, 1973). Most experiments were, therefore, carried out in chloride free solution.

4.2 Solutions and recording conditions

4.2.1 Frog skeletal muscle

Sartorius muscles were dissected in normal frog Ringer as described above. The tissue was pinned to a sylgard sheet, with the inner surface uppermost, and held in a perspex perfusion chamber. Solutions were superfused over the tissue at a rate of 4-5 ml/min. Drugs were applied in the superfusing solution, which could be switched between reservoirs by means of a multi-way tap.
The chloride free solution had the following composition (in mM): Na isethionate 116, K$_2$SO$_4$ 1, CaSO$_4$ 2.4, NaH$_2$PO$_4$ 0.25, Na$_2$HPO$_4$ 0.75. The pH was 7.2. Tetrodotoxin ($10^{-7}$ M) was usually included to prevent the initiation of action potentials. All experiments were carried out at room temperature (20-25 °C).

### 4.2.2 Mouse Diaphragm

Preliminary experiments showed that it was not feasible to record from the soleus muscle using the two-microelectrode technique. It was decided, therefore, to use the diaphragm which, being much thinner than the soleus, has better optical properties. The whole diaphragm was removed and narrow strips were dissected from it. Strips of muscle were mounted as described above.

Most experiments were carried out in a chloride free modified Krebs' solution of the following composition (in mM) Na isethionate 116, K$_2$SO$_4$ 2.31, NaH$_2$PO$_4$ 1.18, MgSO$_4$ 1.16, CaSO$_4$ 5.0, NaHCO$_3$ 25, glucose 11. The solution was gassed with 95% O$_2$/5% CO$_2$ to maintain pH at 7.4. Tetrodotoxin ($10^{-7}$ M) was included to inhibit the setting up of action potentials. The experiments were conducted at room temperature (20-25°C). A few experiments were carried out using the HEPES-buffered solution described in section 3.2

### 4.3 Equipment

Conventional microelectrodes were pulled from 1.5 mm o.d. capillary tubing with a filament (Clarke Electromedical) and had resistances in excess of 20 MΩ. Membrane potential was recorded using an Axoclamp 2A amplifier (Axon Instruments). Current pulses were injected from a second microelectrode. Command pulses were supplied from a square pulse generator (Digitimer). Traces were displayed on a digital storage oscilloscope (Gould) and a chart-recorder (Devices). Data were also digitised (Instrutech VR-10) and stored on betamax video tape.
5 ACTIVITY OF THE ENANTIOMERS OF CETIEDIL, UCL1348 AND UCL1349 AS ANTI-MUSCARINIC AND CALCIUM ENTRY BLOCKING AGENTS

5.1 Calcium entry blocking activity in guinea pig taenia caeci

If smooth muscle is depolarised by exposure to a potassium-rich low-calcium solution it initially contracts and then, with washing, relaxes as the extracellular spaces become depleted of calcium. Restoration of calcium to the bathing solution then causes a maintained dose-dependent contracture. This preparation is frequently used as a screen for calcium entry blocking drugs (e.g. Spedding, 1982).

5.1.1 Preparation of tissue and solutions

Adult guinea pigs of either sex were killed by cervical dislocation. The taeniae were dissected away from the wall of the caecum and 1 cm lengths were mounted in a 2 ml organ bath. The tissue was connected to an isometric transducer (Dynamometer UF1) under an initial tension of 1 g. Tension was monitored using a flat bed potentiometric recorder (Servoscribe 1S). The tissue was allowed to equilibrate for 1 hour in a Krebs' solution containing (mM) NaCl 116, KCl 4.6, MgSO$_4$ 1.18, NaH$_2$PO$_4$ 1.16, CaCl$_2$ 2.5, NaHCO$_3$ 25, glucose 11. This was then replaced by a potassium rich solution containing (mM) KCl 123, MgSO$_4$ 1.2, KH$_2$PO$_4$ 1.2, KHCO$_3$ 25, CaCl$_2$ 0.1, glucose 11. Both solutions were gassed with 95 % O$_2$/5 % CO$_2$ to maintain pH at 7.4. The potassium rich solution also contained mepyramine (1 μM) and atropine (1 μM). This was to block the effect of endogenous histamine and acetylcholine, the release of which might be stimulated by addition of calcium.

Preliminary experiments showed that under these conditions using a 15 min dose cycle and 5 min exposure time, application of 0.3 mM and 3 mM CaCl$_2$ reproducibly caused contractions of about 20 and 60 % of maximum. After obtaining reproducible control responses either UCL1348 or UCL1349 was added to the bathing fluid and increasing concentrations of CaCl$_2$ were applied until the original contractions had been restored.

5.2 Anti-muscarinic activity in guinea pig ileum

Sections of ileum were washed by passing Krebs' solution through the lumen and 1 cm
lengths were mounted in 5 ml organ baths under 1 g tension. Contractions were measured using isotonic transducers. The bathing solution had the same composition as the normal solution described in 5.1.2 except for the addition of hexamethonium (10^{-5} M), which was included in order to prevent the stimulation of autonomic ganglia at high concentrations of ACh.

Reproducible concentration-response curves could be obtained by making cumulative additions of acetylcholine (5-500 nM) at 30 s intervals. This was repeated at 15 min intervals.

Concentration response curves were fitted to the Hill equation and dose ratios calculated from estimates of EC_{50}. Concentrations of the enantiomers were chosen which caused similar shifts in the dose response curve.
6 DATA ANALYSIS

Results are presented as means ± standard error of the mean (s.e.m.) with the number of observations in parentheses. Statistical significance was assessed using paired or unpaired t-tests. Differences were considered significant when p<0.05.

Dose-response curves were fitted to the Hill equation (Eq. 1) by the method of least squares using the CVFIT program written by Professor D. Colquhoun.

\[ y = y_{\text{max}} \cdot \frac{[A]^n}{K^n + [A]^n} \]

where:

\[ y = \text{response as percentage of maximum} \]
\[ y_{\text{max}} = \text{maximum response} \]
\[ [A] = \text{concentration blocker/agonist} \]
\[ K = EC_{50} \text{ or } IC_{50} \]
\[ n_h = \text{Hill slope} \]

The program returns estimates of \( y_{\text{max}}, K \) and \( n_h \) with approximate standard deviations for each. The fit was weighted using 1/var calculated from the standard deviation for each data point. When inhibition curves for blocking agents were constructed, \( y_{\text{max}} \) was usually constrained to be 100%. When several curves were fitted simultaneously in order to calculate potency ratios, the simultaneous fits were first compared to separate fits in which \( n_h \) was allowed to vary. A test for deviation from parallelism was then performed. If the curves were parallel then the Hill slope was constrained to be the same for all data sets.
Section III

The effects of cetiedil, quinine, clotrimazole and related compounds on $K^+$ channels in erythrocytes and in smooth muscle.
7 EFFECTS OF CETIEDIL, QUININE, CLOTRIMAZOLE AND RELATED COMPOUNDS ON THE CALCIUM ACTIVATED POTASSIUM PERMEABILITY OF ERYTHROCYTES

In this section data will be presented from experiments in which several cetiedil analogues were tested for their ability to inhibit A23187-stimulated potassium loss from erythrocytes. In addition to the cetiedil series of compounds, clotrimazole and a series of compounds related to quinine were also investigated.

7.1 Preliminary work on the SAR of cetiedil - the influence of log P

Most of the work presented in this chapter is concerned with the structure activity relationship of cetiedil as a blocker of the erythrocyte $K_{Ca}$. This work was part of a long term project and it is useful to begin by describing some of the results that had already been obtained.

Cetiedil (Fig 7.1.1A) is the 2-(N-hexahydroazepinyl)ethyl ester of $\alpha$-cyclohexyl-$\alpha$-(3-thienyl) acetic acid and the aim of first series of experiments was to explore the effect of making different substitutions at the $\alpha$ carbon atom of the acetic acid residue. The general structure of the first series of compounds tested shown in the inset to Fig. 7.1.2. Table 7.1.1 shows the $R$ groups and $IC_{50}$ for each compound in the series. It can be seen from Table 7.1.1 that substituting lipophilic groups increased activity. This was quantified by using Rekker's fragmental hydrophobic constants (Rekker and de Kort 1979). These empirically derived constants allow the log octanol/water partition coefficient (log P) of different compounds to be compared without the need for direct measurements. A more detailed explanation of the use of the fragmental hydrophobic constant is given in Appendix 1.) The value $\Delta \log P$ shown in table 7.1.1 is the sum of the constants for the groups $R_1$, $R_2$, $R_3$ and is therefore an indication of the contribution these groups make to the total lipophilicity of the molecule. The relationship between the observed activity and predicted $\Delta \log P$ is illustrated in Fig. 7.1.2. In keeping with the apparent importance of lipophilicity, a quaternary derivative of cetiedil, UCL 1285 (cetiedil methiodide) (Fig. 7.1.1B) was found to be considerably less active than cetiedil itself, having an $IC_{50}$ greater than 200 $\mu$M.
Table 7.1.1

<table>
<thead>
<tr>
<th>Compound</th>
<th>R₁</th>
<th>R₂</th>
<th>R₃</th>
<th>IC₅₀ (µM)</th>
<th>Δ Log P</th>
</tr>
</thead>
<tbody>
<tr>
<td>UCL 1267</td>
<td>H</td>
<td>thiényl</td>
<td>H</td>
<td>400</td>
<td>2.14</td>
</tr>
<tr>
<td>UCL 1268</td>
<td>C₆H₁₁</td>
<td>H</td>
<td>H</td>
<td>200</td>
<td>3.45</td>
</tr>
<tr>
<td>UCL 1300</td>
<td>phenyl</td>
<td>phenyl</td>
<td>H</td>
<td>170</td>
<td>4.02</td>
</tr>
<tr>
<td>UCL 1269</td>
<td>C₆H₁₁</td>
<td>thiényl</td>
<td>OH</td>
<td>150</td>
<td>3.94</td>
</tr>
<tr>
<td>UCL 1346</td>
<td>C₆H₁₁</td>
<td>furyl</td>
<td>OH</td>
<td>70</td>
<td>3.54</td>
</tr>
<tr>
<td>UCL 1289</td>
<td>C₆H₁₁</td>
<td>phenyl</td>
<td>H</td>
<td>34</td>
<td>5.11</td>
</tr>
<tr>
<td>Cetiedil</td>
<td>C₆H₁₁</td>
<td>thiényl</td>
<td>H</td>
<td>26</td>
<td>4.89</td>
</tr>
<tr>
<td>UCL 1299</td>
<td>C₆H₁₁</td>
<td>C₆H₁₁</td>
<td>H</td>
<td>13</td>
<td>6.20</td>
</tr>
<tr>
<td>UCL 1274</td>
<td>phenyl</td>
<td>phenyl</td>
<td>phenyl</td>
<td>6</td>
<td>5.68</td>
</tr>
</tbody>
</table>
Fig 7.1.1 Structures of A cetiedil, B UCL 1285, C UCL 1274
Fig. 7.1.2 Correlation between $\Delta \log P$ and $-\log IC_{50}$ for compounds with the general structure shown inset. $\Delta \log P$ is calculated from the sum of the hydrophobic fragmental constants for the groups $R_1$-$R_3$. Linear regression performed on the data yields the relationship:

$$-\log IC_{50} = 0.44 \Delta \log P + 2.3$$

$r=0.91$, s.d.=$0.26$
Fig. 7.1.3 Plot of predicted log P versus -log IC$_{50}$ for 38 compounds with the general structure shown inset. Linear regression of the data gives the relationship:

\[-\log IC_{50} = 0.5\log P + 1.8\]

(R=0.9, s.d.=0.33)
The most potent compound in the series was UCL 1274 (Fig. 7.1.1C), an ester of \(\alpha,\alpha,\alpha\), triphenyl acetic acid, which had an IC\(_{50}\) of 6 \(\mu\)M. Thus UCL 1274 is roughly 4 times more active than cetinedil.

In order to investigate the role of lipophilicity further, a large series of esters of acetic acid with the general structure shown in Fig. 7.1.3 have now been tested. The graph in Fig. 7.1.3 illustrates the relationship between log P and \(-\log IC_{50}\). This plot includes data for the compounds discussed later in this section and also a number of others which had been tested by M. Shiner in the preliminary work. There is clearly a very strong correlation and the regression line fitted to the data gave the following relationship:

\[-\log IC_{50} = 0.5\log P + 1.8\]

The relation between log P and IC\(_{50}\) is such that a ten-fold increase in log P causes only a three-fold increase in activity. Since it is not practical to increase the lipophilicity \textit{ad infinitum} the main aim of the present work was to attempt to identify structural features which influence activity.

Accordingly, in the following sections the compounds tested have been divided into small related groups which have been selected to illustrate a particular aspect of the SAR.

The aqueous solubility of some of the compounds to be tested was very low; in some cases this meant that it was not possible to obtain full inhibition of the response to A23187. In this situation it was not considered appropriate to calculate an IC\(_{50}\) by fitting the Hill equation to the data. Instead the concentration of blocker causing 50% inhibition was estimated directly from the concentration-inhibition curve.

Usually the structures of the compounds are given in a general form for each group and the substituents for each compound given in a table of results. IC\(_{50}\) values were calculated by fitting the Hill equation to the data by the method of least squares. In addition, potency ratios were sometimes calculated by fitting several sets of data.
simultaneously. Since this procedure constrains all the curves in a set to have the same slope there is a small discrepancy between the IC$_{50}$ value from the separate fit and that obtained from the simultaneous fit. Where this is the case concentration-inhibition curves show lines for both separate and simultaneous fits.

In order to avoid unnecessary repetition this section does not contain examples of original records: typical traces are shown in section 8.

7.2 Piperidine derivatives with alkyl substitutions

In this series of experiments the N-ethyl piperidine ester of triphenylacetic acid (UCL 1465) (Fig.7.2.1A) was used as a lead compound. This compound has a similar structure to UCL 1274 (Fig.7.1.1C) (the most potent compound from the previous work) but the nitrogen-containing ring is piperidine as opposed to homopiperidine. Part of the reason for this change was that a greater variety of alkyl substituted piperidines is commercially available and so the synthesis of compounds was simplified. The aim was to prepare a series of compounds with alkyl substitutions at different positions on the piperidine ring to find out if structural features played a part in determining activity.

The compounds in this series had the general structure shown in Fig.7.2.2 and table 7.2.1 lists the R group, IC$_{50}$, r (the potency relative to UCL 1465) and the predicted log P for each compound. The concentration-inhibition curves obtained are shown in Fig.7.2.3 and Fig 7.2.4 shows a plot of -log IC$_{50}$ against predicted log P.

As was expected, changing the ring from one containing 6 carbon atoms to one containing 5 caused a small decrease in activity. Conversely, increasing lipophilicity by substitution of one methyl group (as in UCL 1492) or two (as in UCL 1452 and UCL 1451) caused an increase in activity. This is as would be predicted on the basis of log P. However, when two methyl groups were substituted at the two and six positions of the piperidine ring, adjacent to the nitrogen, there was a decrease in activity although log P is unchanged. When two more methyl groups were added at these positions (as in UCL 1472) activity was reduced still further. In fact, this compound had similar activity to UCL 1465 ($r = 1.1\pm0.25$) despite the fact that log P for this compound is considerably
Fig. 7.2.1. Structures of A UCL 1465 and B UCL 1490. Note that to maintain the separation between the N and O of the ester group in UCL 1490, the ring is attached directly to the oxygen (c.f. UCL 1472)
Fig. 7.2.2 General structure of compounds shown in table 7.2.1
Table 7.2.1

<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>R</th>
<th>IC₅₀(μM) (s.d.)</th>
<th>r (s.d.)</th>
<th>Log P</th>
</tr>
</thead>
<tbody>
<tr>
<td>*UCL 1465</td>
<td>N</td>
<td>9.2 (2.95)</td>
<td>1</td>
<td>5.97</td>
</tr>
<tr>
<td>UCL 1492</td>
<td>CH₃</td>
<td>7.1 (n.c.)</td>
<td>0.78 (0.25)</td>
<td>6.67</td>
</tr>
<tr>
<td>*UCL 1452</td>
<td>CH₃ CH₃</td>
<td>2.25 (0.62)</td>
<td>0.23 (0.08)</td>
<td>7.37</td>
</tr>
<tr>
<td>*UCL 1451</td>
<td>CH₃ CH₃</td>
<td>2.25 (0.58)</td>
<td>0.22 (0.07)</td>
<td>7.37</td>
</tr>
<tr>
<td>*UCL 1453</td>
<td>CH₃ CH₄</td>
<td>6.32 (2.2)</td>
<td>0.72 (0.27)</td>
<td>7.37</td>
</tr>
<tr>
<td>UCL 1495</td>
<td>CH₂ CH₃</td>
<td>1.2 (0.08)</td>
<td>0.12 (0.04)</td>
<td>7.53</td>
</tr>
<tr>
<td>*UCL 1472</td>
<td>CH₃ CH₃ CH₃ CH₃</td>
<td>10.2 (4.4)</td>
<td>1.1 (0.25)</td>
<td>8.77</td>
</tr>
</tbody>
</table>

*tested by M. Shiner
n.c. data did not permit estimate of s.d. for separate fit.
Fig. 7.2.3 Concentration-inhibition curves for alkyl substituted piperidine derivatives.

■ UCL 1465, × UCL1472, ◆ UCL 1492, ▲ UCL1453, ▼ UCL 1451, ♦ UCL 1452, • UCL 1495

Data have been fitted to the Hill equation with $B_{\text{max}}$ constrained to 100%. Solid lines show the curves for the simultaneous fit from which potency ratios relative to UCL 1465 were calculated. The dashed lines show curves from separate fits used to estimate IC$_{50}$'s for each compound. The differences between the two curves result from differences in the estimated value of $n_H$ (see sections 6 and 7.1.2 for further details). Vertical bars indicate s.e.m. Each point is the mean of 3-5 observations.
Fig. 7.2.4 Plot of log P against -log IC\textsubscript{50} for piperidine compounds with alkyl substitutions. The dashed line is the regression line from Fig. 7.1.3. For this series of compounds there is no correlation between log P and activity when all the compounds are considered (dotted line). However, when the compounds UCL 1453 and UCL 1472 in which the N is ‘crowded’ by methyl groups are omitted, the correlation becomes much better and the slope of the line is similar to that in Fig. 7.1.3.
greater. It would appear, therefore, that the relationship between activity and lipophilicity breaks down when groups are substituted either side of the nitrogen in the ring. This may indicate that it is important to have an unhindered nitrogen for interaction with the binding site although an effect on the basicity of the nitrogen may also be a factor.

The most potent compound in this series was UCL 1495. Here lipophilicity was increased by substituting an ethyl group at the 5 position of the ring. This presumably allowed an increase in log P without affecting the nitrogen. UCL 1495 was approximately 20 times more potent than cetiedil.

It is clear from these results that although the role of lipophilicity is important in determining activity the relationship no longer holds when substitutions are made near the nitrogen of the piperidine ring.

Since 'crowding' the N of the piperidine ring in UCL 1472 appeared to reduce activity it was interesting to find out if this could be alleviated by preparing the compound UCL 1490 (Fig. 7.2.1B). In this compound there are 4 methyl groups substituted around the N but the compound is a 4-piperidinino ester as opposed to UCL 1472 which is a 2-[N-piperidino]-ethyl ester. Thus UCL 1490 contains a secondary rather than a tertiary N. This change reduces log P to 7.01. The IC\textsubscript{50} for this compound was 12.0 \mu M (see Fig. 7.2.5) compared with the predicted value of 6\mu M. Thus this compound was also less active than predicted by lipophilicity although only by a factor of approximately 2 compared with UCL 1472 which is roughly 10 times less active than predicted. Furthermore, if one assumes the slope of the relationship between log P and -log IC\textsubscript{50} is the same as for the other compounds one would predict that the change in -log IC\textsubscript{50} (\Delta log IC\textsubscript{50}) would be 0.5\times(8.77-7.01)=0.89 and so the predicted potency ratio (IC\textsubscript{50}(1490)/IC\textsubscript{50}(1472)) would be 7.8. The observed potency ratio was in fact 1.3\pm 0.2. Thus it would appear that the difference in activity between these two compounds is considerably less than would be predicted from the change in log P. Thus altering the position of the N in the ring may indeed help to alleviate the effect of crowding.
Fig. 7.2.5 Comparison of the effect of UCL 1472 (■) and UCL 1490 (●). The data for UCL 1472 are the same as shown in Fig. 7.2.3. Solid lines show simultaneous fit, dashed lines show separate fit. The IC_{50} for UCL 1490 from the separate fit is 12.0±4.3 μM and the potency ratio (IC_{50(1490)}/IC_{50(1472)}) is 1.3±0.2. Vertical bars indicate s.e.m. Each point is the mean of 3-5 observations.
7.3 Compounds with polar and non-polar substitutions at position 4 of the piperidine ring

UCL 1475 (Fig. 7.3.1A), in which a benzyl group is substituted at the 4 position of the piperidine ring of UCL 1465 (Fig. 7.2.1A), was found to be quite active with an IC₅₀ of 1.3 µM. In this series of compounds various substitutions were made at the 4 position of the piperidine ring. Unlike the previous series, some of these substitutions included polar groups. The general structure of the compounds is shown in Fig. 7.3.2 and the substituents R₁ and R₂, IC₅₀, r (potency relative to UCL 1475) and predicted log P values for each compound in the series are given in table 7.3.1.

Results with this series of compounds were complicated by the fact that some of them, in particular UCL 1539, had very poor aqueous solubility. This compound caused a maximum of approximately 30% inhibition which did not appear to be concentration-dependent. UCL 1512 and UCL 1510 were also found to give very variable results and caused less than 50% inhibition at 50 µM. The most likely explanation for these observations is that the solubility limit of these compounds is close to the concentration required for channel blockade.

Nevertheless, it is possible to draw some conclusions about the activity of these compounds. Fig. 7.3.3 shows concentration-inhibition curves for compounds for which reliable data could be obtained. A plot of -log IC₅₀ against log P is shown in Fig. 7.3.6. Compounds for which no reliable estimate of IC₅₀ could be made have been excluded. It can be seen that UCL 1475 and UCL 1500 lie close on the predicted line. The prediction is less good however for the three compounds with polar substitutions (UCL 1523 UCL 1506 and UCL 1527). In particular, UCL 1506 is more active than predicted by almost a factor of 6. The IC₅₀ of this compound is similar to that of UCL 1475 despite having a much lower log P value. This suggests that adding polar residues at this position is less detrimental than would be expected. However, because of the limited solubility of these compounds they were not studied further.

Since UCL 1475 was found to be quite active the compound UCL 1489 (Fig. 7.3.1B), in which the phenyl group is substituted onto the N of the piperidine ring and the ester
Fig. 7.3.1 Structures of A UCL 1475 and B UCL 1489
Fig. 7.3.2 General structure of compounds with substitutions at position 4 of the piperidine ring.

**Table 7.3.1**

<table>
<thead>
<tr>
<th>Compound</th>
<th>R&lt;sub&gt;1&lt;/sub&gt;</th>
<th>R&lt;sub&gt;2&lt;/sub&gt;</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt;(µM) (s.d.)</th>
<th>r (s.d.)</th>
<th>Log P</th>
</tr>
</thead>
<tbody>
<tr>
<td>1475</td>
<td>H</td>
<td>CH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>1.33 (0.19)</td>
<td>1</td>
<td>8.15</td>
</tr>
<tr>
<td>1500</td>
<td>H</td>
<td>CH&lt;sub&gt;2&lt;/sub&gt;-CH&lt;sub&gt;2&lt;/sub&gt;-CH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>2.64 (0.47)</td>
<td>2.05 (0.44)</td>
<td>7.552</td>
</tr>
<tr>
<td>1523</td>
<td>H</td>
<td>OCH&lt;sub&gt;2&lt;/sub&gt;-CH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>6.84 (5.25)</td>
<td>5.01 (1.9)</td>
<td>5.759</td>
</tr>
<tr>
<td>1506</td>
<td>CN</td>
<td></td>
<td>1.78 (0.08)</td>
<td>1.34 (0.19)</td>
<td>6.580</td>
</tr>
<tr>
<td>1512</td>
<td>H</td>
<td>Cl</td>
<td>40% inhibition at 20 µM</td>
<td>n.c.</td>
<td>6.580</td>
</tr>
<tr>
<td>1527</td>
<td>OH</td>
<td>Cl</td>
<td>3.43 (0.78)</td>
<td>2.74 (0.66)</td>
<td>6.900</td>
</tr>
<tr>
<td>1510</td>
<td>H</td>
<td></td>
<td>40% inhibition at 10 µM</td>
<td>n.c.</td>
<td>6.249</td>
</tr>
<tr>
<td>1539</td>
<td>C—O—CH&lt;sub&gt;3&lt;/sub&gt;</td>
<td></td>
<td>max inhibition 25 %</td>
<td>n.c.</td>
<td>8.047</td>
</tr>
</tbody>
</table>
Fig. 7.3.3 Concentration-inhibition curves for 4-substituted piperidine series.

UCL 1475 (■), UCL 1506 (▼), UCL 1500 (●), UCL 1527 (◆) and UCL 1523 (▲).

Data have been fitted to the Hill equation. Solid lines show simultaneous fit, dotted lines show separate fits. Vertical bars show s.e.m. Each point is the mean of 3-5 observation.
Fig 7.3.4 Plot of predicted log P against -log IC\textsubscript{50} for 4-substituted piperidine series. The solid line is plotted from data in Fig. 7.1.3. Note that the compounds with a polar substitution, especially UCL 1506, deviate from the predicted value.
linkage is made to the 4 position, was prepared. This compound did not produce 100% inhibition, again because of low solubility. The concentration required for 50% inhibition was 4 μM. This is close to the predicted value of 3.8 μM. It would appear, therefore, that the position of the N in the piperidine ring is not critical.

7.4 Congeners of UCL 1495
Since UCL 1495 (Fig. 7.4.1A) was the most active of the substituted piperidine series it was decided that this structure warranted further study. In this series of experiments 4 analogues of UCL 1495 were prepared. These were UCL 1617 (Fig. 7.4.1B), an amide derivative, UCL 1631 (Fig. 7.4.1C) the quaternary derivative and UCL 1644 and 1645 which were the resolved cis and trans isomers of UCL 1495. These last two compounds were prepared from UCL 1495 by column chromatography and although each was believed to be isomerically pure, it was not known which was cis and which was trans.

The results obtained are shown in table 7.4.1 and the dose response curves in Fig. 7.4.2. UCL 1495 was included for comparison.

The amide UCL 1617 was less active than UCL 1495. However, the reduction in activity was no greater than would be expected from the change in log P resulting from changing the molecule from an ester to an amide. The predicted IC₅₀ was 10.7 μM (c.f. the observed value of 6.6 ± 0.5 μM). It seems likely, therefore, that the reduction in activity results mainly from a change in log P rather than from a requirement for an ester linkage.

Previous work had suggested that UCL 1285, the quaternary derivative of cetiedil had little activity. However, since cetiedil lacks potency (IC₅₀=30 μM) it seemed worthwhile to test the effect of quaternising a more potent analogue and so UCL 1631, the quaternary derivative of UCL 1495 was prepared. UCL 1631 was active with an IC₅₀ of 5.9 μM and the potency ratio relative to UCL 1495 was calculated to be 4.5. Since the IC₅₀ for cetiedil is 30 μM one might, on the basis of the results with UCL 1285, predict that the IC₅₀ of UCL 1285 would be about 140 μM. With this in mind UCL 1285 was
Fig. 7.4.1 Structures of A UCL 1495, B UCL 1617 and C UCL 1631
Table 7.4.1

<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>IC$_{50}$ (s.d.) (µM)</th>
<th>r (s.d.)</th>
<th>log P</th>
</tr>
</thead>
<tbody>
<tr>
<td>1495</td>
<td>1.3(0.23)</td>
<td>1</td>
<td>7.53</td>
</tr>
<tr>
<td>1617</td>
<td>6.6(0.52)</td>
<td>4.3(0.64)</td>
<td>6.34</td>
</tr>
<tr>
<td>1631</td>
<td>5.9(0.99)</td>
<td>4.5(0.74)</td>
<td>n.a.</td>
</tr>
<tr>
<td>1644</td>
<td>2.47(0.8)</td>
<td>1.6(0.22)</td>
<td>7.53</td>
</tr>
<tr>
<td>1645</td>
<td>1.16(0.22)</td>
<td>0.9(0.15)</td>
<td>7.53</td>
</tr>
</tbody>
</table>
Fig. 7.4.2 Concentration-inhibition curves for the UCL 1495 series. UCL 1495 (■), UCL 1645 (▼), UCL 1644 (▲), UCL 1617 (●) and UCL 1631 (◆). Data were fitted to the Hill equation. Solid lines show simultaneous fit, dotted lines show separate fits. Each point is the mean of three observations. Vertical bars indicate s.e.m.
Fig. 7.4.3 Effect of 100 μM UCL 1285 on A23187-stimulated K⁺ loss from rabbit erythrocytes after 3 and 60 min pre-incubations. This concentration of UCL 1285 had no effect on K⁺ loss when applied three minutes before A23187 but after one hour it caused a 20 % reduction. Vertical bars show s.e.m. Mean of 3 observations. * denotes significant difference at the 5% level.
re-tested at 100\mu M (as was described in section 2.2.1, compounds containing a quaternary nitrogen had a detrimental effect on the K⁺-sensitive electrode and so higher concentrations of UCL 1285 were not tested). The results are shown in Fig. 7.4.3. While UCL 1285 at this concentration had no effect after the standard 3 minute incubation, after 1 hour a small inhibition of 20% was observed. This suggests that UCL 1285 does have some activity but a very slow onset. If quaternisation reduces the speed of onset as well as reducing activity the possibility arose that UCL 1631 may also have a slow onset and that the IC₅₀ had been overestimated. However, it was found that there was no significant difference between the inhibition caused by UCL 1631 after 1 and 3 minutes incubation, suggesting that the onset of this compound is rapid.

Finally, we turn to the isomers of UCL 1495, UCL 1644 and UCL 1645. These were found to differ in activity. A more detailed analysis of the data obtained with these compounds allowed the potency ratio to be estimated as 2.2 ± 0.35 (see Fig. 7.4.4).

Assuming that UCL 1495 contains equal amounts of the two isomers it is possible to predict the IC₅₀ of UCL 1495 from the data obtained for the isomers. The method employed for this calculation is shown below. This approach has the advantage that it is not necessary to assume that the equilibrium dissociation constants for the compounds have the same value as the observed IC₅₀ values. In fact it would be inappropriate to make such an assumption and the reasons for this are further discussed in section 8.4.

When the two compounds are applied separately at concentrations A and B, the proportion of occupied binding sites (p) will be:

\[ p = \frac{A}{K_A + A} \]

and

\[ p = \frac{B}{K_B + B} \]
Fig. 7.4.4 Comparison of UCL 1644 with UCL 1645. Data is as shown in Fig. 7.4.2 but fitted simultaneously to give a better estimate of the relative potencies of the two isomers. Data fitted to the Hill equation. Solid lines show simultaneous fits, dotted lines show separate fit. Each point is the mean of 3 observations. Vertical bars indicate s.e.m. UCL 1645 is the more potent of the two isomers. From this analysis the IC₅₀'s for UCL 1644 and UCL 1645 were 2.36 ± 0.17 and 1.14 ± 0.4 μM respectively and the potency ratio was 2.2 ± 0.35.
When two blockers act together the proportion of occupied binding sites should be given by:

\[ p = \frac{K_A B + K_B A}{K_A K_B + K_B A + K_A B} \]

where: \( K_A \) and \( K_B \) are the equilibrium dissociation constants of the blockers and \( A \) and \( B \) are the concentrations of each.

Since the unresolved mixture contains equal quantities of the two isomers, \( A = B = M/2 \) where \( M \) is the concentration of the mixture.

Thus when the mixture and the resolved isomers are applied at concentrations which give an equal degree of block (say 50%) then:

\[ \frac{K_A \frac{M}{2} + K_B \frac{M}{2}}{K_A K_B + K_B \frac{M}{2} + K_A \frac{M}{2}} = \frac{A}{K_A + A} = \frac{B}{K_B + B} \]

The potency ratio, \( r = B/A = K_B/K_A \) and so \( K_B = r K_A \).

Thus,

\[ \frac{K_A \frac{M}{2} + rK_B \frac{M}{2}}{rK_A^2 + K_A \frac{M}{2} + rK_A \frac{M}{2}} = \frac{A}{K_A + A} \]

This expression can be simplified to give:

\[ M = \frac{2rA}{1+r} \]

Since \( r = B/A \),

\[ M = \frac{2B}{1 + \frac{B}{A}} \]

and,

\[ M = \frac{2AB}{A + B} \]

If \( A \) and \( B \) are the IC\(_{50}\)s of UCL 1644 and UCL 1645 then the IC\(_{50}\) of the mixture, UCL
1495 will be $2 \times 1.14 \times 2.36/(1.14+2.36)=1.54 \text{ M}$. This value is reasonably close to the value of $1.3 \pm 0.23 \text{ M}$ obtained in the same series of experiments.

### 7.5 Quinoline and isoquinoline derivatives

A further strategy for increasing lipophilicity was to add a second ring to the piperidine. UCL 1537 (a N-decahydro quinoline derivative), UCL 1526 (an isoquinoline) and UCL 1640 (a N-decahydro isoquinoline) were therefore synthesised. These compounds had the general structure given in Fig. 7.5.1. Table 7.5.1 shows the R group for each compound and summarises the results obtained.

Concentration-inhibition curves for UCL 1537 and UCL 1640 are shown in Fig. 7.5.2. Both compounds were somewhat more active than might have been expected; the predicted IC$_{50}$ for both compounds was 2.7 μM. Simultaneous fitting of the data for UCL 1537 and UCL 1640 suggested a potency ratio of 0.65±0.06. Although this is a small difference it is, nevertheless, surprising in view of the fact that the compounds have the same log P. It is interesting to speculate that lower activity of UCL 1537 results from hindrance by the C at position 8 of the quinoline.

In preliminary experiments UCL 1526 appeared to be completely inactive at 2 and 20 μM (Fig. 7.5.3A). This result was rather surprising since the log P of this compound is 6.6 and so the IC$_{50}$ was predicted to be 8 μM.

Since it clearly had very low aqueous solubility it was decided to test the effect of incubating the cells with a saturated solution of UCL 1526 (nominally 20 μM) after 1 hour of incubation it was found that 84±2.3%( Fig. 7.5.3B) inhibition had occurred. This suggests that the compound is not completely inactive. Even when the free concentration in the aqueous phase is very low, given time enough drug will partition into a lipid phase to produce inhibition. However, when UCL 1526 was tested at 5 μM under these conditions a smaller degree of inhibition (28 ± 5.6 %) was seen. This implies that the solution was not saturated at this concentration so it is possible that the slow onset of action of UCL 1526 cannot be explained solely by this mechanism.
Fig. 7.5.1 General structure of compounds in table 7.5.1

Table 7.5.1

<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>R</th>
<th>IC(_{50})(s.d.)</th>
<th>r(s.d.)</th>
<th>Log P</th>
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<td><img src="image" alt="Structure" /></td>
<td>1.6(0.08)</td>
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<td>7.65</td>
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<tr>
<td>1640</td>
<td><img src="image" alt="Structure" /></td>
<td>1.0(0.17)</td>
<td>0.7(0.06)</td>
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<tr>
<td>1526</td>
<td><img src="image" alt="Structure" /></td>
<td>n.a.</td>
<td>n.a.</td>
<td>6.6</td>
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</tbody>
</table>
Fig. 7.5.2 Concentration-inhibition curves for UCL 1640 (●) and UCL 1537 (■). Data have been fitted to the Hill equation. Solid lines show simultaneous fit, dotted lines show separate fits. Each point is the mean of 3 observations. Vertical bars indicate s.e.m.
Fig. 7.5.3 Effect of UCL 1526 on A23187 stimulated K⁺ loss from rabbit erythrocytes after 3 min (A) and 60 min (B) pre-incubation. Vertical bars show s.e.m. Each is the mean of 3 observations. UCL 1526 has no significant effect on A23187-stimulated K⁺ loss after three minutes pre-incubation but causes substantial inhibition after one hour.
7.6 'Open-ring' derivatives of UCL 1274

In all the compounds discussed so far the nitrogen atom is contained in a six or seven-membered ring. In order to assess the importance of the ring structure two alkylamino derivatives (UCL 1556 and UCL 1557) and a benzylamine derivative (UCL 1566) were prepared. The compounds UCL 1555 and UCL 1557 have very high log P values (8.93 and 9.97 respectively) and would be expected to have IC₅₀'s of 540 and 160 nM. UCL 1566 has a lower log P (7.99) which should give a better comparison with piperidine compounds having a similar log P and was expected to have an IC₅₀ of approximately 1.6 μM. The general structure of these compounds is shown in Fig. 7.6.1 and table 7.6.1 shows the R groups and summarises the data obtained.

Interestingly, preliminary experiments showed that the onset of action of these compounds was rather slow; whereas most compounds tested produced their maximum effect within three minutes, these compounds seemed to take much longer. The time course of the onset of action of these compounds is illustrated in Fig. 7.6.2. Some of the less lipophilic compounds tested previously had also been shown to have a slow onset suggesting that the rate of onset was related to lipophilicity. This is clearly incorrect as a generalisation.

In order to construct concentration-inhibition curves the cells were, therefore, pre-incubated with these compounds for 1 hr. The curve obtained for UCL 1557 was very poorly defined and no attempt was made to fit the data. The IC₅₀ for this compound would appear to be approximately 1 μM. The IC₅₀'s for UCL 1556 and UCL 1566 were 1.45 and 6.2 μM respectively. These values are both considerably less than predicted from the predicted log P value. This suggests that having a ring in the structure does confer some advantage.

7.7 9-benzyl 9-flourenyl analogues of cetiedil

All the compounds discussed so far were esters of triphenyl acetic acid. However, preliminary work had shown that UCL 1422, a 9 benzyl 9 fluorenyl carboxylic acid ester derivative of cetiedil was also more active than cetiedil itself. In this series of experiments the effect of making polar substitutions to the benzyl group of UCL 1422
**Fig. 7.6.1** General structure of compounds shown in Table 7.6.1

### Table 7.6.1

<table>
<thead>
<tr>
<th>UCL number</th>
<th>$R_1$</th>
<th>$R_2$</th>
<th>$IC_{50}$ (µM)</th>
<th>log P</th>
</tr>
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<td>1556</td>
<td>--(CH$_2$)$_4$CH$_3$</td>
<td>--(CH$_2$)$_4$CH$_3$</td>
<td>1.5(0.2)</td>
<td>8.93</td>
</tr>
<tr>
<td>1557</td>
<td>--(CH$_2$)$_5$CH$_3$</td>
<td>--(CH$_2$)$_5$CH$_3$</td>
<td>$\approx 1^*$</td>
<td>9.97</td>
</tr>
<tr>
<td>1566</td>
<td>--(CH$_2$)$_2$CH$_3$</td>
<td>CH_CH$_3$</td>
<td>6.2(0.4)</td>
<td>7.99</td>
</tr>
</tbody>
</table>

* A precise $IC_{50}$ value was not calculated for compound.
Fig. 7.6.2 Time-course of the onset of inhibition by UCL 1556 (■), UCL 1557 (●) and UCL 1566 (▲). All compounds were tested at 10 µM. For UCL 1566 each point is the mean of three observations and the vertical bars indicate s.e.m.. Data for UCL 1556 and UCL 1557 are from a single experiment. The data have been fitted to a single exponential, constrained to give 0 % inhibition at time=0. The rate constants for the onset of inhibition for UCL 1556, UCL 1557 and UCL 1566 were 0.13, 0.13 and 0.14 min⁻¹ respectively.
Fig. 7.6.3 Concentration-inhibition curves for UCL 1556 (■), UCL 1557 (♦) and UCL 1566 (●). Each point is the mean of 3 observations. Vertical bars indicate s.e.m. Because the onset of the inhibition caused by these compounds was slow (see Fig. 7.6.2.) the cells were pre-incubated with test compound for 1 hour instead of the usual 3 min. Data for UCL 1556 and UCL 1566 were fitted to the Hill equation. The curve for UCL 1557 was not well enough defined for fitting.
was examined. The general structure of this series of compounds is given in Fig. 7.7.1. and the results obtained are shown if Fig.7.7.2 and table 7.7.1. This approach was of interest since it had been found that making polar substitutions on the piperidine ring of compounds in the UCL 1465 series resulted in compounds which were more active than would be predicted from their log P values (see section 7.3).

UCL 1422 was found to have an IC$_{50}$ of 4 μM. However, making the substitutions shown in table 7.7.1 did not have a large effect on potency and as can be seen from Fig.7.7.3 all the compounds had activities close to the value predicted from log P.

In order to assess further the importance of the ester linkage the compound UCL 1608 (Fig.7.7.4), in which the ester function of UCL 1422 is replaced by ethyne, was prepared. The IC$_{50}$ of this compound was 1.5 μM. Log P for this compound is 8.5 and therefore would be expected to be more active than UCL 1422; as can be seen from Fig.7.7.3 the observed IC$_{50}$ for this compound is close to the predicted value. This result supports the observation with UCL 1617 that the ester linkage is not needed for activity.

### 7.8 Effects of quinine analogues

Quinine is a moderately effective blocker of the red cell K$_{(Ca)}$ and its action has certain features in common with cetiedil (see sections 3.5.4 and 8.3). It seemed interesting, therefore, to find out if lipophilicity was important in determining the potency of quinine and related compounds.

The compounds tested had the general structure shown in Fig.7.8.1. Table 7.8.1 shows the R groups and summarises the data obtained. Concentration-inhibition curves are shown in Fig.7.8.2.

Some of the compounds tested were actually analogues of hydroquinine and so this compound was also included.

Hydroquinine was found to be slightly less active than quinine. Adding lipophilic groups to the position R2 increased activity as in UCL 1496 and UCL 1499. Increasing
Fig. 7.7.1 General structure of compounds shown in table 7.7.1

Table 7.7.1

<table>
<thead>
<tr>
<th>UCL Number</th>
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<th>IC&lt;sub&gt;50&lt;/sub&gt;(s.d.) (µM)</th>
<th>r(s.d.)</th>
<th>log P</th>
</tr>
</thead>
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<td>1</td>
<td>6.95</td>
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<tr>
<td>1587</td>
<td>NO&lt;sub&gt;2&lt;/sub&gt;</td>
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<td>1.26 (0.10)</td>
<td>6.72</td>
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<tr>
<td>1588</td>
<td>OCH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>2.8 (0.29)</td>
<td>0.60 (0.06)</td>
<td>7.03</td>
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<td>1589</td>
<td>F</td>
<td>4.1 (0.81)</td>
<td>1.08 (0.04)</td>
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<td>1590</td>
<td>Cl</td>
<td>2.9 (0.32)</td>
<td>0.70 (0.37)</td>
<td>7.69</td>
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Fig. 7.7.2 Concentration-inhibition curves for UCL 1422 series.

UCL 1422 (■), UCL 1587 (●), UCL 1588 (▲), UCL 1589 (▼) and UCL 1590 (◆).

Each point is the mean of 3 observations, vertical bars indicate s.e.m. Data have been fitted to the Hill equation. Solid lines show simultaneous fit, dashed lines show separate fits.
Fig. 7.7.3 Plot of -log $IC_{50}$ against predicted log P for UCL 1422 series. The solid line is the regression line from Fig 7.1.3.
Fig. 7.7.4 UCL 1608. This compound is the acetylene derivative of UCL 1422. The predicted log of this compound was 8.5 and the IC$_{50}$ was estimated to be 1.5±0.1 μM.
lipophilicity still further as in UCL 1505 and UCL 1528 apparently increased activity but these compounds were unable to produce 100% inhibition. As had been found with UCL 1539 (Table 7.3.1), UCL 1505 proved to be so insoluble that it was not possible to estimate an $IC_{50}$.

A plot of log P against $-\log IC_{50}$ is shown in Fig.7.8.3. When compared with the plot for the cetiedil series of compounds it can be seen that the relationship is shallower and that the intercept is greater: since only a few data points are available for the quinine series it is not possible to say whether this difference is significant.

7.9 Effects of imidazole derivatives

The anti-iyhycotic agent clotrimazole (Fig. 7.9.1 A) has recently been shown to be a potent inhibitor of $K_{Ca}$ in human erythrocytes (see section 1.2.6.3) and it was therefore of interest to test this compound. Data obtained are shown in Fig.7.9.2. The $IC_{50}$ for clotrimazole was found to be $2.5\pm0.5\ \mu M$. This is considerably greater than the estimate of $0.1\ \mu M$ for inhibition of $^{86}Rb$ uptake in human erythrocytes (Alvarez et al. 1992, Ellory et al. 1993). To test the possibility that this discrepancy might arise from a species difference clotrimazole was also tested in human erythrocytes. The data obtained are shown in Fig.7.9.3. The $IC_{50}$ was found to be $0.9\pm0.05\ \mu M$. From this it would appear that the human cells are more sensitive to blockade by this compound, although differences in technique cannot be ruled out.

Work with cetiedil analogues had shown that quaternisation causes a decrease in activity. The quaternary derivative of clotrimazole, UCL 1559 (Fig.7.9.1B), was prepared to see if this also applied here. The results are shown in Fig.7.9.2. The $IC_{50}$ for UCL 1559 was $16.3\pm3.7\ \mu M$, considerably less active than clotrimazole, and the potency ratio was calculated to be $6.6\pm2.2$. Thus it would appear that, like the cetiedil compounds, quaternisation of the nitrogen reduces activity.

Since clotrimazole was found to be active, it was decided to test the activity of an imidazole analogue of UCL 1274 (the starting compound of the triphenyl acetic acid series; Fig.7.1.1C). The first compound to be tested was UCL 1565 (Fig.7.9.1C). The
Fig. 7.8.1 General structure of compounds shown in table 7.8.1

<table>
<thead>
<tr>
<th>Compound</th>
<th>$R_1$</th>
<th>$R_2$</th>
<th>IC_{50}(s.d.) (μM)</th>
<th>$r$ (s.d.)</th>
<th>log P</th>
</tr>
</thead>
<tbody>
<tr>
<td>quinine</td>
<td>CH₂—CH</td>
<td>OH</td>
<td>26.0 (6.1)</td>
<td>1</td>
<td>3.16</td>
</tr>
<tr>
<td>hydroquinine</td>
<td>CH₃—CH₂</td>
<td>OH</td>
<td>27.3 (8.6)</td>
<td>1.2 (0.33)</td>
<td>3.52</td>
</tr>
<tr>
<td>1496</td>
<td>CH₃—CH₂</td>
<td>Cl—C—CO—O—</td>
<td>7.9 (0.86)</td>
<td>0.29 (0.06)</td>
<td>7.19</td>
</tr>
<tr>
<td>1499</td>
<td>CH₃—CH₂</td>
<td>Cl—C—N—O—CH₃</td>
<td>4.6 (1.9)</td>
<td>0.2 (0.05)</td>
<td>6.87</td>
</tr>
<tr>
<td>1505</td>
<td>CH₃—CH₂</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>9.11</td>
</tr>
<tr>
<td>1528</td>
<td>CH₂—CH</td>
<td>Cl—C—CO—O-</td>
<td>1.4 (1.3)</td>
<td>0.06 (0.02)</td>
<td>8.97</td>
</tr>
</tbody>
</table>

*These compounds failed to cause full inhibition.
Fig. 7.8.2 Concentration-inhibition curves for quinine series.
Quinine (■), hydroquinine (○), UCL 1496 (▲), UCL 1499 (◆) and UCL 1528 (▼).
Data have been fitted to the Hill equation. Solid lines show simultaneous fit and dashed lines show separate fits. Each point is the mean of 3-5 observations. Vertical bars indicate s.e.m.
Fig. 7.8.3 Plot of -log IC$_{50}$ versus predicted log P for the quinine series of compounds. Linear regression of the data gave the relationship:

\[-\log \text{IC}_{50} = 0.2 \log P + 3.9\]

R = 0.97, s.d. = 0.15
IC$_{50}$ was found to be 9.4±0.4 µM (Fig. 7.9.2). The predicted log P of this compound is 4.86 and so the predicted IC$_{50}$ is 76 µM. This compound is, therefore considerably more active than would be expected. This result was encouraging and the next step was to synthesise compounds with an imidazole ring but containing lipophilic substituents. Accordingly the compounds UCL 1628 and UCL 1629 were prepared (7.9.1D,E). These compounds have log P values considerably greater than UCL 1565 and would be expected to be very active. However, both compounds proved to be too insoluble to test, as was found with UCL 1526 UCL 1539 and UCL 1505. Thus although the imidazole derivative of UCL 1274 was more active than predicted by its log P value, it was less potent than the most active piperidine compounds (e.g. UCL 1495) and it remains to be seen whether or not this structure can be used to develop even more active compounds.

### 7.10 Summary

The results presented in this chapter demonstrate that lipophilicity, as indicated by the predicted log octanol/water partition coefficient (log P), is a major factor in determining the potency of the cetiedil series of compounds. Furthermore, it appears that log P plays a similar role in determining the activity of compounds related to quinine. Exploiting the relationship between log P and potency resulted in the development of compounds such as UCL 1495, UCL 1537, UCL 1640 and UCL 1608 which are approximately 25 times more potent than cetiedil.

However, an approach based solely on increasing lipophilicity has obvious limitations. Some of the compounds tested were poorly soluble, and in addition the most lipophilic compounds tested (UCL 1556 and UCL 1557) were less active than would be predicted from their log P values. It is therefore very important that considerable evidence for the importance of the structure of the compounds has been obtained. In section 7.2 it was found that making substitutions close to the N of the piperidine ring caused a marked reduction in potency. Further evidence for structural determinants of activity comes from the observation that the compounds UCL 1506 (section 7.3) and UCL 1565 (section 7.9) were more active than predicted.
Fig. 7.9.1 Structures of A clotrimazole, B UCL 1559, C UCL 1565, D UCL 1528, E UCL 1529.
Table 7.9.1

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC_{50} (s.d.) (μM)</th>
<th>Log P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clotrimazole</td>
<td>2.50(0.5)</td>
<td>-</td>
</tr>
<tr>
<td>UCL 1559</td>
<td>16.3 (3.7)</td>
<td>-</td>
</tr>
<tr>
<td>UCL 1565</td>
<td>9.5 (0.4)</td>
<td>4.86</td>
</tr>
<tr>
<td>UCL 1528*</td>
<td>n.a.</td>
<td>6.71</td>
</tr>
<tr>
<td>UCL 1529*</td>
<td>n.a.</td>
<td>9.30</td>
</tr>
</tbody>
</table>

*These compounds were too insoluble to test.
Fig. 7.9.2 Comparison of the effect of clotrimazole (●) and its quaternary derivative, UCL 1559 (■) on A23187 stimulated K⁺ loss from rabbit erythrocytes. Data have been fitted to the Hill equation. Fitted lines for simultaneous and separate fits for clotrimazole are superimposed. For UCL 1559 the dashed line shows the separate fit and the solid line shows the simultaneous fit. Each point is the mean of three observations. Vertical bars indicate s.e.m.
Fig. 7.9.3 Effect of clotrimazole on A23187-stimulated K⁺ loss from human erythrocytes. Data have been fitted to the Hill equation. Each point is the mean of three observations. Vertical bars indicate s.e.m.
Fig. 7.9.4 Effect of UCL 1565 on rabbit erythrocytes. Data have been fitted to the Hill equation. Each point is the mean of 3-4 observations. Vertical bars indicate s.e.m.
Finally, the difference in activity between UCL 1644 and UCL 1645 (the cis and trans isomers of UCL 1495) (section 7.4) also underlines the importance of structure.

The implications of these results and the possible direction of further work are discussed in more detail in Chapter 11.
8 MECHANISTIC STUDIES OF THE ACTION OF CETIEDIL ANALOGUES AND CLOTРИMAZOLE ON THE CALCIUM ACTIVATED POTASSIUM PERMEABILITY OF ERYTHROCYTES

This section describes a series of experiments which were designed to explore the mechanism by which the cetiedil compounds inhibit erythrocyte $K_{Ca}$. In addition the actions of UCL 1495, one of the most active of the new cetiedil analogues, are compared with those of another $K_{Ca}$ blocker, clotrimazole.

8.1 Do the cetiedil analogues act directly on $K^+$ channels?

Since the assay system used to test compounds for the ability to block $K_{Ca}$ is indirect, the possibility existed that these agents act not by blocking the channel but either by interfering with the action of the calcium ionophore or by preventing the activation of the channels by interfering with a calcium binding site. The following experiments were designed to investigate these possibilities. UCL 1274 was chosen for these experiments since it was the most active compound available at the time.

8.1.1 Effect of UCL 1274 on net $K^+$ loss stimulated by A23187 and Pb$^{2+}$ ions

In order to rule out the possibility that UCL 1274 might act by somehow interfering with the action of A23187, the ability of this compound to inhibit the activation of these channels by Pb$^{2+}$ ions was tested. Lead ions are thought to enter erythrocytes via the anion exchanger (Simons 1984) and to activate the channels by a direct mechanism not involving Ca$^{2+}$ (Shields et al. 1985). It seemed reasonable to argue, therefore, that if UCL 1274 was able to inhibit the action of lead as it does that of A23187 then it, and by inference the other compounds, were not acting by interfering directly with the action of the ionophore.

The action of Pb$^{2+}$ was somewhat slower than that of A23187 and so the percentage $K^+$ loss was measured after five min instead of three. The mean percentage $K^+$ loss stimulated by the standard (maximal) application of ionophore was $58 \pm 5.4\%$ ($n=6$) and that stimulated by Pb$^{2+}$ (10 μM, also maximal) was $53 \pm 1.2\%$ ($n=6$). Examples of the records obtained are shown in Fig.8.1.1.
Fig. 8.1.1 Inhibition of K+ loss stimulated by 2 μM A23187 and 10 μM Pb2+ from rabbit erythrocytes. A and C show the effect of A23187 and Pb2+ alone. B and D show the effect of A23187 and Pb2+ in the presence of 10 μM UCL 1274, which was added 3 min prior to A23187 or Pb2+. After the exposure to A23187 or Pb2+ 100 μM digitonin was added to cause cell lysis and so allow the total K+ content of the cells to be estimated. In the examples shown A23187 and Pb2+ caused 42 and 56 % of total K+ to be released. In the presence of 10 μM UCL 1274 this was reduced to 10 and 6 %.
Fig. 8.1.2 Effect of UCL 1274 on K⁺ loss from erythrocytes stimulated by either 2μM A23187 (●) or 10 μM Pb²⁺ (■). The IC₅₀ for inhibition of the response to A23187 was 5.3 ± 0.8 μM and for Pb²⁺ 5.5 ± 0.8 μM. Each point is the mean of 3-4 observations. Vertical bars indicate s.e.m.
Fig. 8.1.2 shows concentration-response curves for UCL 1274 as an inhibitor of Pb$^{2+}$ and A23187. The calculated IC$_{50}$'s for inhibition of the response to Pb$^{2+}$ and A23187 were $5.5 \pm 0.8 \mu$M and $5.3 \pm 0.8 \mu$M respectively, strongly suggesting that the effectiveness of UCL 1274 does not depend on the methods used to activate the channels.

### 8.1.2 Effect of UCL 1274 on the Ca$^{2+}$ concentration-response curve

The results described in the previous section seem to rule out the possibility that the compounds act by inhibiting the increase in Ca$^{2+}$ caused by A23187. The possibility remains that they may inhibit the interaction between calcium and its binding site. If this were the case one might expect there to be competition between Ca$^{2+}$ and the blocker, so that the block should be surmountable by an increase in [Ca$^{2+}$]. To test this idea the effect of UCL 1274 on ionophore-stimulated K$^+$ loss in solutions containing different buffered concentrations of Ca$^{2+}$ was examined.

Concentration-response curves to Ca$^{2+}$ were constructed in the presence and absence of 6 µM UCL 1274. Fig. 8.1.3 shows examples of original recordings and the concentration-response curves obtained are presented in Fig. 8.1.4. As expected the proportion of cellular K$^+$ lost following exposure to 2 µM A23187 was dependent on extracellular Ca$^{2+}$ concentration. The data were fitted to the Hill equation and the maximal response, $y_{max}$, for the control curve was estimated to be 69±4.6%. This is in keeping with the size of response seen in the condition used in the rest of this study (when [Ca$^{2+}$] = 1.8 mM). The EC$_{50}$ for the effect of Ca$^{2+}$ was 2.2 ± 0.6 µM and this is in keeping with previously reported values (see e.g. Simons 1976). The Hill slope, $n_H$ was 1.9 ± 0.56 which is also consistent with previous work. In the presence of 6 µM UCL 1274, $y_{max}$ was reduced to 38 ± 2.8 %. This corresponds to 45 % inhibition which is reasonably close to 50 % as expected (the previous estimate of the IC$_{50}$ of UCL 1274 was 6 µM- see section 7.1 ). The unsurmountable nature of the block indicated that the inhibition produced by UCL 1274 is not competitive and is in fact more consistent with open channel block. Similar results with nifedipine have been previously reported by Kaji (1990).
Fig. 8.1.3 Original records showing A23187-stimulated K⁺ loss from rabbit erythrocytes suspended in EGTA buffered solutions containing 1 µM or 100 µM (C) free Ca²⁺. B and D show the effect of A23187 in the presence of 6 µM UCL 1274, which was added 3 min previously. I denotes addition to the bath of 2 µM A23187. After 3 min 100 µM digitonin (dig.) is added to lyse the cells and allow the total K⁺ content of the cells to be estimated.
Fig. 8.1.4 Concentration-response curves for calcium in the absence (■) and presence (●) of 6 μM UCL 1274. Erythrocytes were suspended in solutions containing varying concentrations of calcium prior to exposure to A23187. The data were fitted to the Hill equation. In the absence of UCL 1274 the maximum K⁺ loss was 69 ± 5 %. In the presence of UCL 1274 this was reduced to 38 ± 3 %. The EC₅₀ and nᵢ were 2.2 ± 0.6 μM and 1.9 ± 0.6 respectively in the absence of UCL 1274 and 1.5 ± 0.5 μM and 2.6 ± 2 in its presence.
8.2 Effect of extracellular [K⁺] on potency of blocking agents

Previously published work has shown that blockade of erythrocyte K⁺ by quinine is reduced when extracellular K⁺ is elevated (Reichstein and Rothstein 1981). Cetiedil has also been shown to exhibit this behaviour and it has been found that raising extracellular [K⁺] from 0.1 to 5.4 mM causes an approximately 3-fold decrease in activity. In contrast, neither charybdotoxin nor nitrendipine show this behaviour (M. Shiner, personal communication). It seemed important, therefore, to find out if the blocking action of the more potent cetiedil analogues was also sensitive to extracellular [K⁺]. Since clotrimazole was also found to be effective (see section 7.9) it was also tested in order to determine whether it behaved like cetiedil or like nitrendipine and charybdotoxin.

Concentration-inhibition curves were constructed using cells suspended in either the normal 0.1 mM K⁺ bathing solution or in 5.4 mM K⁺ solution. The action of clotrimazole was quite clearly little affected by raising extracellular [K⁺] (Fig.8.2.1). In contrast, raising extracellular K⁺ caused a marked reduction in the potency of UCL 1495 (Fig.8.2.2). The IC₅₀ was 1.5 µM in 0.1 mM K⁺ and 32 µM in 5.4 mM K⁺, a 21-fold decrease in potency. This change was rather greater than the 3-fold decrease in activity seen for cetiedil.

8.3 Slope of concentration response curves for UCL 1495 and clotrimazole

An incidental observation from the data presented in section 7 was that the concentration-inhibition curves for cetiedil analogues tended to be rather steep, with nH ranging from 2 to 3. In contrast the curves obtained for clotrimazole had shallower slopes with nH approximately 1. However, for most of the curves obtained there were too few points to obtain a precise estimate of the slope. In order to clarify this, detailed curves for clotrimazole and UCL 1495 were constructed simultaneously.

The curves obtained are shown in Fig.8.3.1. The IC₅₀ values for UCL 1495 and clotrimazole were 0.9 and 1.3 µM respectively. Both these values are slightly lower than those reported in section 7. This partly because y_max was not constrained to 100 as in the previous instance. The slopes of the curves were quite clearly different. For
clotrimazole the Hill slope was close to 1 suggesting a simple interaction with its binding site. The higher value of 2.8 for UCL 1495 suggests a more complicated mechanism.

8.4 Comparison of effects of blocking agents on kinetics of ionophore stimulated $K^+$ loss

A further qualitative difference between action of clotrimazole and of UCL 1495 was that the two compounds had different effects on the time course of $K^+$ loss from the erythrocytes. As can be seen from Fig. 8.4.1A, exposure of the cell suspension to A23187 in the absence of blocker elicits a rapid loss of $K^+$ which declines with time. In the presence of clotrimazole, addition of A23187 causes a slower loss of $K^+$ at a fairly uniform rate (Fig.8.4.1c). In contrast, it can be seen that in the presence of a concentration of UCL 1495 which produces a similar degree of inhibition, A23187 causes an initial rapid loss of $K^+$ which rapidly declines, and indeed almost ceases (Fig.8.4.1b). This phenomenon is more clearly illustrated in Fig.8.4.2.

In discussing these findings we have first to consider the time course of the loss of $K^+$ induced by A23187. The simplest expectation is that it should be describable by a simple exponential. This is unlikely to obtain for several reasons. First, as the $K^+$ permeability of the cell membrane increases so the cells hyperpolarise, tending to reduce $K^+$ loss. Also, the cells shrink as $K^+$ is lost into the bathing solution, tending to oppose the decline in $[K^+]_i$. In addition, under the present experimental conditions, the outward movement of $K^+$ is great enough to increase extracellular $K^+$ significantly so that the gradient for $K^+$ loss is reduced. In the face of these complications, only an empirical description can be attempted. The data shown in Fig.8.4.2 were accordingly fitted to a 3rd order polynomial and the expression obtained was differentiated to allow the rate of $K^+$ loss to be calculated. This is illustrated in Fig.8.4.3. Again it can be seen that in the presence of UCL 1495 the rate of $K^+$ loss declines markedly with time whereas in the presence of clotrimazole it remains relatively constant. The difference in action of clotrimazole and UCL 1495 is further illustrated in Fig.8.4.4. Here the rate of $K^+$ loss has been expressed as a rate coefficient. It can be seen that the rate coefficient is almost constant in the presence of clotrimazole but declines with time in the presence of UCL 1495.
Fig. 8.2.1 Concentration-inhibition curves for clotrimazole in 0.1 mM (■) and 5.4 mM (○) K⁺ containing solutions. The data were fitted to the Hill equation and the IC₅₀ for clotrimazole was 2.5 ± 0.5 µM in 0.1 mM K⁺ and 2.9 ± 0.5 µM in 5.4 mM K⁺. Each point is the mean of 3-4 observations and the vertical bars indicate s.e.m.
Fig. 8.2.2 Concentration-inhibition curves for UCL 1495 in 0.1 mM K⁺ (■) and 5.4 mM K⁺ (●) containing solutions. The data have been fitted to the Hill equation. The IC₅₀ for UCL 1495 was 1.5 ± 0.4 μM in 0.1 mM K⁺ and 32.0 ± 7.7 μM in 5.4 mM K⁺. The potency ratio estimated from a simultaneous fit was 21 ± 7.5.

Each point is the mean of 3 observations and the vertical bars indicate s.e.m.
Fig. 8.3.1 Concentration-inhibition curves for UCL 1495 (■) and clotrimazole (●).
The data has been fitted to the Hill equation with $y_{\text{max}}$ unconstrained in order to obtain
the best estimate of the slope, $n_H$. The fitted parameters for UCL 1495 were $y_{\text{max}} = 95.6$
$\pm 0.05\%$, $IC_{50} = 0.92 \pm 0.06 \mu M$, $n_H = 2.8 \pm 0.3$ and for clotrimazole, $y_{\text{max}} = 103.0 \pm 2.8$
$\%$, $IC_{50} = 1.3 \pm 0.2 \mu M$, $n_H = 1.0 \pm 0.1$. Thus the curve for UCL 1495 is considerably
steeper than that for clotrimazole.
Each point is the mean of three observations and the vertical bars indicate s.e.m.
One possible explanation of the decline in rate of $K^+$ loss with time in the presence of UCL 1495 is that this compound is an open channel blocker which associates relatively slowly with the channels only after they have been activated. In contrast, clotrimazole may either bind to closed channels or it may associate so rapidly with open channels that it is not possible to resolve the onset of block with the present technique. Similarly, the fact that UCL 1495 does cause some reduction of the initial rate of $K^+$ loss (Figs. 8.4.3 and 8.4.4) may indicate either that it also has some affinity for closed channels or that the early part of the onset of block is unresolvable (presumably because of relatively slow mixing of the erythrocyte suspension).

The different effect the two compounds have on the kinetics of $K^+$ has a practical implication in that it poses a problem as how best to express the inhibition caused by test compounds. The procedure used in the experiments described in the previous chapter was to measure the reduction in the amount of $K^+$ lost three minutes after addition of ionophore. This choice may be part of the reason why the $IC_{50}$ for clotrimazole measured in this study was greater than that reported by other workers using different techniques.

8.5 Summary

The aim of the experiments described in this chapter was to examine the mechanism of action of the cetiedil series of compounds. Since the test system used in chapter 7 to assess the cetiedil compounds is indirect it was important to be sure that the compounds were indeed acting as $K^+$ channel blockers. This view is supported by the observations that UCL 1274 inhibited the activation of $K_{Ca}$ by $Ca^{2+}$ in a non-competitive manner and that it inhibited $K^+$ loss via $K_{Ca}$ with similar potency regardless of the method used to activate the channels.

The $K_{Ca}$ blocking actions of UCL 1495 were found to differ from those of clotrimazole in three respects. Firstly, the action of UCL 1495 but not that of clotrimazole was greatly diminished when $[K^+]_o$ was increased. Secondly, the concentration-inhibition curve for UCL 1495 was markedly steeper than that for clotrimazole.
Fig. 8.4.1 Original records showing the effect of UCL 1495 and of clotrimazole on the time-course of A23187-stimulated K⁺ loss from rabbit erythrocytes. In the absence of blocker (A), A23187 causes a rapid loss of K⁺ which declines slowly. In the presence of 5 µM UCL 1495, A23187-stimulated K⁺ loss is initially rapid but the rate declines abruptly a few seconds after addition of ionophore. In contrast, in the presence of 10 µM clotrimazole (C), addition of A23187 causes K⁺ to be released at a reduced but almost constant rate.
Fig. 8.4.2 The effect of UCL 1495 (A) and clotrimazole (B) on the time-course of A23187-stimulated K⁺ loss from erythrocytes. The abscissa shows the percentage of total K⁺ remaining in the cells. The solid lines show the K⁺ content of the cells measured at 10 s intervals. The dashed lines were drawn from a 3rd order polynomial fit to the data. This expression was also used to calculate the data shown in Figs. 8.4.3 and 8.4.4. The concentrations of blocker used are shown on the graphs. Controls are marked ‘c’.
Fig. 8.4.3 The polynomial expressions obtained from Fig. 8.4.2 have been differentiated to allow the rate of A23187-stimulated K+ loss to be calculated. The effect of UCL 1495 is shown in A and the effect of clotrimazole in B. Note that in the presence of UCL 1495 the rate declines with time whereas in the presence of clotrimazole it remains relatively constant.
Fig. 8.4.4 The data from Fig. 8.4.2 have been used to express the loss of $K^+$ from the cells as a rate coefficient. Note that UCL 1495 (A) causes a steady decline in rate coefficient with time whereas in the presence of clotrimazole (B) the rate coefficient is nearly constant.
Finally, the two agents had different effects of the time course of ionophore-stimulated K⁺ loss from erythrocytes. Taken together these findings indicate that clotrimazole and UCL 1495 act in different ways. Possible mechanisms of channel block by these compounds are discussed in Chapter 11.
9 EFFECTS OF CETIEDIL AND ITS ANALOGUES ON POTASSIUM CHANNEL OPENER-STIMULATED $^{86}$RB EFFLUX FROM SMOOTH MUSCLE

In order to gain further information about the K⁺ channel selectivity of the compounds tested on erythrocytes, some of them were also assessed for their ability to inhibit the increase in $^{86}$Rb efflux from smooth muscle (rat aorta and rat anococcygeus) treated with potassium channel opener (KCO) drugs.

9.1 Effects of potassium channel openers on $^{86}$Rb efflux from rat aorta

In preliminary experiments carried out using a HEPES buffered bathing solution, it was found that responses to levromakalim and RP52891 were frustratingly variable and somewhat lower than previously reported in the literature. The mean increases in $^{86}$Rb caused by RP52981 and levromakalim (each at 10 µM) were 51 ± 9% (n=7) and 24 ± 7% (n = 7) respectively. However, responses to levromakalim in a bicarbonate buffered Krebs' solution were larger and somewhat more reproducible; the mean response to 10 µM levromakalim was now 81 ± 10% (n = 23). Accordingly, the experiments to be described here were performed in a bicarbonate buffered solution except where stated otherwise. Nevertheless, although the difference between the mean responses to levromakalim in HEPES and in bicarbonate buffered solutions was significant at the 5 % level (unpaired t-test), the responses still remained rather variable in the bicarbonate buffered solution.

Fig. 9.1A illustrates the effect of levromakalim on $^{86}$Rb efflux and the concentration dependence of the effect is shown in Fig. 9.1B. Though the magnitude of the response varied considerably between preparations it appears that 10 µM produced a maximal effect. The data could be fitted to the Hill equation and the EC₅₀ was estimated thereby to be 0.6 ± 0.3 µM. It can be seen from Fig. 9.1A that the rate of onset of the effect was concentration dependent though it is not possible from the present results to say whether the rate was determined by the diffusion of the drug into the tissue or by the kinetics of either binding or action. At high concentrations some desensitisation occurred so it is likely that the true maximum is greater than observed. Since the IC₅₀ for an open channel blocker can be shown to depend on the open probability of the channel and only

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Fig. 9.1 A Stimulation of $^{86}$Rb efflux from rat aorta by 0.3 (○), 1 (●) and 10 (●) μM levcromakalim. Levcromakalim was present during the period indicated by the horizontal bar. The number of observations is as indicated in the graph below. B Concentration-response curve for levcromakalim. Each point is the mean of the number of observations shown in parentheses. The data were fitted to the Hill equation and the EC$_{50}$ was estimated to be 0.6 ± 0.3 μM with $n_H = 2.1 ± 1.2$ and $B_{Max} = 81 ± 36 \%$. In both graphs vertical bars indicate s.e.m.
approaches its limiting value when $P_{\text{open}}$ is high, a near maximal concentration of levomakalim was chosen for the studies on blocking agents.

9.2 Effect of cetiedil, UCL 1285 and UCL 1495

In preliminary experiments using a HEPES buffered solution, cetiedil inhibited the effects of both levomakalim and RP52891 (Fig. 9.2A, B). Cetiedil appeared to be rather more effective as an inhibitor of the response to RP52891 since whereas 3µM cetiedil caused complete abolition (105 ± 7 %, n = 4) of the response to RP52891 the same concentration produced only 53 ± 14 % (n=3) inhibition of the response to levomakalim. Since a full dose response curve using RP52891 was not obtained the significance of this finding is uncertain. It could perhaps reflect the greater degree of block to be expected when more channels are open (the increase in $^{86}\text{Rb}$ efflux caused by RP52891 was somewhat greater and more sustained than that caused by levomakalim).

In experiments using the bicarbonate-buffered solution, both cetiedil and UCL 1285 (cetiedil methiodide) inhibited the action of levomakalim. The curves shown in Fig. 9.3 illustrate the concentration dependence of the inhibition and examples of the data obtained are shown in Fig. 9.4A and B. The curve for cetiedil was fitted to the Hill equation yielding an estimate for the $IC_{50}$ of 1.3 ± 0.4 µM with $n_H = 0.9 ± 0.1$. The curve for UCL 1285 was rather poorly defined and no attempt was made to fit the data. Nevertheless it would appear that UCL 1285 was less active than cetiedil, with an $IC_{50}$ between 3 and 10 µM.

UCL 1495 was tested at a single concentration of 10 µM and did not cause any significant inhibition (-10±31%, n=3, p>0.05), (Fig. 9.4C). Because of its poor aqueous solubility, higher concentrations of UCL 1495 were not tested. None of the compounds caused any observable inhibition of the resting rate of efflux.

9.3 Effect of levomakalim on $^{86}\text{Rb}$ efflux from rat anococygeus

Since cetiedil proved to be a potent inhibitor of levomakalim-stimulated $^{86}\text{Rb}$ efflux in the aorta it seemed worth testing it in a non-vascular smooth muscle preparation. The
Fig. 9.2 Effect of 3 μM cetiedil on $^{86}$Rb efflux from rat aorta stimulated by either 10 μM RP52891 (A) or 10 μM levromakalim (B). These experiments were carried out in HEPES buffered saline. Drugs were present during the periods indicated by the horizontal bars. Open symbols show the effect of KCO alone and filled symbols show the effect of KCO in the presence of cetiedil. Each point is the mean of the number of experiment shown above each graph. Vertical bars indicate s.e.m. Note that the data have been normalised as described in the methods section.
Fig. 9.3 Inhibition of levocromakalim-stimulated $^{86}$Rb efflux from rat aorta by cetiedil (O) and UCL 1285 (□). Each point is the mean of the number of observations shown in parentheses. Vertical bars indicate s.e.m. The data for cetiedil were fitted to the Hill equation. The IC$_{50}$ for cetiedil was $1.3 \pm 0.4$ $\mu$M with $n_H = 0.9 \pm 0.1$. 
Fig. 9.4 Effect of 10 μM cetiedil (A), UCL 1285 (B) and UCL 1495 (C) on levocromakalim-stimulated ⁸⁶Rb efflux from rat aorta. Test compounds were present during the periods indicated by the horizontal bars. Each point is the mean of 3 observations. Vertical bars indicate s.e.m. Data from control tissues are shown by hollow symbols and filled symbols show data from test tissues.
rat anococcygeus was chosen for these experiments because at the time there were no reports of the actions of levromakalim in this tissue.

In the anococcygeus 10 μM levromakalim caused a 42.5 ± 6 % (n = 4) increase in \(^{86}\text{Rb}\) efflux (Fig. 9.5). This is rather less than was observed in the aorta, and again the response was very variable. Cetiedil was tested at a single concentration of 10 μM which, as can be seen in Fig 9.5, caused clear-cut inhibition (67 ± 11 %, n = 4, p<0.05).

9.4 Summary

In Chapter 7 it was shown that the potency of compounds in the cetiedil series increased with lipophilicity. Since cetiedil is now known to block a number of K\(^+\) channels it seemed important to establish whether or not the correlation with lipophilicity extended to the blocking action on these other channels.

In this series of experiments it was found that cetiedil blocked levromakalim-stimulated \(^{86}\text{Rb}\) efflux from rat aorta with an IC\(_{50}\) of 1.4 μM. Thus it is one of the most potent blockers of this channel. In contrast, the lipophilic analogue of cetiedil, UCL 1495 which is 26 times more active than cetiedil as a blocker of erythrocyte K\(_\text{Ca}\) was much less potent as an inhibitor of the actions of levromakalim. It would appear, therefore, that the structural requirements for blockade of the KCO-sensitive channel are different from those of K\(_\text{Ca}\) in erythrocytes.
Fig. 9.5 The effect of 10 μM cetiedil on levromakalim-stimulated ⁸⁶Rb efflux from rat anococcygeus. Test substances were present for the periods indicated by the horizontal bars. Open symbols show the effect of levromakalim alone. Filled symbols show the effect of levromakalim in the presence of cetiedil. Each point is the mean of four observations. Vertical bars indicate s.e.m.
10 ANTI-MUSCARINIC AND CALCIUM ENTRY BLOCKING ACTIONS OF
UCL 1348 AND UCL 1349, THE OPTICAL ENANTIOMERS OF CETIEDIL

Cetiedil has been shown to possess both anti-muscarinic and calcium entry blocking actions (D. Benton, S. Kamboj and D. H. Jenkinson, unpublished results). The former action is likely to be responsible for some of the side-effects of cetiedil (e.g. dryness of the mouth) (Benjamin et al. 1986) and it is possible that both actions could be involved in its ability to alleviate the symptoms of a sickle cell crisis \textit{in vivo}.

Cetiedil possesses a chiral centre (Fig. 10.1) and hence it exists in two enantiomeric forms. It seemed of interest to examine the pharmacological actions of these enantiomers. The compounds UCL 1348 ((+)-cetiedil) and UCL 1349 ((-)-cetiedil) were, therefore, prepared by Dr C. J. Roxburgh. In the series of experiments to be described the effectiveness of the enantiomers of cetiedil as calcium entry blockers and as anti-muscarinic agents were tested. In parallel studies it was found that the enantiomers of cetiedil did not differ in their ability to block A23187-stimulated K$^+$ efflux from rabbit erythrocytes (M. Shiner, personal communication) but that there was a 2-fold difference in their ability to block the volume-activated K$^+$ conductance of rat hepatocytes (P. M. Dunn, personal communication).

10.1 Calcium entry blocking actions of UCL 1348 and UCL 1349

The calcium entry blocking actions of UCL 1348 and UCL 1349 were assessed using depolarised smooth muscle (guinea pig taenia caeci). Changing the normal Krebs' solution for one containing 150 mM K$^+$ and 0.1 mM Ca$^{2+}$ caused a contracture which reversed after repeated washing. Addition of calcium to the bath now caused a well maintained, reproducible contraction. Fig. 10.1.1 shows examples of the recordings obtained.

UCL 1348 and UCL 1349 were both tested at a single concentration of 3 μM. At this concentration they caused a rightward shift in the calcium concentration-response curve (Fig. 10.1.2). The data could be fitted by the Hill equation and dose-ratios were calculated by assuming that the curves in the presence and absence of blocker were parallel. There was no difference in the dose ratios calculated from the curves in Fig.
Fig. 10.1 Structure of cetiedil showing position of the chiral carbon (*). The + and - enantiomers of α-cyclohexyl-α-(3-thienyl) acetic acid were separated on a chiral column and used to prepare UCL 1348, (+)-cetiedil and UCL 1349, (-)-cetiedil. The absolute stereochemistry is unknown.
Fig. 10.1.1 Effect of 3 μM UCL 1349 on Ca^{2+}-induced contractions of depolarised guinea pig taenia caeci. The upper traces show contractions produced by 0.3 and 3 mM Ca^{2+}. In the presence of UCL 1349 (lower traces), much higher concentrations of Ca^{2+} are required to match the original contractions.
Effect of UCL 1348 (A) and UCL 1349 (B) on the calcium concentration-response curve in K⁺ depolarised guinea pig taenia. Both compounds were applied at 3 μM. Each graph shows the size of contraction caused by Ca²⁺ in the absence (■) and presence (●) of test compound. The data were fitted to the Hill equation to estimate the dose ratio in the presence of blocking agent. The dose-ratio was 7.3 ± 4.4 for UCL 1348 and 6.7 ± 2.5 for UCL 1349. Each point is the mean of 4 observations. Vertical bars indicate s.e.m.
10.2 Anti-muscarinic activity of UCL 1348 and UCL 1349

The anti-muscarinic activity of UCL 1348 and UCL 1349 was assessed using the isolated guinea pig ileum preparation. Cumulative concentration-response relationships for acetylcholine were determined and it was found that using a 30 s exposure period with 15 min between cycles it was possible to construct reproducible concentration-response curves (Fig. 10.2.1). UCL 1348 was tested at concentrations of 10 and 30 nM, and UCL 1349 at 100 and 300 nM. The data obtained were fitted to the Hill equation. The dose-ratio was calculated in two ways: firstly, by assuming that the Schild equation applies (i.e. by constraining the potency ratios to give a Schild plot with a slope of 1) and secondly by allowing the slope of the Schild plot to vary. An example of the dose-response curves obtained in the absence and presence of UCL 1348 is shown in Fig. 10.2.2.

Fig. 10.2.3 shows Schild plots of the data obtained. When constrained to fit the Schild equation the estimated $K_b$ values for UCL 1348 and UCL 1349 were $1.6 \pm 0.25$ nM and $14.3 \pm 2.4$ nM respectively. When the slope of the Schild plot was not constrained to unity, the lines intersected the concentration axis at 4.4 and 28.3 nM for UCL 1348 and UCL 1349 respectively. Thus using either analysis it appears that UCL 1348 is 6-8 times more active than UCL 1349.

The slopes of the Schild plots were 1.7 for UCL 1348 and 1.4 for UCL 1349. The significance of the deviation of the slope of the Schild plot from unity might seem uncertain because only two points were obtained for each compound. However, the same feature was seen on repeating the experiment using individual rather than cumulative applications of agonist and at lower concentrations of UCL 1348 and UCL 1349 (Y. Ayalew and D.H. Jenkinson, unpublished results). It is clear therefore that the conditions of competitive antagonism have not been met. All that may be concluded is that UCL 1349 is approximately 7 times less effective in blocking the response to ACh.
Fig. 10.2.1 Effect of UCL 1348 on the ACh concentration-response curve in guinea pig ileum. ACh was added cumulatively to the organ bath to give the concentrations shown by each trace. There is an interval of 45 min between each curve. Three curves were meaned to give the data shown in Fig. 10.2.2.
Fig. 10.2: Effect of UCL 1348 on ACh concentration-response curve. Data are taken from a single experiment. Curves were constructed in the absence (■) and presence of either 10 nM (●) or 30 nM (♦) UCL 1348. The data were fitted to the Hill equation either separately (dashed lines) or constrained to obey the Schild equation (solid lines).
Fig. 10.3 Schild plots for UCL 1348 (■) and UCL 1349 (●). Solid lines show plot for the data not constrained to obey the Schild equation. Dashed lines show Schild plot constrained to give a slope of 1.

Each point is the mean of three estimates of the dose ratio (less unity); vertical bars show s.e.m.
under the present experimental conditions. Since only a limited quantity of the enantiomers was available, these experiments were not taken further.

10.3 Summary
In this series of experiments it was found that the enantiomers of cetiedil, UCL 1348 and UCL 1349, did not differ in their effectiveness as calcium entry blocking agents but that there was an 8-fold difference in their potency as anti-muscarinic agents.

It should be noted that since the slope of the Schild plots for both enantiomers differed significantly from unity the precise mechanism of the anti-muscarinic action of these compounds remains uncertain. Nevertheless, it is clear that the anti-muscarinic effect is manifest at lower concentrations than are required for K⁺ channel block. A study of the anti-muscarinic activity of the compounds described in Chapter 7 would, therefore, be interesting.

In Chapter 11 the relevance of these findings to the use of cetiedil as an anti-sickling agent is considered.
11 DISCUSSION OF THE RESULTS PRESENTED IN SECTION III

11.1 Studies on the relation between structure and activity of the cetiedil series

11.1.1 Log $P$ and the activity of cetiedil analogues.

The relationship between predicted log $P$ and $IC_{50}$ was found to apply for compounds in the cetiedil series over a wide range of log $P$ values (Fig. 7.1.3). The more limited data obtained with the quinine analogues suggested that here too log $P$ influences activity, although the number of compounds tested was too small to allow firm conclusions to be drawn.

Although log $P$ is a useful predictor of the activity of these compounds, some of the limitations of the approach should not be overlooked. Firstly, the relationship in Fig. 7.1.3 is relatively shallow and many of the more lipophilic compounds tested were acting at concentrations near to the limit of solubility. Secondly, as will be discussed in later sections, it is unclear whether the increase in potency seen with increasing log $P$ actually corresponds to an increase in affinity.

It was important to identify compounds which were significantly more active than predicted by log $P$. In the present study two compounds fell into this category, the substituted piperidine UCL 1506 and the imidazole UCL 1565. These compounds were found to be 5 and 8 times more potent than predicted and it will be interesting to use these structures as a basis for further compounds.

11.1.2 The role of the nitrogen atom; structural influences

The compound UCL 1453, in which two methyl groups were substituted adjacent to the N in the piperidine ring, was found to be less active than its structural isomers UCL 1452 and UCL 1451 in which the methyls were substituted at more distant positions on the ring. These compounds have the same predicted log $P$ and so it may be inferred that the difference in activity arises from a structural effect. In keeping with this, UCL 1472, in which four methyls were adjacent to the nitrogen, was even less active despite having a much higher log $P$. The presence of these groups close to the nitrogen may have a number of effects. Firstly it is possible that their close proximity to a polar group may cause log $P$ to be underestimated, though this does not seem likely. Alternatively, it may
imply that the N has to come into close proximity with a group in the channel protein and that the presence of immediately adjacent methyl groups hinders this process. The finding that the quaternary compound UCL 1631 was only six times less active than its non-quaternary analogue, UCL 1495, suggests that a lone electron pair on the nitrogen is not needed. This rules out the possibility that the compounds hydrogen bond with their site of action.

It is unclear whether the compounds act in the protonated or unprotonated form, though the former predominates (the pKₐ of cetiedil is 9.3; Chrzanowski and Ulissi, 1987), so that 99% would be in the protonated form at physiological pH. Clearly, since the quaternary compound UCL 1631 is active, channel block can occur when the nitrogen is charged. A possible explanation for the six-fold lower potency of UCL 1631 compared with UCL 1495 is that quaternisation of the N interferes with access to the site of action rather than binding. It is interesting in this context that UCL 1559 (quaternised clotrimazole) was also less active than clotrimazole itself.

The compounds in which the piperidine N was not attached to the ethyl group of the ester had IC₅₀'s consistent with the predicted log P suggesting that the position of the N is not critical.

11.1.3 Importance of the ester function
Nearly all the compounds tested in the cetiedil series were esters. The only exceptions were UCL 1617 (an amide) and UCL 1608 (an acetylene compound). Interestingly, both these compounds had IC₅₀'s quite close to the predicted value, demonstrating that the ester function is not essential. This also rules out the admittedly unlikely possibility that hydrolysis of the esters by the esterases possessed by erythrocytes was needed for activity.

Replacing the ester of UCL 1422 by acetylene (UCL 1608) is, however, a potentially useful strategy for increasing log P and would be worth exploring with other compounds in the series. It would also be interesting to establish the effect of replacing the ester function in the action of the compounds as anti-muscarinic and calcium entry blocking
agents (and indeed as blockers of other K⁺ channels) since it may be possible thereby to increase selectivity if not potency.

### 11.1.4 Stereoisomers

The optical enantiomers of cetiedil (UCL 1348 and UCL 1349) have been found to be equipotent as blockers of the red cell K⁺ₕ (M. Shiner, personal communication). In view of the lack of structural effects seen when substitutions are made at the chiral carbon of cetiedil this is perhaps unsurprising. The finding that there was a two-fold difference in potency when the same enantiomers were tested for their ability to block the hepatocyte volume-activated K⁺ channel adds to the evidence that these channels have a different pharmacology (Sandford et al. 1992).

The cis and trans isomers of UCL 1495 (UCL 1644 and UCL 1645) did however differ in activity. Though the difference was only two-fold it provides further evidence that structure is important in determining activity. It is conceivable that the true log P might differ between the isomers because of the different spatial arrangement of the side-chains, though this does not seem likely. Because UCL 1495 is much more potent than cetiedil it would not be surprising if stereoselectivity was to become more pronounced. This would be in keeping with the evidence (above, section 11.1.2) that the spatial arrangement of the groups near the nitrogen is important, possibly because this part of the molecule is more directly concerned with binding than is the acidic end of the molecule.

### 11.1.5 The design of more active compounds.

It is clear that increasing lipophilicity increases the activity of compounds in this series and it appears that this applies to substitutions made at any part of the molecule. However the importance of the distribution of lipophilic groups has not been studied systematically. It would be interesting to compare the activity of compounds with similar log P values but with lipophilic groups substituted at different positions. Examples of structures which would allow this to be done are shown in Fig. 11.1.1.
Fig. 11.1.1 Proposed structures to investigate the effect of altering the distribution of lipophilic groups within a molecule while keeping the total log P constant. Both molecules have a log P value of 7.03. Exchanging the methyl and benzyl groups between the α carbon of the acetic acid and 4 position of the piperidine ring corresponds to shifting 1.7 log P units from one end of the molecule to the other.
Fig. 11.1.2 Structure of UCL 1710. This compound was designed to combine features of UCL 1495 and UCL 1608. The predicted log P of this compound is 9.5. The IC$_{50}$ was found to be 0.31 µM (Data from experiments by Ms M. Malik).
Experiments with the UCL 1422 series showed that the presence of electron-releasing or withdrawing groups did not greatly affect activity. However, UCL 1506 which contains CN (an electron withdrawing group) at position 4 of the piperidine ring was significantly more active (by a factor of 6) than predicted by its log P value. A study of electronic effects on the piperidine ring may, therefore, be rewarding.

Preliminary work with compounds based on UCL 1565 was not successful in that the compounds prepared (UCL 1526 and UCL 1527) were not sufficiently soluble to test. However further variations on the structure are possible.

Replacement of the ester group of UCL 1422 by acetylene (UCL 1608) resulted in an increase in activity, presumably mainly by virtue of an increase in log P. This prompted the synthesis of UCL 1710 (Fig. 11.1.2) which, like UCL 1608 contains an acetylene rather than an ester but also has the 2-methyl 5-ethyl piperidine group of UCL 1495. This compound has a log P of 9.5. It has now been tested by Ms M. Malik (unpublished results) and found to have an IC₅₀ of 0.31 µM. Hence it is approximately 100 times more potent than cetiedil.

11.2 The mechanism of action of the cetiedil series on erythrocytes
The experiments on the effect of UCL 1274 on K⁺ loss produced by Pb²⁺ (Fig. 8.1.2) and on the Ca²⁺ concentration-response curve (Fig. 8.1.4) suggested that the action of the cetiedil compounds is either directly or very closely linked to the K⁺ channel. Assuming that these compounds do indeed act directly on K⁺ channels what deductions can be made about their mechanism of action? The following sections outline some possible mechanisms by which cetiedil might block Kᵣ.

11.2.1 Do the compounds act by disrupting the cell membrane?
The relative lack of structural effects and the importance of lipophilicity might suggest that the interaction between these compounds and their site of action is rather non-specific. Is it possible that the agents simply act by diffusing into and disrupting the cell
membrane? If this were the case it is possible that channel block occurs when the concentration of blocker in the membrane reaches a particular level, regardless of structure. In these circumstances the importance of log P would simply be that the total amount of drug which must be added to the suspension to reach the required concentration in the membrane would decrease as log P increased. A number of observations seem to make this unlikely. Firstly, cetiedil was found to be ineffective as a blocker of the apamin-sensitive K⁺ channel (IC₅₀ ≈ 1 mM) in guinea pig hepatocytes although in the same tissue it potently inhibited volume activated K⁺ channels (Sandford et al. 1992). Secondly, as will be discussed in section 11.5, the SAR seems to be different for different cetiedil-sensitive K⁺ channels. Thirdly, cetiedil had no effect on membrane fluidity in erythrocytes at concentrations as high as 250 μM (Giannettini et al. 1992). Finally, the effect of increasing extracellular K⁺ on the potency of cetiedil and of UCL 1495 is difficult to explain in terms of an action solely on the cell membrane.

It might be argued, however, that the effect of increasing lipophilicity is simply to increase the equilibrium concentration attained at the site of action rather than to increase the compound's affinity for its binding site. It is interesting to note that a similar relationship between activity and log P has been described for certain calcium entry blockers (Rojratanakiat and Hansch 1990; Kimball et al. 1992). Here it has also been suggested that the effect of increasing log P is to increase the concentration of drug at the site of action. However, there is evidence that structure does play a role in determining the activity of the cetiedil compounds (e.g. the results with the isomers of UCL 1495 and with the compounds shown in Table 7.2.1) so it is unlikely that this is the sole explanation.

11.2.2 Comparison of the actions of clotrimazole and UCL 1495

Experiments in which the action of UCL 1495 were compared with those of clotrimazole provided some further clues as to the mechanism of block by each compound.

The actions of clotrimazole and UCL 1495 were found to differ in three respects. Firstly, the blocking action of UCL 1495 but not that of clotrimazole was markedly reduced by increasing [K⁺]₀ from 0.1 to 5.4 mM. Secondly, the Hill slope of the
concentration-inhibition curve for UCL 1495 was 2.8 ± 0.3 compared with 1.0 ± 0.1 for clotrimazole. Thirdly, the time course of the onset of inhibition by UCL 1495 and clotrimazole were found to differ: the inhibition by UCL 1495 developed steadily during the exposure to A23187 whereas clotrimazole reduced the initial rate of K loss (see Fig. 8.4.1). This latter observation suggests that UCL 1495 cause open channel block, though other possibilities have not been excluded.

Taken together these findings strongly suggest that the mechanism of action of UCL 1495 differs from that of clotrimazole. It has previously been shown that increasing extracellular[K⁺] also reduces the effectiveness of cetiedil and quinine whereas the actions of nitrendipine and charybdotoxin were, like clotrimazole, relatively insensitive to changes in [K⁺]₀ (M. Shiner, unpublished observations). Interestingly, the concentration-inhibition curve for quinine (Reichstein and Rothstein 1981) is steep whereas the curves for nitrendipine and charybdotoxin are shallow (M. Shiner, personal communication). It is likely, therefore, that clotrimazole and UCL 1495 and their congeners have distinct mechanisms of action.

Since clotrimazole has been shown to inhibit the binding of ¹²⁵I-charybdotoxin (Brugnara et al. 1993b) it seems reasonable to suppose that the two compounds have a similar mechanism and perhaps act at the same site. Although the actions of charybdotoxin on erythrocyte Kᵥ SRC have not been studied at the single channel level it seems unlikely that such a large molecule would act from within the cell or penetrate very deeply into the pore. Indeed, evidence from experiments on the BK channel and on the shaker Kᵥ channel strongly suggests that charybdotoxin acts at the outer mouth of the pore (MacKinnon and Miller 1988, 1989; Miller, 1988). It seems likely therefore that the same is true for clotrimazole. This would be consistent with the observations made in the present study.

As was stated in the introduction, the flux ratio experiments of Vestergaard-Bogind and colleagues (Vestergaard-Bogind et al. 1985) suggested that the erythrocyte Kᵥ SRC behaves as a multi-ion channel which can hold at least 3 K⁺ ions at a time. The effects of UCL 1495 and its congeners may be understood in terms of the behaviour of a single-file
multi-ion channel (Hille and Schwarz 1978). In this model the channel can be occupied by several ions at once but they are constrained to move in single file. Furthermore, a $K^+$ ion (or blocking molecule) can only move if an adjacent site is vacant. Assuming that the cetiedil compounds can enter at the cytoplasmic side but not pass through the channel one might expect that the concentration-inhibition curve would have a Hill slope greater than one since entry of a second molecule of blocker would reduce the probability of a molecule already in the channel leaving. It would also be expected that raising the external $K^+$ concentration would alleviate block by speeding the exit of blocking molecule. The apparent use-dependence of the block caused by UCL 1495 is also consistent with a model which requires the drug to enter the open ion channel. However, the techniques used in the present study did not allow a more rigorous analysis of the differences between UCL 1495 and clotrimazole. Studies at the level of single channels are clearly needed.

Assuming that the cetiedil compounds do block the channel in this way it is possible to make other predictions about their behaviour. Firstly, it would be expected that the blockade would be steeply dependent on voltage. There is evidence that blockade of erythrocyte $K_{Ca}$ by quinine is voltage dependent (Grygorczyk and Schwarz 1985). Secondly, the blocking action should be enhanced by an increase in intracellular $K^+$ or the presence of another blocking ion such as $Na^+$. In fact, blockade of the channel by quinine, which appears to act in a manner similar to that of cetiedil, is enhanced by raising the concentration of intracellular $Na^+$ (Reichstein and Rothstein 1981).

In the model outlined above the importance of lipophilicity might be two-fold. Firstly, as pointed out in the previous section, high lipophilicity may simply increase the effective concentration at the site of action. Secondly, it might be that a hydrophobic interaction occurs between the blocker and the ion channel protein. In this connection it is interesting to note that increasing chain length, and therefore hydrophobicity, of symmetrical quaternary alkyl ammonium ions increases potency in some systems (e.g. the delayed rectifier in squid axon, French and Shoukimas, 1981).
11.3 Suitability of K⁺-sensitive electrode technique as a screening system

11.3.1 The choice of an appropriate measure of the inhibition caused by $K_{Ca}$ blockers

The K⁺ electrode system was chosen for this study because it allows compounds to be
screened fairly rapidly and because it was possible to obtain reproducible data.
However, it was found that clotrimazole was less active in this system than was reported
by other workers who measured inhibition of tracer flux (Alvarez et al. 1992). Although
this is partly due to a small difference in the sensitivity of human and rabbit erythrocytes
to clotrimazole, the main factor is likely to be differences in the techniques applied. As
was shown in section 8.4 clotrimazole altered the time course of A23187 induced K⁺ loss
from erythrocytes. When the cell suspension was exposed to A23187 alone the rate of
K⁺ loss from the cells declined gradually with time and by 3 min (the point at which K⁺
loss was measured for quantifying the size of the response) was considerably less than
the initial rate. However, when A23187 was applied to the suspension in the presence of
clotrimazole the initial rate of K⁺ loss was reduced but remained relatively constant.
Because of this, the degree of the inhibition of total K⁺ loss depends partly on the time
point at which the total K⁺ loss is measured. It is likely therefore that a tracer flux
method which measures the initial rate would give a lower IC₅₀. In the case of the
cetiedil analogues it was found that the initial rate of K⁺ loss was not greatly affected but
declined dramatically with time. In this case the IC₅₀ for inhibition of the steady state
rate coefficient of tracer efflux would also be lower than the IC₅₀ for inhibition of net loss
of K⁺. There is, however, no obvious 'best' way to express the results and the arbitrary
choice of a 3 min period had the advantage that it allowed the findings with the cetiedil
compounds and with clotrimazole to be expressed in the same way.

11.3.2 Implications of the high lipid solubility of some of the compounds:

depletion into lipid

A further possible source of error, particularly with very lipophilic compounds, might
arise from partitioning of the drug into the erythrocyte membrane thereby reducing the
free concentration of drug in the medium used to suspend the cells in the experiments
with K⁺-sensitive electrodes.
The most active compounds used in this study had predicted log P values between 7 and 8. Thus for an octanol/water system one would expect that the concentration in octanol of say, UCL 1495 (log P= 7.5) would be 3.5x10^7 times greater than in water! It is essential, therefore, to consider some of the possible effects of partitioning of the compounds into the membrane under the conditions of the present experiments.

When the cells are at equilibrium with the blocker, the concentration of blocker in the lipid phase will be :-

$$C_t = \frac{PC_t}{1 + Pkh}$$

Equation 1

Similarly, the concentration of drug remaining in the aqueous phase is given by :-

$$C_{aq} = \frac{C_t}{1 + Pkh}$$

Equation 2

Where

- $C_t$ = concentration in lipid phase
- $C_{aq}$ = concentration in aqueous phase
- $C_t$ = total concentration
- $P$ = molar partition coefficient
- $h$=haematocrit
- $k$ = constant of proportionality relating volume of lipid to haematocrit
  
  ($=\text{volume lipid per unit volume cells}/100$)

In order to estimate the extent to which depletion occurs it is necessary to have an estimate of $k$. The total lipid content of erythrocytes is 512 mg/100 ml cells and most of this is associated with the cell membrane (Altman and Dittmer 1974). If we assume that the density of the lipid is 1 g l^-1 then the cells will contain 5.1 x 10^-3 l lipid per l cells. Thus $k$ will be value of 5.1 x 10^5. By using this value of $k$ in equation 2 it can be seen that if log P = 7.5 and $h = 1$ % (the standard haematocrit used in these experiments) then the concentration of drug in the medium used to suspend the cells ($C_{aq}$) will only be 0.06 % of the total concentration. This would have two consequences. Firstly, incubation of cells with the compound should cause depletion of the compound and secondly, the
degree of inhibition caused by a particular concentration should depend upon haematocrit.

Re-arranging equation 1 in terms of $C_t$ gives:

$$C_t = \frac{C}{P} + C_j kh$$  \hspace{1cm} \text{Equation 3}$$

If it is assumed that equal degrees of inhibition correspond to equal concentrations in the lipid then a plot of $IC_{50}$ versus $h$ should give a straight line with slope $C_j k$. The intercept, $C/P$, gives the $IC_{50}$ for a very dilute cell suspension in which no depletion of drug from the aqueous phase occurs.

Recently these ideas have been tested in experiments by M. Malik in our laboratory. The effect of varying haematocrit on $IC_{50}$ of UCL 1495 is shown in Fig. 11.3.1. The intercept on the abscissa, $C/P$, suggests that at a very low haematocrit, when no depletion occurred, the $IC_{50}$ for UCL 1495 would be 300 nM, compared with 1 µM at a haematocrit of 1%. The value for the slope of the line, $C_j k$, was $6.7 \times 10^{-7}$ M.

Using the predicted values of $k$ (5.1 x $10^{-5}$) and $P$ (3.1 x $10^{-7}$) and the observed values of the intercept and the slope it should be possible to estimate $C_h$, the concentration of drug in the lipid phase required to produce 50% inhibition. However, when this is done $C_i$ is calculated to be either 13 mM (from the slope) or 9.3 M (from the intercept): a 700 fold difference! So although the results agree qualitatively with the predictions, the effects of varying haematocrit on the $IC_{50}$ are not consistent with the predicted values of $k$ and $P$.

How can this be explained? Firstly, the estimate of $k$ was based on the published value of the total lipid content of erythrocytes. Since some of this will be associated with proteins the amount available for partitioning is likely to be rather less. A second source of error is that the calculated log P is likely to be an over-estimate. There are in fact a number of reasons why one might expect the calculated log P value to differ from that observed for the saline solution/membrane system. Firstly, the predicted log P only applies to the unionised molecule. Cetiedil has a pKa of 9.3 and so at physiological pH most of the compound would be in the ionized form. This would have the effect of
Fig 11.3.1 Effect of varying haematocrit on the IC$_{50}$ of UCL 1495. The extrapolated IC$_{50}$ at 0% haematocrit (i.e. when no depletion occurs) is 0.3 µM compared with 1 µM at 1%.

These results were kindly supplied by Ms M. Malik.
reducing the apparent partition coefficient and it would also reduce the discrepancy in the estimates of $C_t$ mentioned above. Secondly, the calculated values are for an octanol/water system and although this is a widely used system for measuring P values it is known that partition coefficients differ according to the composition of the lipid phase (Luxnat and Galla 1986). Finally, the partition of solutes into a bilayer differs in many respects from partition into a bulk phase. Measurements have shown that the observed partition coefficients of many compounds are lower for bilayers than for bulk phases (see e.g. De-Young and Dill 1988). It is likely, therefore, that the amount of partitioning into the cell lipid was considerably less than the theoretical value and this is borne out by experimental observation.

Nevertheless, under the conditions used in the present study it seems likely that partitioning of highly lipophilic compounds into the cell membrane does cause the $IC_{50}$ to be over-estimated. This problem can be overcome by using a lower haematocrit but this introduces the difficulty of measuring very small changes in $[K^+]_o$.

It is apparent, therefore, that the $IC_{50}$ values presented in Section III are more likely to understate rather than overstate the true potency of the compounds tested. Clearly for this and indeed many other reasons the $IC_{50}$ values quoted in this work are not equivalent to the dissociation equilibrium constant for the combination with a binding site. However, it is seems unlikely that the relative potencies of the compounds have been estimated wrongly.

It is worthy of mention here that Boer et al. (1989) found that the apparent $K_i$ for displacement of $[^3H]$isradipine by (+)-niguldipine was also dependent on the quantity of membrane lipid in the sample and that this could be analysed by using similar reasoning to that described above.

11.4 Relevance of the present work to anti-sickling actions of cetiedil

It has been suggested that the anti-sickling action of cetiedil results from its ability to inhibit $K_{Ca}$ (Berkowitz and Orringer 1982). In the present work it has been found that in physiological $K^+$ concentrations the $IC_{50}$ of cetiedil was 100 $\mu$M. This is consistent with
previously published estimates and in the range required to prevent cell-sickling \textit{in vitro}. It would be very interesting, therefore, to test the more potent analogues of cetiedil for their effect on sickle cells. The existence of a correlation between anti-sickling and $K^+$ channel blocking activity would be good evidence in support of the hypothesis that cell-sickling involves the activation of $K_{Ca}$.

In this study it was also shown that UCL 1349, (-)-cetiedil, was less active as an anti-muscarinic agent than UCL 1348, (+)-cetiedil. In reports of clinical trials subjects reported experiencing side effects typical of administration of an anti-muscarinic agent (e.g. dry mouth, Benjamin et al., 1986). Since the enantiomers of cetiedil did not differ in their ability to block $K_{Ca}$ it is possible that the use of (-)-cetiedil would reduce the incidence these side effects.

11.5 Action of cetiedil and related compounds at other $K^+$ channels

Cetiedil and a limited number of its analogues have been tested in our laboratory for their ability to block a wide range of $K^+$ channels. These are summarised in table 11.1.

Some patterns do seem to emerge. Firstly the quaternary derivative UCL 1285 is less active than cetiedil in all systems in which both were tested. Secondly, whereas as the lipophilic UCL 1495 is considerably more active than cetiedil in the erythrocyte it is much less active than cetiedil as an inhibitor of levocromakalim stimulated $^{86}$Rb efflux from rat aorta. Similarly the compound UCL 1459 which is also more lipophilic than cetiedil and more active in the erythrocyte is much less active than cetiedil as an inhibitor of volume activated $K^+$ channel in liver cells. It would appear, therefore, that lipophilicity is not important in these systems. A further difference between the erythrocyte and liver cell channels was that whereas UCL 1269, the hydroxy derivative of cetiedil, is less active than cetiedil in the erythrocyte it possesses similar activity in hepatocytes (P.M. Dunn and K. Burke, personal communication). It is unlikely that the low activity of the lipophilic analogues of cetiedil as blockers of the hepatocyte volume-activated $K^+$ channel can be explained by a reduction in concentration as a consequence of partitioning into the membrane because these experiments were done with isolated cells bathed in a flowing solution so that little depletion could occur.
11.6 Further work

The present study has shown that it is possible to use cetiedil as a starting point to develop compounds with increased selectivity and potency for erythrocyte $K_{Ca}$. Since cetiedil is now known to block several $K^+$ channels it is possible that further studies on the SAR of cetiedil will result in the development of compounds with selectivity for other channels.

Preliminary data have been presented which suggest that the action of the cetiedil series of compounds differs from other agents such as clotrimazole. Further studies at the single channel level will be needed to verify this. As yet no information is available concerning the mechanism of action of these compounds at other $K^+$ channels. This information would be of interest in its own right but if differences were found it may also give clues as to how more selective agents may be developed.
### Table 11.1 Cetiedil - sensitive K⁺ channels

<table>
<thead>
<tr>
<th>Compound</th>
<th>IK(RBC)₁ᵃ</th>
<th>K_{ACH} ²ᵇ (atria)</th>
<th>S.M.P. ³ᵇ (aorta)</th>
<th>K_{KCO} ⁴ᶜ (annecoc.)</th>
<th>K_{KCO} ⁴ᶜ (frog sart.)</th>
<th>K_{ATP} ⁴ᶜ</th>
<th>K_{vol} ⁴ᵈ (liver)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cetiedil</td>
<td>26(100*)</td>
<td>2</td>
<td>6</td>
<td>1.4</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>2.2</td>
</tr>
<tr>
<td>UCL 1495</td>
<td>1.2</td>
<td>0.4</td>
<td>&lt;10</td>
<td>&gt;10</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>UCL 1285</td>
<td>&gt;100</td>
<td>-</td>
<td>-</td>
<td>≈4</td>
<td>-</td>
<td>-</td>
<td>&gt;10</td>
</tr>
<tr>
<td>UCL 1459</td>
<td>1.7</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>&gt;10</td>
</tr>
<tr>
<td>UCL 1269</td>
<td>150</td>
<td>&lt;10</td>
<td>&gt;10</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2</td>
</tr>
</tbody>
</table>

**Notes**

1. K⁺ efflux stimulated by A23187 in 0.1mM K⁺ solution
2. Change in conductance induced by adenosine
3. Change in conductance induced by nor-adrenaline
4. Stimulation of Rb efflux by levocromakalim
5. Inhibition of change in conductance induced by cell swelling

*IC₅₀ in 5.4mM K⁺*

₁ᵃ M. Shiner./D.C.H.B.

₁ᵇ A. G. Jones.

₁ᶜ D.C.H.B.

₁ᵈ P. M. Dunn/K. S. Burkes./C. A. Sanford.
Section IV

The effects of KCO’s and metabolic inhibition on the \( K^+ \) permeability of skeletal muscle
12 EFFECTS OF POTASSIUM CHANNEL OPENERS AND METABOLIC INHIBITION ON THE POTASSIUM PERMEABILITY OF SKELETAL MUSCLE

Potassium channel opener drugs such as cromakalim have been shown to act at ATP-sensitive K⁺ channels in many tissues (see Chapter 1.4 for a more detailed discussion of this). Since these channels are present in frog skeletal muscle (Spruce et al. 1985) and are conveniently studied by using ^86^Rb as a tracer for K⁺ (Castle and Haylett 1987) an investigation of the effects of cromakalim in this tissue and the effects of blockers thereon seemed worth undertaking. The experiments also provided the opportunity to test the blocking action of cetiedil on this subtype of K⁺ permeability.

12.1 Effects of potassium channel openers on frog skeletal muscle

12.1.1 Changes in ^86^Rb efflux

Cromakalim was found to cause a large increase in the rate of ^86^Rb efflux from the frog sartorius muscle (Fig. 12.1A). The concentration-dependence of the effect is shown in Fig. 12.1B. Taken at face value, the curve suggests that the effect of 300 μM cromakalim was considerably greater than 100 μM and that even 300 μM did not cause a maximal response. However, this may misrepresent the true situation. The response to cromakalim was highly variable and the point on the curve for 100 μM represents the mean of 30 observations whereas the value for 300 μM is based on only 5. On three occasions pairs of muscle from the same frog were exposed to 100 and 300 μM cromakalim and the mean responses were found to be 155 ± 58 % and 189 ± 69 % respectively. A two-tailed paired t-test showed that these values are not significantly different at the 5 % level (Fig. 12.2). It is likely, therefore, that 100 μM is closer to the maximally effective concentration than Fig. 12.1B might suggest.

Generally, the effect took about 4 min to reach a peak and started to decline before the end of the exposure period (Fig. 12.1A). On a few occasions the muscle was exposed to two successive doses of cromakalim. It was observed that the second response was always markedly reduced (see Fig. 12.3 for an example).
Fig. 12.1 A Stimulation of $^{86}$Rb efflux from frog sartorius by 100 μM cromakalim. The drug was present during the period indicated by the horizontal bar. Each point is the mean of 30 observations. Vertical bars indicate s.e.m. B Concentration dependence of the effect of cromakalim. Each point is the mean of the number of observations shown in parentheses.
Fig. 12.2 Comparison of the effect of 100 μM (○) and 300 μM (●) cromakalim on paired sartorius muscles. Each point is the mean of 3 observations. Vertical bars indicate s.e.m. Note that the data have been normalised as described in the methods section.
The effect of cromakalim on the semitendinosus muscle was also tested in a few preliminary experiments. A comparison of the action of 30 and 100 µM cromakalim is shown in Fig. 12.4. The mean increases in rate constant caused by these concentrations of cromakalim were 35.6 ± 10.3 % (n = 4) and 82.6 ± 12% (n = 14). These values are in keeping with those found for sartorius.

Levcromakalim at 50 µM caused an increase of 92 ± 28% (n = 3) in 86Rb efflux from the frog sartorius (Fig. 12.6). This is similar to the effect of 100 µM cromakalim and is consistent with other evidence that the activity of cromakalim resides in the (-) enantiomer (Hof et al. 1988).

In three tests diazoxide at a concentration of 600 µM had no significant effect on 86Rb efflux from sartorius muscle; the mean change in rate constant was -5.4 ± 3 %.

12.1.2 Inhibition of cromakalim stimulated efflux by glibenclamide
Glibenclamide reduced the response to 100 µM cromakalim at concentrations as low as 3 nM. Fig. 12.5A illustrates the inhibition of cromakalim induced efflux caused by 30 nM glibenclamide. Cromakalim alone caused a mean increase in rate constant of 63 ± 11 % (n = 5) and glibenclamide caused 82 ± 6 % inhibition. Glibenclamide was tested over the range from 3 to 100 nM and the concentration-effect curve is shown in Fig. 12.5B. The data were fitted to the Hill equation and the IC<sub>50</sub> was estimated to be 5.5 ± 5 nM, with n<sub>H</sub> = 0.8 ± 0.3. Glibenclamide had no obvious effect on resting efflux.

12.1.3 Effect of cetiedil on levcromakalim-stimulated 86Rb efflux from frog sartorius
Cetiedil had been found to be an effective inhibitor of levcromakalim stimulated efflux from rat aorta (see section 9.2) and so it was of interest to test it in this system. Cetiedil was tested at a single concentration of 10 µM which caused 84 ± 3.5 % inhibition. The results obtained are shown in Fig. 12.6. This compound too had no obvious effect on the resting efflux. Although a full dose-response relationship was not determined the degree of inhibition caused by 10 µM cetiedil was similar to that seen in the aorta.
Fig. 12.3 Effect of 2 applications of 100 μM cromakalim to frog sartorius. Cromakalim was present during the periods indicated by the horizontal bars. Note the reduced magnitude of the second response. Data from a single experiment.
Fig. 12.4 Comparison of the effect of 30 μM (●, n=4) and 100 μM (〇, n=14) cromakalim of frog semitendinosus. Vertical bars indicate s.e.m.
Fig. 12.5 A Effect of 30 nM glibenclamide on the increase in $^{86}$Rb efflux from frog sartorius stimulated by 100 μM cromakalim. Drugs were present during the periods indicated by the horizontal bars. Each point is the mean of 5 observations. B Concentration-inhibition curve for glibenclamide. Each point is the mean of the number of observations shown in parentheses. The data were fitted to the Hill equation and the IC$_{50}$ was estimated to be 5.5 ±5 nM with $n_H = 0.8 ± 0.3$. In both graphs vertical bars indicate s.e.m.
Fig. 12.6 Effect of 50 μM levromakalim on $^{86}$Rb efflux from frog sartorius in the absence (O) and presence (●) of 10 μM cetiedil. Each point is the mean of 3 observations. Vertical bars indicate s.e.m.
12.2.1 Microelectrode recording from frog skeletal muscle
The effects of levcromakalim on membrane potassium permeability were further investigated by using intracellular microelectrodes to measure changes in input resistance and membrane potential of single fibres of frog sartorius bathed in Cl⁻ free solution.

The fibres used had membrane potentials close to -80 mV (mean -80 ± 2.3 mV, n = 5). Levcromakalim (100 μM) caused a mean hyperpolarisation of 7 ± 3 mV (n = 5) and a decrease in input resistance which was manifest as a decrease in the size of the electrotonic potential of 49 ± 4.6 % (n = 5).

Pinacidil was also tested and at 300 μM had no effect on either resistance or membrane potential in frog sartorius fibres (n = 4). The trace shown in Fig. 12.7 shows a recording from a fibre which was insensitive to pinacidil but subsequently responded to levcromakalim (100 μM). However, in a single experiment using the frog cutaneous pectoris muscle, the same concentration of pinacidil caused hyperpolarisation and a decrease in input resistance in two fibres (Fig. 12.8). It should be mentioned that the fibres in this preparation had resting potentials rather lower than those used in sartorius preparations. The fibre used in Fig. 12.8 had a resting potential of -67 mV and application of pinacidil caused a hyperpolarisation of 16 mV accompanied by a 64 % decrease in the amplitude of the electrotonic potential. A second fibre with a resting potential of -51 mV showed a hyperpolarisation of 17 mV and a 74 % decrease in electrotonic potential. Further work would be needed to clarify these differences in the response to pinacidil. One possibility to explore is that the effect of pinacidil is greater in fibres with lower membrane potentials.

12.2.2 Effects of tolbutamide and phentolamine on the response to levcromakalim
Phentolamine at either 100 μM (n=1) or 300 μM (n=3) was unable to reverse the effects of 100 μM levcromakalim (n=3). Tolbutamide (300 μM), in contrast, caused almost complete inhibition (Fig. 12.9). These concentrations of phentolamine would be expected
Fig. 12.7 The effect of pinacidil (300 μM) (A) and levocromakalim (100μM) (B) on the membrane potential and input resistance of a single fibre of frog sartorius muscle. The traces are segments of a continuous recording. Pinacidil has no visible effect whereas levocromakalim, applied 7 min. after washout of pinacidil, causes a hyperpolarisation of 5 mV accompanied by a 57% decrease in the size of the electrotonic potential. The membrane potential of this fibre was -85 mV.
Fig. 12.8 The effect of 300 \( \mu \text{M} \) pinacidil on the membrane potential and input resistance of a single fibre from frog cutaneous pectoris muscle. This fibre had a resting membrane potential of -67 mV. Application of pinacidil caused a hyperpolarisation of 16 mV accompanied by a 64% decrease in the amplitude of the electrotonic potential.
Fig. 12.9 The effect of 300 μM phentolamine (A) and 300 μM tolbutamide (B) on the changes in membrane potential and input resistance produced by 100 μM levromakalim. The effect of levromakalim alone is shown in C. The traces are from a continuous recording from a single fibre in the order A, B, C. The record is broken for 17 min. between A and B and for 17 min. between B and C. Phentolamine has no visible effect on the response to levromakalim whereas tolbutamide causes almost complete inhibition. Subsequent application of levromakalim demonstrates the reversibility of the effect of tolbutamide.
to cause almost complete inhibition of the effect of levromakalim in smooth muscle (McPherson and Angus 1989).

12.3 Effects of potassium channel openers on mammalian skeletal muscle
Since cromakalim was found to be active in frog skeletal muscle it was considered of interest to extend the study to mammalian muscle. In addition to levromakalim two other potassium channel openers, pinacidil and RP52891 (the active enantiomer of RP49356), were also tested. Although the results of these experiments were ultimately to prove disappointing, a substantial amount of work was undertaken to establish whether the techniques used in the frog could be applied to studies of mammalian muscle.

12.3.1 Effects of potassium channel openers on \(^{86}\text{Rb}\) efflux from mouse soleus
The results obtained are summarised in Table 12.1 and examples of the effect of the three KCOs tested are shown in Fig. 12.10. Preliminary experiments at room temperature showed that the levromakalim-stimulated increase in tracer efflux seen with mammalian muscle was rather less than observed in the frog. Since Sanguinetti et al. (1988) have reported that cromakalim was ineffective in experiments on cardiac muscle carried out at room temperature, subsequent experiments were carried out at 37 °C. Although no attempt was made to study the effect of varying temperature in detail, a comparison of the effect of 100 μM levromakalim at room temperature and at 37 °C is shown in Fig. 12.11 and from this it is clear that increasing temperature did not significantly alter the magnitude of the response to levromakalim although the onset was somewhat more rapid at 37°C.

Returning to Fig. 12.10, RP52891 caused a small increase in tracer efflux but this was also highly variable and rather smaller than observed with levromakalim.

The results with pinacidil are more difficult to interpret. At 100 μM pinacidil clearly had no effect (5/5 muscles) in experiments carried out at 37 °C (Fig. 12.10C). However in
Table 12.1

<table>
<thead>
<tr>
<th>KCO</th>
<th>Conc. (µM)</th>
<th>Temp.(°C)</th>
<th>% Inc.</th>
<th>s.e.m.</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>LK</td>
<td>30</td>
<td>20-25</td>
<td>23</td>
<td>6</td>
<td>3</td>
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<tr>
<td></td>
<td>100</td>
<td>20-25</td>
<td>32</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>20-25</td>
<td>24</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>37</td>
<td>13</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>37</td>
<td>35</td>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td>RP52891</td>
<td>30</td>
<td>37</td>
<td>16</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>37</td>
<td>20</td>
<td>5</td>
<td>14</td>
</tr>
<tr>
<td>Pinacidil</td>
<td>30</td>
<td>20-25</td>
<td>2.3</td>
<td>11</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>20-25</td>
<td>26</td>
<td>20</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>37</td>
<td>2</td>
<td>4</td>
<td>5</td>
</tr>
</tbody>
</table>
Fig. 12.10 Effect of levromakalim (A), RP52891 (B) and pinacidil (C) at 100 μM on ⁸⁶Rb efflux from mouse soleus. The compounds were present during the period indicated by the horizontal bars. Each point is the mean of the number of observations shown above each graph. Vertical bars indicate s.e.m. All experiments were carried out at 37°C.
Fig. 12.11 Comparison of the effect of 100 μM levocromakalim (present during the period indicated by the horizontal bar) on $^{86}$Rb efflux from mouse soleus at room temperature (20-25°C) (●) and 37°C (○). Each point is the mean of 3 observations. Vertical bars indicate s.e.m.
some experiments at room temperature a small and highly variable response was observed at 100 μM.

It was evident from these results that the efflux stimulated by levocromakalim in mouse soleus was much less than seen in frog skeletal muscle. As can be seen in Table 12.1 increasing the concentration of KCO had little effect so the responses were probably near maximal. Since the responses to KCO's were so small and so variable it was decided that this preparation was not suitable for the study of K⁺ channel blocking agents.

12.3.2 Effects of potassium channel openers on membrane properties of mouse diaphragm

In the next series of experiments attempts were made to examine the effects of potassium channel openers on the membrane properties of the mouse diaphragm using the electrophysiological techniques which had been successful in the frog. The effects of levocromakalim and RP52891 were tested in both chloride free and normal solutions.

Levcromakalim (100 & 300 μM) had no appreciable effect on either membrane potential or input resistance (n=3). RP52891 had no effect at 100 μM (n=2) but at 300 μM it caused hyperpolarisation in a single fibre in one out of three preparations (Fig 12.12). In the single fibre which did respond, two successive applications of RP52891 elicited hyperpolarisations of 25 and 27 mV. The resting membrane potential of this fibre was only -50 mV which is rather lower than the mean of -66 ± 3(n = 11) mV. There was no accompanying change in resistance, possibly because this experiment was carried out in normal chloride so that the high chloride conductance of the membrane could have masked a change in potassium conductance.

It is difficult to account for the discrepancy between the electrophysiological experiments and the flux studies. One possible explanation is that only a small proportion of fibres in mammalian muscle are able to respond. This would be consistent with the small size of the responses seen in the flux studies.
Fig. 12.12 The effect of 300 μM RP 52891 on the membrane potential and input resistance of a single fibre from mouse diaphragm. The fibre had a resting membrane potential of only -48 mV. Application of RP 52891 caused a hyperpolarisation of 27 mV. There is, however, no detectable change in input resistance. Note: this recording was made in Cl- containing solution.
12.4 Metabolically poisoned mouse skeletal muscle

Since results with potassium channels openers were rather disappointing it was next decided to attempt to use treatment with metabolic inhibitors as a possible way of activating ATP-sensitive K⁺ channels in mammalian skeletal muscle, as has previously been achieved in frog skeletal muscle (Castle and Haylett 1987).

Exposure of soleus muscle to solution containing cyanide and iodoacetate caused the rate coefficient for $^{86}$Rb efflux to rise from a mean resting level of $4.4 \times 10^{-3}$ min⁻¹ to a peak of $3.5 \times 10^{-2}$. This represents an increase of $707 \pm 26$ % (n = 18). As can be seen in Fig. 12.13 the effect of the metabolic inhibitors developed slowly over a period of approximately 6 min and tended to decline toward the end of the experiment.

12.4.1 Effects of metabolic inhibition on mouse diaphragm

Since the diaphragm was used in electrophysiological experiments it was of interest to compare its behaviour with the soleus in a flux study. Fig 12.14 shows the results from 5 experiments testing the effect of treatment with CN/IAA on $^{86}$Rb efflux from strips of diaphragm. The mean resting rate coefficient was $0.007 \pm 0.0005$ min⁻¹ which reached a peak of $0.039 \pm 0.003$ min⁻¹. The resting rate was higher than for soleus and this is presumably a consequence of the much greater proportion of damaged fibres. The maximal increase in rate coefficient stimulated by poisoning was similar to that seen for soleus. However, the results obtained were much more variable and it was decided that the preparation was not suitable for experiments with K⁺ channel blockers. Similarly it seemed unlikely that the effects of KCO's could be studied by this method.

12.4.2 Effects of potassium channel blockers on $^{86}$Rb efflux

The effects of all the compounds tested are illustrated in Fig.12.15 and the results have been summarised in Table 12.2. It can be seen that effective blockers not only reduced the maximum increase in efflux caused by poisoning but also altered the time course of the effect: the rate at which efflux increased after poisoning was reduced and whereas in control tissues the rate constant decreased towards the end of the experiment, in muscles exposed to blocker it tended to increase.

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Fig. 12.13 Effect on $^{86}$Rb efflux from mouse soleus of treatment with 2 mM CN$^-$ and 1 mM iodoacetate. Metabolic inhibitors were present during the period indicated by the horizontal bar. Each point is the mean of 18 observations. Vertical bars indicate s.e.m.
Fig. 12.14 Effect of treatment with 1 mM iodoacetate and 2 mM CN⁻ on \(^{86}\)Rb efflux from mouse diaphragm. Metabolic inhibitors were present during the period indicated by the horizontal bar. Each point is the mean of 5 observations. Vertical bars indicate s.e.m.
Fig. 12.15 Effect of various agents of IAA/CN⁻ stimulated $^{86}$Rb efflux from mouse soleus. IAA and CN⁻ were present during the period indicated by the upper horizontal bars. Test compounds were present during the period indicated by the lower horizontal bars. The open symbols show the effect of metabolic inhibition alone and the filled symbols show the effect of metabolic inhibition in the presence of test compound. Each point is the mean of 3-4 observations. Vertical bars indicate s.e.m.
Table 12.2

<table>
<thead>
<tr>
<th>Drug</th>
<th>Conc</th>
<th>% Inhibition</th>
<th>s.e.m.</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glibenclamide</td>
<td>10 μM</td>
<td>35</td>
<td>6</td>
<td>4*</td>
</tr>
<tr>
<td>Glibenclamide</td>
<td>30</td>
<td>25</td>
<td>6</td>
<td>4*</td>
</tr>
<tr>
<td>4-aminopyridine</td>
<td>3 mM</td>
<td>-12</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>$\text{Ba}^{2+}$</td>
<td>1 mM</td>
<td>78</td>
<td>1</td>
<td>3*</td>
</tr>
</tbody>
</table>

(* denotes significant inhibition at 5% level as assessed by a paired t-test)
Although glibenclamide caused a significant degree of inhibition at both concentrations tested this did not appear to be dose dependent. The simplest explanation is that both concentrations maximally inhibited a glibenclamide-sensitive component of the efflux but that the magnitude of this varied between experiments. In keeping with this, the maximum rate of efflux attained in the experiments testing 10 μM glibenclamide was rather greater than those testing 30 μM. The observation that Ba\(^{2+}\) caused more inhibition than glibenclamide suggests that a large proportion of the remaining, glibenclamide insensitive component, was carried by potassium channels, albeit of a different kind. The ineffectiveness of 4-aminopyridine would seem to rule out the possibility that any of the increase in efflux is the result of activation of the delayed rectifier secondary to depolarisation caused by the cessation of electrogenic pumping and the loss of intracellular potassium.

Ba\(^{2+}\) caused an inhibition of resting efflux of 35.6 ± 2.0 %. None of the other compounds tested had an appreciable effect on resting efflux.
13 DISCUSSION OF RESULTS PRESENTED IN SECTION IV

13.1 The effect of KCO's on skeletal muscle

In frog skeletal muscle cromakalim and levocromakalim caused an increase in $^{86}$Rb efflux, membrane hyperpolarisation and a decrease in input resistance. This is good evidence of the activation of potassium channels and is in keeping with observations on the effects of levocromakalim in excised patches (Forestier et al. 1993). The concentrations of cromakalim required to open K$^+$ channels in this tissue are, however, rather higher than in smooth muscle for example, the EC$_{50}$ for stimulation of $^{86}$Rb efflux by cromakalim is typically 4-6 μM in rat aorta (Quast and Baumlin 1988). Presumably this indicates differences in the channel type and/or binding site between tissues and this is supported by evidence from blocking agents (see next section). The concentrations of cromakalim used in this study are, however, in keeping with those reported by Spuler et al. (1989) working on human skeletal muscle.

In mammalian muscle levocromakalim was found to stimulate $^{86}$Rb efflux but the magnitude of the response was rather smaller than seen in the frog. Furthermore, it was not possible to demonstrate the expected effects on membrane potential and input resistance. This is perhaps surprising since cromakalim has been shown to activate ATP-sensitive channels in excised patches (Weik and Neumcke 1990, Hussain et al. 1994) and to cause hyperpolarisation of human skeletal muscle (Spuler et al. 1989). Possible explanations are considered below.

Turning to the other KCO's tested in this study, pinacidil had no electrophysiological effects in either frog sartorius or mouse diaphragm and the effect of this compound on $^{86}$Rb efflux from mouse EDL were highly variable. In one experiment, however, pinacidil did have the expected effect on fibres from frog cutaneous pectoris. RP52891 was only tested in mouse skeletal muscle. In the flux study experiments RP52891 consistently caused an increase in tracer efflux but only caused hyperpolarisation in one diaphragm preparation.
Diazoxide was tested in the frog sartorius alone and was found to be inactive at 600 μM. This is perhaps less surprising since at similar concentrations it also failed to activate $K_{\text{ATP}}$ in membrane patches from mouse skeletal muscle (Weik and Neumcke 1990).

There is clearly a discrepancy between the activity of cromakalim and levcromakalim in the frog and mouse and between intact tissue and excised patches. One possible explanation for the difference between experiments on whole muscles and single fibres is that only a small proportion of fibres in the mammalian muscle were sensitive to levcromakalim. Since it has been found that in excised patches cromakalim was ineffective in the presence of high concentrations of ATP (>1 mM) (Weik and Neumcke 1990) it is possible that $^{86}\text{Rb}$ efflux was stimulated only in fibres which had a reduced ATP content. Since this could result from hypoxia one might then expect that the fibres contributing to the increase in tracer efflux were deep in the muscle and therefore not accessible in the electrophysiological experiments. In the same way it is possible that the difference between mammalian and frog skeletal muscle arose from differences in the metabolic condition of the tissues in the conditions used rather than from differences in the channels themselves.

RP 52891 and pinacidil have also been shown to be effective in excised patches and to hyperpolarise human skeletal muscle. In the present work, the only occasions when RP52891 and pinacidil were found to have an effect on membrane potential was when relatively depolarised fibres were used. Interestingly, a number of workers using intact tissue have reported the effects of KCO's depends on membrane potential. Thus Hong and Chang (1991), using mouse diaphragm, reported that levcromakalim only reduced the input resistance of mouse diaphragm when the fibres were deliberately depolarised by current injection. Similarly, Spuler et al. (1989) found that cromakalim had a more marked effect on healthy human skeletal muscle when it was deliberately depolarised. It would, of course, be expected that the degree of hyperpolarisation would be greater in fibres with membrane potentials further away from $E_K$, however, the effect of deliberately causing depolarisation on the response to KCO's applied equally to the initiation of changes in input resistance. Although the current findings are in keeping with this observation it is not clear what mechanism could account for it.
A further observation was that the maximal rate of efflux stimulated by KCO's was rather less than seen in metabolically poisoned muscle. This can partly be accounted for by the observation that only a proportion of the efflux stimulated by metabolic inhibition was sensitive to glibenclamide. In addition it is again possible that the intracellular ATP concentration was too high to allow a full activation of the channels in the non-poisoned muscle. Also, it is known that $K_{\text{ATP}}$, is regulated by other factors such as pH; and concentrations of nucleotide diphosphates (Allard and Lazdunski 1992, Davies et al. 1992) which would differ between the metabolically poisoned and normal muscle preparations.

In intact mammalian skeletal muscle, levromakalim had no effect of the contractions of normoxic muscle but did increase the rate of decline of contraction seen in hypoxic conditions (Weselcouch et al. 1993). This is further evidence that the response of intact muscle to KCO's is dependent on the metabolic state of the tissue.

13.2 Effects of blockers on responses to cromakalim and levromakalim
Glibenclamide and tolbutamide both inhibited responses to cromakalim, as would be expected given the likely involvement of $K_{\text{ATP}}$. The IC$_{50}$ for inhibition of responses to cromakalim was 8 nM. In vascular smooth muscle the IC$_{50}$ for inhibition of the actions of cromakalim is typically of the order of 100 nM. For inhibition of tracer efflux from exhausted frog semitedinosus muscle the IC$_{50}$ was between 3 and 10 μM (Castle and Haylett 1987). Estimates of the IC$_{50}$ for inhibition of $K_{\text{ATP}}$ in excised patches vary from 190 nM in mouse skeletal muscle (Allard and Lazdunski 1993) to 3 μM in frog skeletal muscle (Standen et al. 1992). Thus glibenclamide appears to be particularly potent in this system. A possible explanation for this is that the process of metabolic exhaustion alters the affinity of glibenclamide for its receptor. Studies with β-cell membranes have shown that both MgATP and MgADP, but not their non-hydrolysable analogues, inhibit the binding of $^3$H- glibenclamide (Schwanstecher et al. 1991). Since the levels of both ATP and ADP would be altered by metabolic inhibition it is difficult to predict the effect of this on the affinity of glibenclamide for its receptor. A further possible explanation for the discrepancy is that binding of cromakalim and glibenclamide interact. In smooth muscle the antagonism by glibenclamide is apparently competitive (see e.g. Eltze, 1989) and
evidence from binding studies with 3-H P1075 suggests that glibenclamide may inhibit the binding of KCO's albeit by an allosteric mechanism (Quast et al. 1993).

Phentolamine failed to inhibit the action of levocromakalim at concentrations which would be expected to cause complete inhibition in vascular smooth muscle (McPherson and Angus 1989). This is an indication that the two channel types differ in their pharmacology. Phentolamine has also been found to block $K_{\text{ATP}}$ in pancreatic β cells (Dunne 1991) suggesting further differences in the pharmacology of these channels. It has recently been suggested that the sulphonylureas and imidazolines inhibit the actions of KCO's in vascular smooth muscle by binding to different sites (Challinor and McPherson 1993). Cetiedil, on the other hand did not appear to discriminate between skeletal muscle and rat aorta and it would be rewarding to identify which (if indeed either) of the sites identified by Challinor and McPherson is involved in its action on skeletal muscle.

13.3 Metabolically poisoned mouse skeletal muscle
Exposure of mouse soleus to CN⁻ and iodoacetate caused a large increase in tracer efflux. In previous experiments on frog skeletal muscle increases of this order were only seen after the muscle had been stimulated to the point of developing rigor (Castle and Haylett 1987). Thus the mammalian muscle appears to be more sensitive to metabolic inhibition. Only a minor component of the efflux was sensitive to glibenclamide. The concentrations of glibenclamide required were similar to those used in frog skeletal muscle (Castle and Haylett 1987). The glibenclamide-sensitive component of poisoning-induced efflux was smaller than previously reported for the frog. This may indicate either a lower density of channels or that full activation of the channels was not achieved in the experiments on mammalian muscle. The extent of ATP-depletion was not measured and it is possible that more extensive depletion could have been achieved by combining metabolic inhibition with electrical stimulation. However, under these conditions it has been found that a non-specific increase in membrane permeability occurs, as indicated by efflux of lactate dehydrogenase occurs (Jones et al. 1983).

The possibility that the remaining tracer efflux was due to membrane damage can be ruled out by the observation that $\text{Ba}^{2+}$ ions caused almost complete inhibition. What then could
be the identity of the remaining component? The ineffectiveness of 4-aminopyridine excludes an involvement of the delayed rectifier. The exposure to metabolic inhibitors would also be expected to cause an increase in intracellular Ca$^{2+}$ and so it is possible that BK channels would be activated. However, Castle and Haylett (1987) reported that venom of *Leirius Quinquestriatus* did not inhibit $^{86}$Rb efflux from metabolically exhausted frog skeletal muscle. Furthermore, since BK channels are also voltage-dependent and since the membrane potential of the exhausted muscle was not known it is difficult to predict what contribution the BK channels would make. A further possible explanation for the glibenclamide-insensitive component is that $K_{ATP}$ channels can under, certain circumstances, become insensitive to glibenclamide. Findlay (1993) has reported that $K_{ATP}$ current induced by metabolic inhibitors in ventricular myocytes is only transiently inhibited by sulphonylureas. This led the suggestion that $K_{ATP}$ can become dissociated from the sulphonylurea receptor during prolonged metabolic stress. Such a mechanism might also be applied to the present findings. However, further work would be needed to verify this.

13.4 Conclusions from work presented in section IV

The actions of KCO's reported here are consistent with effects mediated via $K_{ATP}$ and add to the evidence for the presence of these channels in skeletal muscle. Further, the inability of phentolamine to inhibit the action of levocromakalim in frog sartorius indicates that it is possible to distinguish between types of channel acted on by KCO's. Clearly, the discovery of selective blockers of the action of KCO's will be useful in the further characterisation of KCO-sensitive channels.

The KCO's were found to be relatively ineffective in mammalian muscle. However, the present results strongly suggest that the effects of these compounds may depend on the metabolic state of the fibres. Identification of the mechanism of this dependence could throw light on the actions of the KCO's.

An incidental finding was that the use of metabolic inhibition to activate $K_{ATP}$ is likely to be of limited use as a procedure for screening for blockers. However, these experiments brought to light the existence of a large glibenclamide and 4-AP-insensitive $K^+$

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permeability in mammalian skeletal muscle that becomes activated under these conditions. It would be interesting to characterise this further.
14 Concluding remarks

The results presented in this thesis suggest various directions for future research.

Work with the cetiedil compounds has shown that it is possible to use this molecule as a starting point for the development of more potent agents which block the erythrocyte $K_{Ca}$. It is also clear that cetiedil is able to block several other $K^+$ channels and that the structural requirements for block of these other $K^+$ channels are different. A systematic study of the effects of cetiedil and its congeners on these other channels could well result in the discovery of potent and selective $K^+$ channel blockers.

The effectiveness of clotrimazole as a blocker of the erythrocyte $K_{Ca}$ has been verified and evidence has been produced to suggest that the mechanism of channel block by this agent differs from that of the cetiedil compounds. These findings clearly merit further study, preferably by applying the techniques of single channel recording. It would also be of interest to establish whether or not the differences in the mechanisms of action of these compounds are reflected in their effects on sickle cells.

The studies on the effects of KCO's on skeletal muscle add to the evidence that these compounds modulate $K_{ATP}$. It is clear, however, that the pharmacological properties of the channel activated by KCO's in skeletal muscle differ from those of the KCO-sensitive channels in smooth muscle. Further pharmacological characterisation of these channels is now needed. Also the present findings with mammalian muscle suggest that the effect of the KCO's may depend on the metabolic state of the tissue. An investigation of this dependence would throw much needed light on the mechanism of action of these compounds.

In studies on metabolically poisoned muscle the existence of a 4-AP- and glibenclamide-insensitive $K^+$ permeability was demonstrated. Further characterisation of this would be useful in developing an understanding of the events that occur during hypoxia.
Appendix 1 The use of the hydrophobic fragmental constant ($f$) in the prediction of log $P$

The system of hydrophobic fragmental constants ($f$ values) was introduced by Nys & Rekker (1973) as a way of predicting the log octanol/water partition coefficient of organic molecules. This system has the advantage that it is possible to make reasonably accurate predictions about the lipophilicity of compounds without the need for experimental measurements. The $f$ value is the contribution a fragment makes to the total log $P$ of a molecule. Thus the log $P$ of a molecule is given by:

$$\sum_{i=1}^{n} a_n f_n$$

where $f_n$ is the hydrophobic fragmental constant of a given fragment and $a_n$ is the number of times it occurs in the molecule.

The $f$ values for a large number of fragments were deduced by multiple regressional analysis of measured log $P$ values of a large number of compounds. The system was refined and the number of fragments for which $f$ values were calculated was increased by Rekker & de Kort (1979).

An important observation was that under certain circumstances fragments interact in such a way as to produce an excessive log $P$ value. These could be corrected for by the incorporation of a constant, $c_m = 0.28$. The relevant cases here are:

1) Close proximity of 2 electronegative groups - when electronegative groups are separated by 1 or 2 saturated C atoms the two groups hamper the lone pair's tendency to achieve hydration. Thus the log $P$ is higher than would be predicted otherwise. This is accounted for by adding $(3 \times 0.28)$ for a 1 C separation or $(2 \times 0.28)$ for a 2 C separation.

2) Conjugation of aromatic groups (e.g. biphenyl) or cross conjugation of aromatic groups by a double bond (e.g. benzophenone) or by a group with a lone electron pair (e.g. diphenylether) requires the addition of 0.28.

3) Condensation of aromatic groups requires the addition of 0.28 for each pair of shared C atoms (e.g. napthalene $1 \times 0.28$, phenanthrene $2 \times 0.28$)

4) For most functional groups, transition from an aliphatic to an aromatic position increases lipophilicity by $3 \times 0.28$. 

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At the end of this appendix there is a list of all the \( f \) values used to calculate \( \log P \) for the compounds considered in sections 7 and 8.

In this thesis the \( f \) values have been used in two ways, firstly to estimate \( \log P \) values of whole molecules and secondly to estimate the change in lipophilicity (\( \Delta \log P \)) that substituents make to a homologous series.

Below are some worked examples.

1 **Calculation of \( \Delta \log P \) for a homologous series:**
   Consider the general structure:
   \[
   \begin{array}{c}
   \text{R}_1 \quad \text{R}_2 \quad \text{R}_3 \\
   \text{O} \quad \text{O} \quad \text{N}
   \end{array}
   \]
   
   In order to estimate the contribution the \( \text{R} \) groups make to the lipophilicity of the molecule we calculate the sum of the \( f \) values for the fragment \( \text{R}_1 \text{R}_2 \text{R}_3 \text{C} \):

<table>
<thead>
<tr>
<th>compound</th>
<th>( \text{R}_1 )</th>
<th>( \text{R}_2 )</th>
<th>( \text{R}_3 )</th>
<th>( f ) values</th>
<th>( \sum f = \Delta \log P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>UCL1274</td>
<td>( \text{C}_6\text{H}_5 )</td>
<td>( \text{C}_6\text{H}_5 )</td>
<td>( \text{C}_6\text{H}_5 )</td>
<td>3(1.84)+0.155</td>
<td>5.68</td>
</tr>
<tr>
<td>UCL1299</td>
<td>( \text{C}<em>6\text{H}</em>{11} )</td>
<td>( \text{C}<em>6\text{H}</em>{11} )</td>
<td>( \text{H} )</td>
<td>2(2.93)+0.337</td>
<td>6.2</td>
</tr>
<tr>
<td>UCL 1289</td>
<td>( \text{C}<em>6\text{H}</em>{11} )</td>
<td>( \text{C}_6\text{H}_5 )</td>
<td>( \text{H} )</td>
<td>2.93+1.84+0.337</td>
<td>5.11</td>
</tr>
<tr>
<td>cetiedil</td>
<td>( \text{C}<em>6\text{H}</em>{11} )</td>
<td>thienyl</td>
<td>( \text{H} )</td>
<td>2.93+1.62+0.337</td>
<td>4.89</td>
</tr>
</tbody>
</table>

2 **Calculation of \( \log P \) of a whole molecule:**
   Consider UCL 1269:
   \[
   \begin{array}{c}
   \text{S} \quad \text{O} \\
   \text{HO} \quad \text{N}
   \end{array}
   \]
   
   This structure can be broken down as follows:
   
   aliph. \( \text{OH} = -1.17 \)
thienyl = 1.62

\[ C_6H_11 = 5(CH_2) + CH = 5(0.519) + 0.337 = 2.93 \]

C = 0.155

aliph. COO = -1.251

\[ 2(CH_2) = 2 \times 0.519 = 1.04 \]

homopiperidine = 6 (CH_2) + aliph. N = 6(0.519) - 2.085 = 1.03

Since there is a 2 C separation between the \( \alpha \) hydroxyl and the carbonyl a proximity factor of \( 2 \times 0.28 \) must be added. Thus \( \Sigma f(\log P) = 4.9 \).

\textit{f values used in the present study:-}

C 0.155

CH 0.337

CH_2 0.519

CH_3 0.701

C=H 1.84

H 0.182

imidazoly -0.081

thienyl 1.26

COO (aliphatic) -1.25

COO (aromatic) -0.384

N (aliphatic) -2.085

O (aliphatic) -1.595

S (aliphatic) -0.51

F (aliphatic) -0.476

F (aromatic) 0.391

Cl (aliphatic) 0.057

Cl (aromatic) 0.924

NO_2 (aliphatic) -0.920

NO_2 (aromatic) -0.053

CN (aliphatic) -1.041

CN (aromatic) -0.174
Appendix 2
Sources of materials

UCL Compounds
UCL compounds were synthesized by Dr C. Roxburgh, Dr S. Athmani, Dr W. Quaglia and Ms Z. Miscony under the supervision of Prof. C. R. Ganellin in the department of Chemistry, UCL. The only exceptions were:
- UCL 1496 (O-(4-Chlorobenzoyl)hydroquinine)
- UCL 1499 (hydroquinine-4-methyl-2-quinoyl ether)
- UCL 1505 (hydroquinine-9-phenanthryl ether)
- UCL 1547 (N-benzylcincholinium chloride)

which were purchased from Aldrich.

Other reagents were obtained from the sources shown below:

<table>
<thead>
<tr>
<th>Compound</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^8$RbCl</td>
<td>New England Nuclear</td>
</tr>
<tr>
<td>A23187</td>
<td>Sigma</td>
</tr>
<tr>
<td>Acetylcholine Iodide</td>
<td>BDH Laboratory Supplies</td>
</tr>
<tr>
<td>Atropine Methyl Sulphate</td>
<td>Sigma</td>
</tr>
<tr>
<td>Cetiedil citrate</td>
<td>Innothéa (gift)</td>
</tr>
<tr>
<td>Clotrimazole</td>
<td>Sigma</td>
</tr>
<tr>
<td>Cromakalim (BRL 34915)</td>
<td>Smith Kline Beecham (gift)</td>
</tr>
<tr>
<td>di-Butyl sebacate</td>
<td>Sigma</td>
</tr>
<tr>
<td>Diazoxide</td>
<td>Allen and Hanbury (gift)</td>
</tr>
<tr>
<td>Digitonin</td>
<td>BDH</td>
</tr>
<tr>
<td>EDTA</td>
<td>BDH</td>
</tr>
<tr>
<td>EGTA</td>
<td>Sigma</td>
</tr>
<tr>
<td>Glibenclamide</td>
<td>Hoechst (UK) (gift)</td>
</tr>
<tr>
<td>HEPES free acid</td>
<td>Calbiochem</td>
</tr>
<tr>
<td>Hexamethonium Chloride</td>
<td>Sigma</td>
</tr>
<tr>
<td>Hydroquinine</td>
<td>Sigma</td>
</tr>
<tr>
<td>Inosine</td>
<td>Sigma</td>
</tr>
<tr>
<td>Levcromakalim (BRL 38227)</td>
<td>Smith Kline Beecham (gift)</td>
</tr>
<tr>
<td>Mepyramine Maleate</td>
<td>Sigma</td>
</tr>
<tr>
<td>Phentolamine Mesylate</td>
<td>Ciba</td>
</tr>
<tr>
<td>Pinacidil</td>
<td>Leo (gift)</td>
</tr>
<tr>
<td>PVC (high molecular weight)</td>
<td>ICI</td>
</tr>
<tr>
<td>Quinine HCl</td>
<td>Sigma</td>
</tr>
<tr>
<td>RP 52891 (Aprikalim)</td>
<td>Rhône-Poulenc Santé (gift)</td>
</tr>
<tr>
<td>Sodium Isethionate</td>
<td>Fluka Chemie</td>
</tr>
<tr>
<td>Sodium tetrphenyl borate</td>
<td>Koch Light</td>
</tr>
<tr>
<td>Tetrahydrofuran</td>
<td>BDH</td>
</tr>
<tr>
<td>Tetrodotoxin</td>
<td>Calbiochem</td>
</tr>
<tr>
<td>Tolbutamide</td>
<td>Sigma</td>
</tr>
<tr>
<td>Tris HCl</td>
<td>Calbiochem</td>
</tr>
<tr>
<td>Valinomycin</td>
<td>Sigma Chemical</td>
</tr>
</tbody>
</table>

Glucose and all inorganic salts and organic solvents were of Analar quality and purchased from BDH.
REFERENCES


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