A Study of the Distribution and Function of P2 Receptors During Chick Embryogenesis

Written by

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Submitted to the University of London in 1999 for the award of

PhD

University College London
Abstract

The aim of this thesis is to investigate the possibility that extracellular nucleotides, and their receptors (P2 receptors) are involved in regulating developmental processes during chick embryogenesis.

In the first chapter, the classification, structure, and signal transduction mechanisms of P2 receptors are described. The evidence that suggests that this type of signalling has the potential to be important developmentally is also discussed.

In the second chapter, in-situ hybridisation is used to describe the developmentally regulated pattern of expression of cP2Y\textsubscript{1}, a G protein-coupled P2 receptor, in multiple tissue systems during chick embryogenesis. This is the first description of P2 receptor subtype expression during embryonic development of the chick, and only the second time that expression of a specific P2 receptor has been described during the embryonic development of an organism.

The following two chapters are devoted to investigating the function of cP2Y\textsubscript{1} in one of the regions in which it is expressed, the developing limb. In chapter 3 monitoring of intracellular calcium is used to characterise the response of early limb cells to extracellular nucleotides. This technique is used to show that in addition to cP2Y\textsubscript{1}, at least one other P2 receptor subtype is expressed in early limb cells. Monitoring of intracellular calcium, and RNase protection is also used to show that within 24 hours of culturing, expression of cP2Y\textsubscript{1} is lost from limb cells. Monitoring of intracellular calcium was then used as an assay to demonstrate that expression of functional cP2Y\textsubscript{1} could be rescued in these cells by transfection with an expression vector carrying the cP2Y\textsubscript{1} cDNA.

In the fourth chapter, the loss of cP2Y\textsubscript{1} in-vitro is exploited, using a gain-of-function approach, to investigate a function for cP2Y\textsubscript{1} in limb development. Early limb bud cells were transfected as described and characterised above, and an in-
vitro assay of cartilage formation was used to provide evidence that cP2Y₁ may have an important role to play in regulating the formation of the limb skeleton. In the final chapter immunohistochemistry is used to show that two members of the P2X, ligand-gated ion channel family of P2 receptors are expressed during chick skeletal muscle development, and in the embryonic chick nervous system.
Acknowledgments

Far too many people have helped me throughout this Ph.D. to mention them all, but special thanks must go to Jon Clarke for his patience, advice, and always being willing to help. Thanks also to Prof. Geoffrey Burnstock for taking me on in the first place. Ute Groschel-Stewart was also a huge help with the antibody staining, as was Karl Swann with the calcium imaging. For providing endless distractions in the form of beer, coffee, and pool, I’d like to thank Richie and Will, and for their support, encouragement and advice Ketan Patel, Mary Rahman, and Jon Gilthorpe. Lastly I’d like to thanks Lou for putting up with me, and keeping me sane.
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cP2Y, is expressed in the myotome - a compartment of cells within each somite which contains muscle precursors.

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Expression of cP2Y$_1$ reduces cartilage formation in micromass cultures

cP2Y$_1$ may perform multiple roles during limb development

The autocrine nature of P2 receptor signalling could reinforce developmental decisions and coordinate cells to make these decisions at the same time

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CHAPTER 1
General Introduction

Intracellular nucleotides (ATP for example) play fundamental roles in energy metabolism, nucleic acid synthesis, and enzyme regulation. Since the early part of this century there has also been widespread appreciation that extracellular nucleotides and nucleosides (e.g. adenosine) can act as signalling molecules, exerting a wide spectrum of biological effects on many adult tissues and cells. The aim of this thesis is to investigate the possibility that extracellular nucleotides are used as signalling molecules during embryonic development.

In this chapter, the history, classification, molecular structure, and transduction mechanisms of the receptors for extracellular nucleotides is described, and the evidence that suggests that this type of signalling has the potential to be important developmentally is also discussed.

• Early History of Receptors For Extracellular Nucleotides and Nucleosides.

In 1929, Drury and Szent-Gyorgyi observed that extracellular nucleotides and nucleosides, namely adenosine and ATP, exerted profound effects on the mammalian heart. Following this report, there was considerable activity within the field, with particular emphasis on the actions of adenosine and ATP on the cardiovascular system (Gillespie, 1933; Green and Stoner, 1950; Holton, 1959; Berne 1963). In the 1960s, a component that was neither adrenergic nor cholinergic was recognised in the autonomic nerves supplying the gut and bladder (Burnstock et al., 1966), and later tentatively attributed to ATP (Burnstock et al., 1970). As a consequence these nonadrenergic, noncholinergic nerves were termed purinergic (Burnstock et al., 1970; Burnstock, 1972). In the subsequent years, the purinergic theory was strengthened by a body of experimental evidence supporting the role of ATP as a transmitter or co-transmitter with noradrenaline, acetylcholine and other substances (Burnstock, 1976, 1990). Implicit in this concept of purinergic transmission was the existence of post-junctional receptors for ATP, and in 1978 Burnstock proposed a basis for distinguishing two types of purinergic
receptors; P1 receptors, activated by adenosine, and P2 receptors activated by ATP (Burnstock, 1978). The original classification into P1 and P2 receptors was based on 4 criteria: i) The relative potencies of ATP, ADP, AMP and adenosine; ii) the selective actions of antagonists, particularly methylxanthines, which competitively antagonise adenosine but not ATP actions; iii) the modulation of adenylate cyclase with the resultant changes in intracellular cAMP levels by adenosine, but not ATP; iv) the induction of prostaglandin synthesis by ATP, but not by adenosine. Since the time of this proposal, many experiments have been carried out that support the P1/P2 classification, and it is now well established and widely used in the literature (Abbracchio et al., 1993; Fredholm et al., 1994; Fredholm et al., 1997). (Figure 1.1 shows the structure of nucleotides).

- The P1 and P2 Receptor Families

Following Burnstock’s proposal, subsequent work led to the demonstration of both P1 and P2 receptor subtypes, so that these now constitute relatively large receptor families.

Subclasses of the Adenosine (P1) Receptor. The P1 receptor family encompasses four different G protein-coupled receptors, termed A₁, A₂A, A₂B, and A₃, whose activation leads to either stimulation or inhibition of adenylate cyclase, with corresponding changes in the levels of the intracellular second messenger, cAMP (Fredholm et al., 1994). Analysis of experimental evidence supporting the subclassification of P1 receptors, and the physiological roles of the different subtypes is not within the aims of this chapter, but is reviewed in Jacobson et al., 1992, and Linden et al., 1994.

Subclasses of the P2 receptor family. There are now at least 13 different P₂ receptor subtypes (Burnstock and King, 1996). This ever growing family of receptors was initially proposed to include P2X and P2Y receptor subtypes, mediating vasoconstriction and vasodilation respectively, and showing differential responses to various synthetic ATP analogues (Burnstock and Kennedy, 1985). This was soon extended to encompass two further receptor subtypes which did not fit the original P2X and P2Y subclassification (Gordon, 1986): the ADP, P2t receptor, involved in platelet
Figure 1.1. Nucleotides are composed of a base (either purine or pyrimidine) attached to a 5 carbon (pentose) sugar and three phosphate groups. Nucleosides (e.g. adenosine) consist of a base linked to a sugar, with no phosphate groups attached.
aggregation, and a P2z receptor, activated by ATP$^+$ and mediating large increases of plasma membrane conductance in several cell types. Later, responses to diadenosine-polyphosphates (Ap$_r$A) in various biological systems were claimed to involve specific P2 receptors that were different from the already characterised ones, and were consequently termed P2d receptors (Pintor and Miras-Portugal, 1993). Some of these receptors also responded to uridine nucleotides and were termed P2u receptors (O'Conner et al., 1991). Evidence was also accumulating that suggested that different P2 receptor subtypes had different molecular structures and electrophysiological properties, leading to the recognition of both fast ionotropic, and slow metabotropic receptors for nucleotides (Dubyak, 1991). This, and the cloning of the first receptors in 1993 and 1994, led to the proposal of a new system dividing all naturally-occurring P2 receptors into two major families, one a family of ligand-gated ion channels (the P2X receptors), and another family of G protein-coupled receptors (the P2Y receptors) (Abbracchio and Burnstock, 1994). This classification of P2 receptor subtypes is now widely accepted, and at least 7 different ionotropic receptors (P2X$_{1-7}$), and 6 different G protein-coupled receptors (P2Y$_1$, P2Y$_2$, P2Y$_3$, P2Y$_4$, P2Y$_6$ and P2Y$_9$) for nucleotides have been cloned so far from various species (Burnstock and King, 1996; Fredholm et al., 1997).

- **Molecular Structure and Transduction Mechanisms of P2X Receptors.**

Reviews of this class of receptors have been published by Bean, 1992, and in Dubyak and El-Moatassim, 1993, thus in this chapter only the general features of these receptors are discussed.

P2X receptors regulate cell function by gating ion permeability, and for the 7 cloned subtypes, ATP or analogues of ATP are the principal ligands for this family of receptors (Burnstock and King, 1996, and references therein). ATP can therefore be added to the group of physiological compounds (e.g. acetylcholine, glutamate, GABA, glycine, and serotonin) that act as extracellular agonists for ligand-gated ion channels. Activation of P2X receptors typically occurs within milliseconds, is in general followed by a rapid inactivation of the depolarising current, and accounts for fast excitatory neurotransmission
at a number of defined synapses (Edwards et al., 1992; Evans et al., 1992; Dubyak and El-Moatassim, 1993). Neither G proteins nor second messengers appear to be directly involved in the function of these P2X receptors. P2X ion channels exhibit little selectivity for monovalent cations, but under physiological conditions Na$^+$ is the predominant depolarizing ion (Benham, 1989; Evans et al., 1992; Christie et al., 1992). Activation of P2X receptors in many tissues also results in increases in intracellular calcium concentration ([Ca$^{2+}$]) (Benham and Tsien, 1987; Benham, 1989; Schneider et al., 1991; Christie et al., 1992), apparently occurring as a consequence of permeation of the P2X channel(s) by Ca$^{2+}$ (Benham, 1989; Bean, 1992). Activation of voltage-dependent Ca$^{2+}$ channels may also account for secondary changes in [Ca$^{2+}$], following activation of P2X receptors (Dubyak and El-Moatassim, 1993). The recent cloning of P2X receptor subtypes has allowed for detailed analysis of recombinant P2X receptor subtypes in isolation (in Xenopus oocyte expression studies for example), and the pharmacological profile and channel properties of each receptor subtype is detailed in Burnstock and King, 1996. The cloning of P2X receptors has also revealed a new class of ligand-gated receptors showing different features from the already characterised classes of the ligand-gated receptors, such as the nicotinic and the excitatory amino acid receptors, which are all characterised by four hydrophobic transmembrane domains (Evans et al., 1998; North, 1996). Each P2X receptor subtype is thought to have two membrane-spanning domains, both the C- and N- terminals located intracellularly, and a large cysteine-rich extracellular loop (Fig 1.2).

P2X subunits are able to form functional homo-oligomeric channels when expressed in heterologous systems. Functional studies of native receptors, together with patterns of subunit gene expression suggest that hetero-oligomeric assembly among members of this family may also occur. Recently, strong evidence has been provided to support this. For instance, co-expression of P2X$_2$ and P2X$_3$ is required to produce the ATP-activated currents of adult sensory neurons (Lewis et al., 1995), and in rat dorsal root and nodose ganglia P2X$_2$ and P2X$_3$ immunoreactivity were found to be highly colocalised (Vulchanova et al., 1997). It has also been shown that P2X$_3$ can coassemble with P2X$_1$. 
Figure 1.2. Structure of P2X receptors. (Adapted from North, 1996)

Figure 1.3. Schematic structure of P2Y receptors, which contain seven hydrophobic transmembrane domains characteristic of G protein-coupled receptors. The N terminus is located extracellularly, and the C terminus intracellularly.
to form a novel channel (Torres et al., 1998). A recent study examined the ability of all members of the P2X receptor family to interact using a co-immunoprecipitation assay. It was found, that with the exception of P2X6, all P2X subunits can form homo-oligomeric complexes, and when co-assembly between pairs of subunits were examined, all were able to form hetero-oligomeric complexes with the exception of P2X7 (Torres et al., 1999). These results suggest that hetero-oligomeric assembly might underlie functional discrepancies observed between P2X responses seen in the native and recombinant settings, while providing for an increased diversity of signalling by ATP.

- **Molecular Structure and Transduction Mechanisms of P2Y Receptors.**

A broad range of extracellular hormones, neurotransmitters, chemoattractants, and other extracellular stimuli produce their physiological effects by stimulating the second-messenger signalling cascades that involve the action of an intermediate trimeric GTP-binding regulatory protein (G protein). Deduced amino acid sequences based on cloned nucleotide sequences are available for over 150 of these G protein-coupled receptors (Parmentier et al., 1993). All of these proteins have seven transmembrane spanning domains, and share notable conservation of sequence in these regions (Fig 1.3). Agonist-occupied receptors in this superfamily of proteins specifically couple to one of at least 20 different heterotrimeric G proteins (Hepler and Gilman, 1992). Exchange of GTP for GDP is promoted upon receptor activation. This leads to dissociation of the inactive heterotrimeric protein into a GTP bound α-subunit, and a tightly associated dimer of β- and γ-subunits. In most cases the GTP bound α-subunit then interacts with, and activates an effector enzyme or channel. Compelling evidence also exists for the direct regulation of many effector proteins by βγ-subunits (Clapham and Neer, 1993). G protein-regulated effector proteins range from adenylyl cyclase, the classical effector of the cAMP signalling cascade, to inward-rectifying K+ channels. Table 1.1 summarises the major families of trimeric G proteins, their associated effector proteins and functions, and the bacterial toxins that can be used to characterise them.
**Table 1.1 The major families of trimeric G proteins**

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<td>( \alpha_s )</td>
<td>activates adenylate cyclase; activates ( \mathrm{Ca}^{2+} ) channels</td>
<td>cholera activates</td>
</tr>
<tr>
<td></td>
<td>( \mathcal{G}_{o\text{lf}} )</td>
<td>( \alpha_{o\text{lf}} )</td>
<td>activates adenylate cyclase in olfactory sensory neurons</td>
<td>cholera activates</td>
</tr>
<tr>
<td>II</td>
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<td>( \alpha_i )</td>
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<td>pertussis inhibits</td>
</tr>
<tr>
<td></td>
<td>( G_o )</td>
<td>( \alpha_o )</td>
<td>activates ( \mathrm{K}^+ ) channels; inactivates ( \mathrm{Ca}^{2+} ) channels; activates phospholipase C-( \beta )</td>
<td>pertussis inhibits</td>
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<td></td>
<td>( G_t )</td>
<td>( \alpha_t )</td>
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<td>cholera activates and pertussis inhibits</td>
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<td>( \alpha_q )</td>
<td>activates phospholipase C-( \beta )</td>
<td>no effect</td>
</tr>
</tbody>
</table>

*Families are determined by amino acid sequence relatedness of the α subunits. Only selected examples are shown. About 20 α subunits, 4 β subunits, and 7 γ subunits have been described in mammals (Taken from Alberts et al., 1994).

Phospholipase C (PLC) is the effector protein associated with cloned P2Y receptor subtypes (Harden et al., 1998). This enzyme catalyses the hydrolysis of phosphatidylinositol(4,5)-bisphosphate (PtdIns(4,5)P₂) to the \( \mathrm{Ca}^{2+} \) mobilising second messenger inositol(1,4,5)-triphosphate (Ins(1,4,5)P₃), and to diacylglycerol, which activates protein kinase C (PKC) (Berridge and Irvine, 1987). Mobilisation of \( \mathrm{Ca}^{2+} \), and the cascade of intracellular protein phosphorylation initiated by activation of PKC, are important regulators of cell growth, differentiation, transformation, secretion and migration (Berridge 1993; Lauder 1993). From the above table it can be seen that
extracellular stimuli that regulate this pathway do so by activating seven transmembrane spanning receptors that activate one of the four members of the $G_q$ class of G proteins (Hepler and Gilman, 1992; Boyer et al., 1994). An activated $G_q$ $\alpha$-subunit then stimulates one of the multiple members of the phospholipase C-β class of isoenzymes. The typical P2Y receptor transduction pathway is shown in figure 1.4.

To date, 7 P2Y receptors subtypes have been identified. These are P2Y$_1$, P2Y$_2$, P2Y$_3$, P2Y$_4$, P2Y$_6$, P2Y$_8$ and P2Y$_{11}$. The cP2Y$_3$ receptor has only been identified in chick (Webb et al., 1996), but shares 60% amino acid identity with the rat P2Y$_6$ receptor (Chang et al., 1995). However, the agonist potency profiles of P2Y$_3$ and P2Y$_6$ are slightly different, so more studies are needed to determine whether the variations in the agonist selectivities of P2Y$_3$ and P2Y$_6$ are species dependent or due to differences in expression systems before concluding that the P2Y$_3$ and P2Y$_6$ receptor truly represent different subtypes within the P2Y receptor family. Another cDNA (P2Y$_5$) shows 20-30% sequence homology with the other P2Y subtypes, but a question mark remains over its functional identity (Li et al., 1997), and P2Y$_7$, since its initial inclusion into the P2Y family, has been identified as a leukotriene receptor (Yokomizo et al., 1996). In general, adenine and uridine nucleotides are the ligands for these receptors (for detailed activities at each subtype see Burnstock and King, 1996; Communi et al., 1997). However, the P2Y$_8$ receptor is unusual in that it responds equally to ATP, UTP, ITP, CTP, GTP (Bogdanov et al., 1997).

• Control of Cyclic AMP Levels By P2Y Receptors.

Extracellular ATP has been reported to promote a decrease in the levels of intracellular second messenger, cyclic AMP (cAMP) in a number of target tissues (Okajima et al., 1987; Sato et al., 1992; Yamada et al., 1992; Boyer et al., 1993). Such responses could occur secondarily to mobilisation of Ca$^{2+}$, and activation of a Ca$^{2+}$ dependent cyclic nucleotide phosphodiesterase. However, most of the tests of the effects of extracellular adenine nucleotides have been carried out in the presence of phosphodiesterase inhibitors which would mask any Ca$^{2+}$ promoted degradation of cAMP. The most likely mechanism
Figure 1.4. P2Y receptor signalling mechanisms. See text for explanation.
for phosphodiesterase independent decreases in cAMP levels involves a G_i-promoted inhibition of adenylate cyclase activity, and therefore distinguishes these responses from the G_q-mediated regulation of PLC described above. The regulation of cAMP levels by native P2Y receptors have been well characterised in platelets and C6 glioma cells (Reviewed in Boarder, 1998), but as yet the receptors linked directly to the inhibition of adenylate cyclase in these two systems have yet to be cloned.

• **Release, Metabolism and Interconversion of Nucleotides.**

The very presence of receptors for extracellular nucleotides on the surface of cells suggests, that nucleotides are released from cells and exist extracellularly, and that mechanisms must be in place to metabolise and inactivate these molecules. It also follows that given that intracellular nucleotides play fundamental roles in energy metabolism, nucleic acid synthesis, and enzyme regulation, their release from cells must also be tightly regulated.

**Sources of Extracellular ATP.** ATP is present in milimolar (3-5mM) concentrations in the cytosol of all cell types. Extracellular levels of ATP will normally be maintained at extremely low levels due to the minimal permeation of ATP and MgATP (the predominant cytosolic form) across the plasma membrane, and due to the action of ubiquitous enzymes that quickly metabolise extracellular nucleotides. The three routes by which ATP and other nucleotides can be released from cells are: lysis of cells and transmembrane transport, both of which release cytosolic ATP, and exocytosis, in which ATP stored in vesicles or granules is released.

**Packaging of Nucleotides in Exocytotic Granules or Vesicles, and Exocytotic Release of Nucleotides from Cells.** ATP (and ADP) is copackaged with conventional neurotransmitters and local mediators within the exocytotic granules of many cell types. (Dubyak and El-Moatassim, 1993). Some of the earliest speculation regarding the role of ATP as a signalling molecule came from the studies of neurotransmission that was resistant to blockade by cholinergic and adrenergic antagonists (reviewed in Burnstock, 1971; Burnstock and Kennedy, 1986). There is substantial evidence that supports the
hypothesis that ATP is packaged and released as a cotransmitter with noradrenaline (Von Kugelgen et al., 1998; Westfall et al., 1990). However, the most detailed studies concerning exocytotic release of ATP have been performed using nonneuronal secretory cell types that are easily isolated and maintained as single cells. These include platelets (Beigi et al., 1999), adrenal chromaffin cells (Rojas et al., 1985; Rojas et al., 1986; Cheek et al., 1989; Cena and Rojas, 1990), mast cells and basophilic leukocytes (Osipchuk and Cahalan, 1992). As is true for most types of exocytotic secretion, the abilities of various agonists to elicit ATP secretion can be correlated with their abilities to trigger, via different mechanisms, rapid increases in \([\text{Ca}^{2+}]\). Intact single chromaffin cells have been used to characterise the role of \(\text{Ca}^{2+}\) in regulating the exocytotic release of ATP in response to direct membrane depolarisation, or stimulation of nicotinic acetylcholine receptors (Rojas et al., 1985; Rojas et al., 1986; Cheek et al., 1989; Cena and Rojas, 1990). Under all conditions, ATP secretion was blocked by removal of extracellular \(\text{Ca}^{2+}\), or blockade of voltage-dependent \(\text{Ca}^{2+}\) channels. Activation of mast cells by extracellular ATP causes rapid elevation of \([\text{Ca}^{2+}]\), and ATP released from individual mast cells has been shown to be sufficient to trigger the activation of ATP receptors on adjacent mast cells (Osipchuk and Cahalan, 1992). Similar patterns of ATP release and cell-cell communication were observed in a rat basophilic leukemia cell line (Osipchuk and Cahalan, 1992). These data suggest that ATP can be used as a specific extracellular messenger to propagate cell-cell signals in the absence of direct gap junction-based communication.

**Release of Cytosolic ATP via Transmembrane Transport.** There is generally little permeation of ATP (or MgATP) across the plasma membrane. However, there are several lines of evidence to suggest that cytosolic ATP (as opposed to ATP sequestered within intracellular granules or vesicles) can be released from cells in response to physiological or pathological stimuli. ATP is released from intact hearts, isolated cardiac myocytes and erythrocytes following hypoxia (Clemens and Forrester, 1981; Forrester, 1990), and this hypoxia induced increase can be attenuated by inhibitors of anion or nucleoside transporters. This suggests the release of cytosolic ATP through channels or pores, rather
than as a result of lysis or membrane damage. Even higher concentrations of cytosolic ATP are released following tissue trauma. Such release of nucleotides may serve as emergency signals under such conditions, and there is a growing body of evidence that this type of signalling may have a role in the activation and maintenance of healing mechanisms leading to tissue repair and regeneration (Neary et al., 1996b).

Vascular endothelial cells and smooth muscle secrete cytosolic ATP when treated with agonists for Ca$^{2+}$, mobilising receptors (e.g. thrombin, noradrenaline) (Pearson and Gordon, 1979; Westfall et al., 1990). The multidrug resistance (mdr 1) gene product, P glycoprotein, has been shown to function as a channel for ATP (Abraham et al., 1993). This finding raises the possibility that other proteins that belong to the ABC (ATP-binding cassette) superfamily may also play a role in release of cytosolic ATP.

**Metabolism and Interconversion of Extracellular Nucleotides.**

Ecto-enzymes that rapidly hydrolyse nucleoside triphosphates and diphosphates reside on the surface of most cells (Dubyak and El-Moatassim, 1993; Zimmermann, 1996). The expression of individual enzymes can vary greatly between tissues and cells, and the pattern of catalytic activities at the cell surface can be much more complex than the linear hydrolysis of ATP to ADP, AMP and finally adenosine. Figure 1.5 summarises a number of activities of extracellular purine metabolising enzymes and their potential interaction. The presence of these enzymes has severely compromised pharmacological studies. The effects of ATP (and UTP) have been difficult to distinguish from their metabolic products, and the problem is compounded by the absence of hydrolysis resistant, P2 receptor subtype selective agonists or antagonists. A single tissue may also express multiple receptors that recognise ATP, ADP or adenosine as well as receptors for UTP and UDP. Thus, studies of the response of a tissue to ATP itself are compromised by rapid conversion of ATP to ADP, which potentially acts on a different receptor(s), and conversion of ADP to AMP and finally adenosine, which can then activate the P1 family of receptors. (P1 receptor subtype selective antagonists have now been developed that can block the potential contribution of these receptors). The situation is further complicated by the identification of nucleoside diphosphokinase activity that catalyzes the transfer of the
Figure 1.5. Extracellular pathways for the degradation, and interconversion of nucleotides. Numbers indicate the type of enzyme involved in the reaction. Not all cells will posses the same complement of enzymes on their surface. (Adapted from Zimmermann, 1996).
γ-phosphate of nucleoside triphosphates to nucleoside diphosphates. In addition to the transfer between purines, shown in figure 1.5, transfer can also occur between purines and pyrimidines. For example, in the presence of ATP, nucleoside diphosphokinase catalyzes the conversion of UDP to UTP: ATP+UDP→ADP+UTP. (Lazarowski et al., 1997).

- **P2 Receptors as Regulators of Cell Growth and Differentiation.**

  Given that receptors for extracellular nucleotides are found on the surface of so many different tissues, it is not surprising that they regulate a broad range of physiological processes in adult tissues and cells. For example, ATP is involved in short term signalling in the nervous system, exocrine and endocrine secretion, regulation of immune cell function, pain, inflammation, and cardiovascular function (Dubyak and El-Moatassim, 1993; Abbracchio and Burnstock, 1998; Boarder and Hourani, 1998). As well as regulating short term events, there is now considerable evidence that points to a role for this type of signalling in regulating long term changes in cell proliferation and differentiation, processes that are fundamental both during repair and regeneration following tissue trauma, and during embryonic development.

  Stimulation of cell proliferation by nucleotides has been demonstrated in Swiss mouse 3T3 and 3T6 fibroblasts (Huang et al., 1989; Gonzalez et al., 1990; Wang et al., 1994), primary astrocytes and astrocytoma cell lines (Neary et al., 1994a and 1994b, Rathbone et al., 1992; Abbracchio et al., 1994 and 1995; Ciccarelli et al., 1994), and in vascular smooth muscle cells and endothelial cells (Wang et al., 1992; Erling et al., 1995; Satterwhite et al., 1999). These reports suggest that it is the P2Y receptor family that contribute to the mitogenic response. Evidence that P2X receptor activity may also contribute to a mitogenic response is currently lacking. In the study by Huang et al., 1989 the mitogenic effects of ATP were shown to be synergistic with those induced by polypeptide growth factors, so that both epidermal growth factor (EGF) and ATP had little mitogenic effect when tested alone, but synergistically stimulated DNA synthesis when added together to cells. In this study, ATP was also shown to be a competence
factor for stimulating DNA synthesis in these cells. Pre-exposure of cells to ATP for 60 minutes before addition of a low concentration of serum, which was insufficient to be mitogenic by itself, resulted in a 50-60 fold increase of $^3$H-thymidine incorporation into DNA. ATP was also shown to synergise with members of the FGF family in inducing DNA synthesis in astrocytes (Neary et al., 1994a). This interaction was shown to be highly specific for members of the FGF family, since it did not occur when ATP was combined with platelet-derived growth factor, epidermal growth factor, or nerve growth factor.

The signalling pathways underlying these mitogenic effects appear to involve mitogen-activated protein (MAP) kinase, a component of an intracellular protein phosphorylation cascade, that plays an important role in proliferation and differentiation (Avruch et al., 1993; Davis, 1993). Extracellular ATP stimulated MAP kinase in astrocytes (Neary and Zhu, 1994; King et al., 1996), and bFGF stimulated MAP kinase to a similar extent, but did so by a mechanism distinct from ATP. Protein Kinase A significantly reduced the activation of MAP kinase by bFGF but not by ATP; because protein kinase A can block the Ras/Raf pathway, this suggested that bFGF, but not ATP, activates MAP kinase by a Ras/Raf pathway (Neary and Zhu, 1994). Treatment of astrocytes with ATP or UTP also leads to the formation of activator protein (AP)-1 complexes (Neary et al., 1996a), which are transcriptional activators that have been implicated in the expression of a diverse array of genes. bFGF also induced AP-1 complex formation (Zhu et al., 1995). The activation of AP-1 activity by ATP and bFGF, was dependent on PKC, since Ro 31-8220, a PKC inhibitor, abolished AP-1 complex formation. Activation of enzymes in the MAP kinase family leads to increased AP-1 activity (Karin, 1995). Therefore, AP-1 may be a downstream nuclear signal in the P2/MAP kinase pathway. The intracellular signalling pathways downstream of P2Y receptor, and bFGF tyrosine kinase receptor activation may merge at MAP kinase activation and lead to transcriptional changes that underlie the synergistic enhancement of bFGF-induced mitogenesis by ATP. The AP-1 complex is a heterodimer consisting of Fos and Jun family members, and c-Fos and c-Jun levels are
increased in primary striatal cultures treated with P2 receptor agonists (Abbracchio et al., 1995).

As well as regulating proliferation, extracellular nucleotides regulate the differentiation of astrocytes. Extracellular ATP has been shown to induce the expression of the astroglial specific marker glial fibrillary acidic protein (GFAP), promote astrocytic hypertrophy, and elongation of astrocytic processes, events that may participate in the remodelling and functional recovery of brain circuitries following trauma (Neary et al., 1994b; Abbracchio et al., 1995). The mitogenic effect of extracellular nucleotides have also been investigated in vascular smooth muscle cells (VSMCs). ATP added to VSMCs in culture induced activation of both immediate-early and delayed early cell cycle-dependent genes which are usually expressed within 1-23 hours after mitogenic stimulation (Malam-Souley et al., 1993). The pharmacology of the response suggested involvement of a P2Y receptor subtype. Based on the inability of ATP to increase late G1 gene mRNA levels the authors concluded that ATP induces a limited progression through the G1 phase, but is unable by itself to induce crossing over the G1/S phase boundary, and consequently DNA synthesis. This observation would be in agreement with studies done on other cell types which have suggested that ATP may act as a competence factor, and may need to act in combination with other growth factors to trigger proliferation (Huang et al., 1989). A subsequent study using monoclonal antibodies to cell-cycle specific nuclear antigens (PCNA and Ki-67) to identify the cell cycle phase of each cell in primary cultures of VSMCs, showed that activation of a P2Y2/P2Y4 receptor by ATP and UTP in these cells resulted in [Ca2+]i elevation and transition from the G1 to the S and M phases of the cell cycle (Miyagi et al., 1996). The intracellular signalling cascade responsible for ATP induced proliferation of VSMCs is also strikingly similar to the pathway described in astrocytes. In VSMCs, activation of a P2Y receptor by ATP results in the activation of PLC, mobilisation of Ca2+, stimulation of PKC (α and δ), Raf-1 and MAP kinase, and eventually induction of primary response genes such as c-fos and c-myc.
• **Purines as Regulators of Cell Death.**

Apoptosis plays a major role in the shaping of tissues during embryogenesis. As opposed to necrosis, apoptosis progresses through a series of well-defined morphological and biochemical stages occurring in the nucleus and cytoplasm of the dying cell. This includes condensation and margination of the chromatin towards the inner nuclear membrane, followed by nuclear fragmentation into apoptotic bodies and chromatin cleavage into high molecular weight DNA, which is subsequently degraded into smaller DNA fragments.

Adenosine has been shown to induce apoptosis in primary rat astroglial cells, and embryonic chick sympathetic neurons (Abbracchio et al., 1995; Wakade, 1995), endothelial cells (Dawicki et al., 1997), and in mouse and human thymocytes (Kizaki et al., 1990; Szondy, 1994). The effects of adenosine on apoptosis of these cells result from interaction with cell surface P1 receptors, but the receptor subtypes mediating the effect in each case is unknown, and the second messenger system by which adenosine triggers apoptosis is also unclear (Chow et al., 1997). The ability of extracellular ATP to kill cells is well established, particularly in cells of the immune system (Zanovello et al., 1990; Apasov et al., 1995), and hepatocytes (Nicotera et al., 1986; Zoeteweij et al., 1996). ATP is known to cause cell death by apoptosis and necrosis, and two members of the P2X family have been implicated. The first is the receptor for ATP^, P2X, (Suprenant et al., 1996), and the second is P2X, which was suggested to be involved in ATP-induced cell death by virtue of its sequence homology with the RP-2 gene, which is expressed in thymocytes during the onset of apoptosis (Owens et al., 1991; Valera et al., 1994 and 1995). Supporting evidence for the involvement of P2X, was shown by Chvatchko et al., 1996, who detected an increase in P2X, mRNA levels during apoptosis in-vitro, and in-vivo. These data suggest a possible role for P2X, during T cell development in the thymus. Although both P2X, and P2X, are both non-selective cation channels, P2X, is different in that it is capable of forming a pore that allows the passage of large molecules (<900 Dalton), as well as acting as a selective ion channel for small cations (Suprenant et al., 1996). This may explain the observations that activation of P2X, by ATP^ in macrophages results in necrosis rather than apoptosis (Blanchard et al., 1995; Chiozzi et
P2X₇ has also been shown to mediate the cytotoxic effects of ATP in microglia (Ferrari et al., 1997).

**Physiological and Pharmacological Studies have Demonstrated Responses to Extracellular Nucleotides in a Number of Embryonic Cells Types.**

From the examples given in the above section it can be seen that P2 receptor signalling is involved in regulating growth, differentiation and death of adult cells and cell lines. Since these are processes fundamental to embryonic development, it has been postulated that extracellular nucleotides may also be utilised as signalling molecules during embryogenesis. The ability of these receptors to modulate the actions of signalling molecules already known to be important in development, such as FGFs, lends support to this idea. Direct evidence that P2 receptors direct developmental processes is scarce however, but a number of studies have demonstrated responses to extracellular nucleotides in embryonic cells. An ATP-activated Na⁺ channel in egg membranes is involved in sperm-induced fertilization (Kupitz and Atlas, 1993), and extracellular ATP is a trigger for the acrosome reaction in human spermatozoa (Foresta et al., 1992). Together with muscarinic cholinergic receptors, extracellular ATP mobilises Ca²⁺ in cells of the gastrulating chick embryo (Laasberg, 1990). ATP also elicited Ca²⁺ responses in cells dissociated from stage 23-24 chick embryos (Lohmann et al., 1991). Ca²⁺ responses have also been demonstrated in the developing chick otocyst (Nakoaka et al., 1995), cultured astrocytes from embryonic rat spinal cord (Salter and Hicks, 1995), and cultured neurons from embryonic rat brain (Mironov, 1994). Developmentally regulated responses to extracellular nucleotides have also been demonstrated. In the developing chick retina large P2Y₂-like Ca²⁺ responses were demonstrated at embryonic day 3, and the size of the response declined until day 13, when no response could be elicited (Sugioka et al., 1996). In developing chick skeletal muscle it has been shown that at embryonic day 6 all muscles tested contracted in response to ATP, but by day 17 no muscles responded (Wells et al., 1995). The pharmacology of the response of chick skeletal muscle, and of
chick myoblasts and myotubes to extracellular ATP has been studied extensively, and these studies suggest involvement of P2X family members (Kolb and Wakelam, 1983; Haggblad et al., 1985; Hume and Hönig, 1986; Hume and Thomas, 1988; Thomas and Hume, 1990a and 1990b; Thomas et al., 1991; Thomas and Hume, 1993). Extracellular applications of ATP to developing *Xenopus* neuromuscular synapses in culture potentiate ACh responses of developing muscle cells during the early phase of synaptogenesis (Fu and Poo, 1991; Fu, 1994; Fu and Huang, 1994), and a possible role for extracellular ATP co-released with ACh in development of the neuromuscular synapse has also been raised (Fu and Poo, 1991; Fu, 1995).

- **The P2Y₈ Receptor was the first P2 Receptor Subtype Shown to be Expressed During Early Embryonic Development.**

  Confirmation that P2 receptor signalling may be important developmentally, came from the cloning and characterisation of a P2Y receptor (P2Y₈) that is expressed during development of the *Xenopus* embryo. P2Y₈ was found to be expressed at stage 13-14 in an arc corresponding to the anterior ridge of the neural plate. Expression subsequently spread throughout the neural plate, and after neural tube closure P2Y₈ was no longer detectable in caudal regions of the neural tube, but was detectable in neural tissue emerging from the tailbud (Bogdanov et al., 1997). This pattern of expression suggests that P2Y₈ may be involved in regulating early development of the nervous system, but no functional studies have yet been reported.

- **Summary**

  Physiological and pharmacological studies, combined with molecular cloning have identified two families of receptors for extracellular nucleotides: the P2X receptors which constitute a family of ligand-gated ion channels, and the P2Y receptors which are coupled to heterotrimeric G proteins. Extracellular nucleotides, signalling through these receptors have been shown to be regulators of cell proliferation, differentiation, and death, all of which are key processes during embryonic development. Responses to extracellular
nucleotides have also been demonstrated in a number of embryonic cell types suggesting that these receptors are indeed expressed during embryogenesis. These data suggest that this type of signalling has the potential to be important during development, but there is a lack of data that shows developmentally regulated expression of specific members of the P2 receptor families during embryonic development, and almost no data exists that has demonstrated a specific developmental role for this type of signalling during embryogenesis. In this thesis, I describe for the first time developmentally regulated patterns of expression of three members of the P2 receptor families during chick embryogenesis, and evidence is also provided that suggests that one of these receptors plays an important role during development of the chick limb.
Analysis of cP2Y$_1$ Expression During Chick Embryogenesis Using Northern Blotting and In-situ Hybridisation

• Northern blotting shows that cP2Y$_1$ transcripts first appear between stages 7 and 12 of development. Expression persists throughout development, and just prior to hatching there is particularly strong expression in brain and skeletal muscle.

• Wholemount in-situ hybridisation shows that during the first ten days of development cP2Y$_1$ is expressed in the limb buds, mesonephros, somites branchial arches and CNS.

Introduction

• Previous Studies have Shown that P2Y Receptors have the Potential to be Important Developmentally.

As outlined in the general introduction, P2Y receptors have been shown to mediate the effects of extracellular nucleotides on differentiation and proliferation of primary cells and cell lines (for reviews see Abbracchio, 1996; Neary and Burnstock, 1996). Both of these are fundamental during embryonic development. Also physiological and pharmacological studies have demonstrated responses to extracellular nucleotides in a number of embryonic cell types (for review see Burnstock, 1996). This and the identification of a novel P2Y receptor subtype, P2Y$_8$, expressed in the Xenopus embryo neural plate prior to neural tube closure, and later in neural tissue emerging from the tailbud (Bogdanov et al., 1997) suggests that P2, and in particular P2Y receptor signalling may be important developmentally.
The Chick Embryo is a Good System in which to Investigate Potential Developmental Roles for P2Y Receptor Signalling.

In this chapter the possibility that extracellular nucleotides are used as signalling molecules during embryogenesis is explored further by looking for expression of a specific P2Y receptor subtype during development of the chick embryo. The chick embryo was chosen as a model organism to investigate a developmental role for this type of signalling because responses to extracellular ATP have been demonstrated in a number of embryonic chick tissues, isolated from various stages of development. ATP has been shown to cause increase in intracellular calcium (Ca\(^{2+}\)) in cells dissociated from the gastrulating chick embryo (Laasberg, 1990), from embryonic day 4 embryos (Lohmann et al., 1991), from early chick otocyst (Nakoaka and Yamashita, 1995) and retina (Sugioka et al., 1996), and in chick myoblasts, myotubes and intact skeletal muscle (Kolb and Wakelam, 1983; Häggblad et al., 1985, Wells et al., 1995).

There are a Number of Reasons for Choosing \(\text{cP2Y}_1\) in Preference to the Other Chick P2Y Receptors.

Of the eleven proposed P2Y receptor subtypes three have been identified in chick. These are chick P2Y\(_1\) (\(\text{cP2Y}_1\)), chick P2Y\(_3\) (\(\text{cP2Y}_3\)), and chick P2Y\(_5\) (\(\text{cP2Y}_5\)). As well as from chick, P2Y\(_1\) receptors have been cloned from a wide range of species including rat and mouse (Tokuyama et al., 1995), human (Janssens et al., 1996), turkey (Filtz et al., 1994), and bovine (Henderson et al., 1995) tissues. The presence of P2Y\(_1\) in these divergent species suggests that this receptor has been conserved throughout evolution, and its presence in another model developmental organism - the mouse, made \(\text{cP2Y}_1\) an attractive starting point for investigating a role for P2Y receptor signalling during embryonic development. As well as positive reasons for choosing \(\text{cP2Y}_1\), there were also reasons for not choosing the other two chick P2Y receptors. A question mark remains over the functional identity of P2Y\(_5\). An orphan G protein-coupled receptor, termed 6H1, with approximately 30% sequence identity to P2Y receptors has been proposed to be a P2Y receptor (P2Y\(_3\)) based on a radioligand binding assay with
[35S]dATP alphaS (Webb et al., 1996). It has since been shown that [35S]dATP alphaS is not a general ligand for P2Y receptors, and this putative member of the P2Y receptor family did not mediate nucleotide induced second messenger responses when stably expressed in 1321N1 human astrocytoma cells (Li et al., 1997). cP2Y₁ was chosen in preference to cP2Y₃ because homologues of the cP2Y₃ receptor have not been identified in any other species apart from chick.

For the reasons outline above, it was decided to begin to investigate a role for P2Y receptor signalling during embryogenesis by looking for expression of cP2Y₁ during development of the chick embryo.
**Methods**

- **Embryos**
  Fertilised White Leghorn chicken eggs were obtained from Needle farm (Essex) and incubated at 37°C and staged according to the Hamburger and Hamilton (HH) series (Hamburger and Hamilton, 1951). Embryos were removed from the egg, rinsed and dissected free of extra embryonic membranes in sterile phosphate buffered saline (PBS). Embryos were then either fixed in 4% paraformaldehyde for use in wholemount in-situ hybridisation or processed for RNA extraction.

- **Northern Blots**
  Total RNA was isolated from staged embryos and embryo tissues using guanidium isothyocyanate (CP laboratories). Poly A+ mRNA was prepared from the total RNA using a Poly A tract mRNA isolation system (Promega). The Poly A+ mRNA was then electrophoresed through a MOPS formaldehyde 1% agarose gel, and transferred to Hybond N+ membrane (Amersham) using standard techniques (Sambrook et al., 1989). The hybridisation probe was a 1.4kb cP2Y1 cDNA fragment labelled with [a- 32P] dCTP to a specific activity of approximately 1x10^9 dpm/μg. Probe synthesis was carried out using a Ready to Go DNA labelling kit (dCTP), and unincorporated nucleotides were removed using a Sephadex G-50 NICK column (both from Pharmacia). Hybridisation was performed overnight at 42°C in 50% formamide, 5xSSC, 5xDenhardtts, 0.5% SDS, 100μg/ml salmon sperm DNA, 50mM phosphate. The hybridisation filter was subjected to a final wash at 65°C in 0.2xSSC, 0.1% SDS for 20 minutes before being exposed to X-ray film for 24 hours at -70°C with an intensifying screen. The same blot was stripped of bound probe according to manufacturers instructions (Amersham) and probed with a 250bp fragment of chicken GAPDH (glyceraldehyde-3-phosphate dehydrogenase) cDNA as a control (Panabieres et al., 1984).
• Generation of Riboprobes.

A 1.4kb cP2Y₁ cDNA fragment was subcloned into the Hind III site of the eukaryotic expression vector pRCCMV (Invitrogen) to generate pRCCMV-P2Y₁ (see figure 2.1 for map). pRCCMV-P2Y₁ was linearised by digestion with either BssH II to generate an antisense probe, or Not I for generating a sense probe. The DNA template was then cleaned by treatment with proteinase K, followed by phenol:chloroform:isoamyl alcohol (25:24:1) (Sigma) extraction. A final extraction was performed in chloroform alone before ethanol precipitation. Sense and antisense digoxygenin (Boehringer Manheim) labelled probes for cP2Y₁ were made by transcription with T7 (sense) and Sp6 (antisense) RNA polymerase according to the manufacturers instructions (Promega):

The following were mixed in the following order:

4μl Transcription Buffer  
2μl DTT (100mM)  
1μl Rnasin  
4μl NTP mix (2.5mM each ATP, GTP, CTP-1μl of each from 10mM stock+1μlH₂O)  
2.6μl UTP (2.5mM)  
1.4μl DIG-11-UTP (2.5mM)  
1μl linearised DNA template (1μg)  
1μl RNA polymerase

The reaction mix was incubated for 2 hours at 37°C. The DNA template was removed by addition of 1μl RQ1 Dnase (Promega) and incubating for 15 minutes at 37°C. 2μl 0.2M EDTA pH 8.0 was added to terminate both the polymerase reaction and the DNase. Lithium chloride (RNase free, from Sigma) was added to a final concentration of 0.4M prior to ethanol precipitation. The antisense probe was a 1.2kb transcript made from the coding region 161-1407 of the published cP2Y₁ nucleotide sequence (Genbank accession number x73268).
Figure 2.1.
A 1.4Kb fragment (bases 0-1407) encompassing the entire coding region of cP2Y1 (bases 177-1265) was cloned into the Hind III site of the eukaryotic expression vector pRCCMV (Invitrogen). The main features of the vector are outlined below.
1. Promoter sequences from the immediate early gene of the human cytomegalovirus (CMV) for constitutive, high level expression of cP2Y1 in eukaryotic cells.
2. Polyadenylation signal, and transcription termination sequences from the bovine growth hormone (BGH) gene, to enhance RNA stability.
3. T7 and Sp6 RNA promoters flanking cP2Y1 for in-vitro transcription of sense and antisense RNA.
4. The ampicillin resistance gene, and the Col E1 origin for selection, and maintenance in E. coli.
Wholemount In-Situ Hybridisation.

1. Embryos were fixed in 4% paraformaldehyde (PFA) in PBS overnight at 4°C.

2. Embryos were washed 2 x 10 minutes in PBS, 0.1% Tween 20 (PBT)

3. Embryos were then dehydrated:
   - 25% methanol in PBT, 10min
   - 50% methanol in PBT, 10min
   - 75% methanol in PBT, 10min
   - 100% methanol in PBT, 10min.

4. Embryos were then rehydrated by performing the above washes in reverse order followed by a final wash of PBT for 2 x 10 min.

5. Embryos were bleached in 6% hydrogen peroxide for 1 hour

6. Wash in PBT 3 x 10 min.

7. To permeabilise, embryos were treated with 10μg/ml proteinase K in PBT (for approximately 1 minute per embryonic stage.

8. Embryos were washed 2 x 10 min in PBT and were then post-fixed in 0.2% glutaraldehyde/4% PFA in PBT for 20 min.

9. Embryos were then washed 3 x 10 min with PBT.

10. Embryos were rinsed for 10 min in prehybridisation solution:
   - 50% formamide
   - 5 x SSC
   - 50μg/ml yeast RNA
   - 1% SDS
   - 50μg/ml heparin

11. The pre-hybridisation solution was replaced with fresh, and incubated at 70°C for 1 hour.

12. The prehybridisation solution was replaced with hybridisation solution containing 1μg/ml of riboprobe and incubated at 70°C overnight.

13. The embryos were then washed as follows:
   - 50% formamide, 5 x SSC, 1% SDS for 2 x 30 min at 70°C.
   - 50% formamide, 2 x SSC for 2 x 30 min at 65°C
   - 3 x 10 min in 1xTBST containing 2mM levamisole.
   - (10 x TBST: 8g NaCl, 0.2g KCl, 25ml 1M TrisHCl pH 7.5, 10 ml Tween 20)
14. Embryos were preblocked in 10% goat serum in TBST for 1-2 hours.

15. During this time the anti-digoxigenin antibody was preabsorbed with embryo powder: 0.5ml of TBST (no levamisole) was added to 3mg embryo powder in an eppendorf and heated for 30 min at 70°C. This was cooled on ice before adding 5µl goat serum and 1µl anti-digoxigenin, alkaline phosphatase conjugated antibody (Boehringer Manheim). This was shaken for 1-2 hours at 4°C. The eppendorf was then spun for 10 min, the supernatant removed and diluted to 2mls with 1% goat serum in TBST.

Preparation of embryo powder: Day 4-7 embryos were homogenised in a minimum volume of PBS. 4 volumes of ice cold acetone was added and the mixture was placed on ice for 30 minutes. The mixture was spun at 10 000g for 10min and the supernatant removed. The pellet was spread out and ground into a fine powder on a sheet of filter paper and allowed to dry.

16. The 10% goat serum was replaced with the preabsorbed ant-Dig.AP antibody and embryos were rocked overnight at 4°C.

17. Embryos were washed 3 x 15 min in TBST.

18. The TBST was changed hourly and embryos were washed throughout the day and overnight.

19. Embryos were then washed for 3 x 15 min in freshly prepared NTMT:

100mM NaCl
100mM Tris-HCl pH 9.5
50mM MgCl₂
0.1% Tween 20

20. The in-situs were developed by incubating the embryos in NTMT containing 3.5µl/ml each of NBT and BCIP. The reaction product was allowed to develop in the dark at room temperature.

21. The reaction was stopped by washing thoroughly in PBT plus 20mM EDTA.

22. Finally embryos were refixed in 4% PFA.

To ensure complete penetration of the probe at stage 29-36, wholemount in-situ hybridisation was performed on brains dissected free of surrounding head tissue. A
sense riboprobe was used throughout as a negative control. Stained embryos were embedded in gelatine-albumin for cutting 50 µm vibratome sections.
Results

• Northern Blotting Shows that cP2Y₁ is Expressed Almost Continuously Throughout Embryonic Development of the Chick.

Northern blotting of Poly A+ mRNA isolated from different stage embryos and embryo tissues showed that cP2Y₁ transcripts were first detected between stages 7 and 12. Transcripts were not detected in pooled RNA isolated from stages 2-7. A single band of 2.7kb was detected that persists until hatching, with particularly intense expression seen in the brain and skeletal muscle of stage 41 and 45 embryos. Transcripts were also detected in the heart and liver at stage 41, and in the liver and eye at stage 45 (Fig 2.2). High levels of discrete expression are first detected by wholemount in-situ hybridisation at stage 19. The Northern analysis demonstrates expression before this stage but failure to detect it by in-situ suggests that the early expression levels may be low but in many tissues and cells.

Wholemount In-situ Hybridisation Shows a Developmentally Regulated Pattern of cP2Y₁ Expression in Several Tissues During the first Ten Days of Development.

• cP2Y₁ Shows a Dynamic Pattern of Expression in Chick Limb Buds and Seems to be Expressed Preferentially in Undifferentiated Mesenchyme Cells.

Expression of cP2Y₁ was first detected in mesodermal cells in the posterior third of the wing buds in stage 19 embryos. By stage 21 the expression had intensified and spread within the limbs to all but the anterior third of both wing and leg buds (Fig 2.3). Sectioning revealed an asymmetric pattern of expression along the dorso-ventral axis, with expression increased dorsally (Fig 2.3 B and C). cP2Y₁ was absent from a layer of mesodermal cells immediately subjacent to the limb ectoderm and from the limb bud core.

In a stage 25 limb, cP2Y₁ transcripts formed a distal band of expression running anterior to posterior, just proximal to the progress zone which is the most distal strip of
Figure 2.2.
Northern blot analysis of cP2Y1 expression during chick embryogenesis. Stage and tissue are indicated at the top. GAPDH was used as a control. cP2Y1 transcripts first appear between stages 7 and 12, and persist until hatching. Particularly strong expression was seen in the brain and skeletal muscle at stages 41 and 45.
mesenchyme underlying the apical ridge (Fig 2.3 D). This pattern was seen on both the dorsal and ventral surfaces of the limb. cP2Y1 was not expressed in the ectoderm or immediately subjacent mesenchyme, nor in the cartilage condensations (Fig 2.3 F). However cP2Y1 was expressed in the rest of the limb mesenchyme which surrounded the condensing cartilaginous elements. Proximal expression formed a uniform ring around the limb bud core (Fig 2.3 E).

By stage 29 expression was strongest around the distal periphery (Fig 2.3 G). At the proximal limit of a stage 29 wing (not shown) the expression pattern was very similar to that seen in equivalent proximal sections at stage 25 (Fig 2.3 E). At the level of both ulna and radius (Fig 2.3 H) and wrist (Fig 2.3 I) expression was strong in the anterior and posterior mesenchyme but was less intense dorsally and absent ventrally. In a section through the developing digits, strong expression surrounded the cartilage condensations (Fig 2.3 J) the strongest expression being at the posterior margin.

In stage 31 limbs the relationship of cP2Y1 expression to the developing skeleton and tendons was visible, particularly in the digits (Fig 2.3 K). In a proximal section (Fig 2.3 L) there was uniform, but faint expression in the dorsal and ventral mesenchyme. A section through the proximal digit region (Fig 2.3 M) revealed faint expression in dorsal mesenchyme, but ventrally expression of cP2Y1 was absent. Expression was strongest in the mesenchyme surrounding the most posterior digit. Strong expression was also seen in the other digits (Fig 2.3 N and O) surrounding cartilage and tendon condensations (arrowed). cP2Y1 expression was absent from the core mesenchyme in the interdigital regions.

Expression of cP2Y1 in a stage 35 leg (Fig 2.3 P) bordered the rods of cartilage that will later form the bones of the digits but there were gaps in expression which correspond to the sites of joint formation.

In summary, cP2Y1 was expressed in limb bud mesenchyme, but was absent from ectoderm, developing cartilage condensations, tendons and areas of joint formation. Expression within mesenchyme was dynamic; position and strength of expression varied according to stage and position within the limb. Particularly strong expression
Figure 2.3. Expression of cP2Y_1 during limb development visualised by wholemount in-situ hybridisation. In (A,D,G,K and P) specimens are oriented with anterior towards the top. All other specimens are oriented with the dorsal surface uppermost and anterior to the right. Stage is indicated in the bottom right hand corner and the dotted lines indicate the level at which sections were taken. (A) Dorsal view showing expression of cP2Y_1 in the wing buds. (B) Section through wing demonstrating dorso-ventral difference in cP2Y_1 expression. (C) Longitudinal section through stage 21 wing showing expression beneath distal tip. (D) Dorsal view of stage 25 wing showing a more restricted pattern of expression of cP2Y_1. (E and F) Proximal and distal sections through a stage 25 wing showing expression in mesenchyme surrounding cartilage condensations, c. (G) Dorsal view of stage 29 wing showing strong expression of cP2Y_1 around the distal periphery. (H, I and J) Proximo distal series of transverse sections through a stage 29 wing. Expression is strong in the anterior and posterior margins in (I), and was absent from the ventral surfaces of (H and I). (K-P) These specimens show expression in the leg. The expression pattern was the same in the wing, but the leg better demonstrates the pattern of expression in the digits. (K) Dorsal view of stage 31 leg, showing strong expression of cP2Y_1 surrounding the digits, joints and tendons, and weaker expression in the inter digital areas. (L,M,N and O) Proximo distal series through a stage 31 leg. The arrows in (N and M) indicate the tendons. Expression in the core of the inter digital areas is absent in specimen (N). (P) Expression of cP2Y_1 in the digits of a stage 35 leg. Areas of joint formation did not express cP2Y_1. Bars indicate 100μm.
Figure 2.3.
was seen in the mesenchyme surrounding the most distal cartilaginous elements and tendons.

- **The Pattern of cP2Y₁ Expression in the Mesonephros Suggests a Possible role for this Receptor in Development of the Glomeruli.**

Expression of cP2Y₁ in the mesonephros was first detected at stage 19 level with somites 14-17. There was a cephalocaudal gradient of expression, and by stage 20 transcripts were detected at the level of somites 16-22, while expression anterior to somite 16 was now absent (Fig 2.4 A). Expression was characterised by rows of discrete oval shaped areas of intense staining in cells that lie at the proximal end of the s-shaped mesonephric tubules (Fig 2.4 B). This area of mesenchyme is the mesonephric corpuscle and will become the glomerulus. By stage 25 expression of cP2Y₁ in the mesonephric corpuscle had reached the level of the leg buds but was absent at the level of the wing buds (Fig 2.4 C). The pattern of expression reflects the maturity of the developing mesonephros. Rostrally, expression of cP2Y₁ was more diffuse and less intense. The close proximity of cells expressing cP2Y₁ to the vasculature of the developing glomeruli was clearly visible in the more mature rostral mesonephros (Fig 2.4 E). Meanwhile the more recently established areas of expression, located more caudally (Fig 2.4 D) were confined to the discrete oval clusters of cells described above. The glomeruli at this caudal position had not yet begun to form. By stage 29 expression of cP2Y₁ was detected in only the most caudal region of the mesonephros.

- **cP2Y₁ Expression in the Branchial Arches - The facial Primordia.**

In stage 21 embryos cP2Y₁ was expressed in a crescent shaped distribution of cells in the mandibular process of the 1st arch, and to a lesser extent the 2nd arch. (Fig 2.5 A). Transcripts were restricted to the mesenchyme in these structures. In the anterior half of the mandibular process expression of cP2Y₁ was intense and confined to a triangular shaped group of cells on the dorsal surface immediately subjacent to the endoderm lining the pharynx (Fig 2.5 B). In the posterior half of the mandibular process
Figure 2.4.
Expression of cP2Y1 in the mesonephros. Stage is indicated in the bottom right hand corner. Dotted lines indicate the position from which sections were taken.
(A) Ventral view of stage 20 embryo showing expression in the mesonephros (and limb buds). (B) High power photo showing localisation of expression to mesonephric corpuscle (mc); mt, mesonephric tubule. (C) Ventral view of stage 25 embryo showing that expression of cP2Y1 in the mesonephros had progressed caudally, and was down-regulated rostrally. (D) cP2Y1 in the most recently established areas of expression located caudally. Expression was strong, and restricted to tight oval clusters of cells. (E) The most rostrally located areas of cP2Y1 expression were associated with the newly formed glomeruli, g. The bright areas are blood cells within the glomerulus. Expression of cP2Y1 here was weaker and less restricted than seen caudally. Bars indicate 200\(\mu\)m in (A) and (D), 100\(\mu\)m in (B) and 50\(\mu\)m in all other specimens.
Figure 2.5.
Expression of cP2Y1 in the branchial arches. Stage is indicated in the bottom right hand corner. (A) Lateral view of expression in the mandibular (man) process of the first arch, and the second branchial arch (2nd ba). The line drawing indicates the region photographed, and anterior is to the right. (B) Transverse section through the anterior mandibular process. Dorsal is uppermost. nc, notochord; ph, pharynx. Expression was confined to a triangular shaped group of cells on the dorsal surface. Bars indicate 100μM.

Figure 2.6
Expression of cP2Y1 in the somites. Stage is indicated in the bottom right hand corner. (A) Lateral view showing expression in the anterior somites. (The dark area in the head region is due to an artefact of photography, and slight trapping of probe in head mesenchyme, which also occurred with the sense control probe) (B) Transverse section showing localisation of expression to the myotome, m. sc, spinal cord. (C) Embryo probed with sense control. Bars equal 30μm.
expression was less strong and here expression extended across to the ventral surface. cP2Y₁ was not expressed in the branchial arches prior to stage 21, and by stage 24 expression of cP2Y₁ in the branchial arches was no longer detectable.

- **cP2Y₁ is Expressed in the Myotome - a Compartment of Cells Within Each Somite Which Contains Muscle Precursors.**

  cP2Y₁ expression in the somites was first detected at stage 21. Expression was strongest in the 6 most anterior somites (Fig 2.6 A), but by stage 25 all but the most posterior somites at the tip of the tail expressed cP2Y₁. Sectioning revealed that expression was confined to the myotome, within which expression was stronger ventrally (Fig 2.6 B).

- **cP2Y₁ Expression in the Central Nervous System Appears Later in Development at Embryonic day 8.**

  Expression in the central nervous system (CNS) was first detected by in-situ hybridisation at approximately stage 33. The largest area of expression was in the telencephalon, with smaller patches in dorsal diencephalon, and posterior midbrain (Fig 2.7 A and B). A small triangular patch of expression was also seen in the anterior hindbrain either side of the midline (Fig 2.7 B) which is tentatively identified as the trochlear motor nucleus. Expression in the telencephalon, dorsal diencephalon and posterior midbrain strengthened by stage 36 (Fig 2.6 C). Telencephalic expression was fairly uniform within the superficial cell layers of the pallium (Fig 2.7 D) and extended up to but not into the striatum. cP2Y₁ was also found in a small patch of the ventricular zone close to the septal regions and extended from just the lateral aspect of the ventricle at stage 33 to include its medial aspect by stage 36. The dorsal diencephalic expression lay within developing grey matter and did not include ventricular zone cells (Fig 2.7 E).
Figure 2.7.
Expression of cP2Y1 in the developing CNS. In A-C specimens are orientated with anterior to the left, and dorsal surface uppermost. In figures D and E dorsal is at the top. Stage is indicated in the bottom right hand corner, and dotted lines indicate the region from which sections were taken. (A) Dorsal view of forebrain showing expression in telencephalon and dorsal diencephalon. (B) Dorsal view of midbrain and hindbrain. cP2Y1 was expressed in posterior midbrain, and in triangular patches of cells either side of the midline in anterior hindbrain (arrowed); hb, hindbrain. (C) Dorsal view of stage 36 brain showing increased levels of expression in telencephalon, dorsal diencephalon, and posterior midbrain; tel, telencephalon; mes, mesencephalon; cb, cerebellum. Figures D and E are sections through the telencephalon at stage 33. (D) Expression is confined to the superficial layers of the pallium; sp, striatum-pallium boundary. Expression close to the septal region (sr) is also shown. (E) Dorsal diencephalic expression; vz, ventricular zone. The scale bar in C equals 1mm (for figures A-D), and the scale bar in E represents 300μm.
Discussion

In this chapter the expression of a G protein-coupled receptor for extracellular nucleotides, cP2Y₁, in the developing chick embryo is described for the first time. Northern Blotting of Poly A+ mRNA isolated from embryos at different stages of development showed that cP2Y₁ transcripts were first detected between stages 7 and 12, but were not detected in RNA isolated from stage 2-7 embryos. However ATP, and to a lesser degree ADP have been shown to mobilise Ca²⁺ in cells of the gastrulating chick embryo (stages 3-5) in a manner consistent with activation of a P2Y receptor (Laasberg, 1990). The pharmacology of the response suggests that the receptor involved could well be cP2Y₁. A more sensitive assay for detecting cP2Y₁ expression such as RNase protection or RT-PCR, could be used to verify the presence or absence of this receptor at these earliest stages of chick development. Expression of cP2Y₁ persisted throughout development, and at stages just prior to hatching there was particularly strong expression in brain and skeletal muscle. cP2Y₁ has also been shown to be expressed in the brain and skeletal muscle of adult and one day post-hatch chicks (Webb et al., 1993), suggesting that at these later stages of development cP2Y₁ may fulfill a physiological/adult role, rather than a putative role in development.

- A Dual Role for cP2Y₁ in Regulating Proliferation and Differentiation of Cells in the Developing Limb?

Having established when cP2Y₁ is expressed during embryonic development of the chick, wholemount in-situ hybridisation was used to identify discrete domains of receptor expression. The P2Y receptor family have been shown to be regulators of proliferation (Huang et al., 1989; Rathbone et al., 1992; Abbracchio et al., 1994; Ciccarelli et al., 1994; Neary et al., 1994), and some aspects of the cP2Y₁ expression pattern in the limb suggest that this receptor may fulfill this function. In the early limb bud (stage 20) cP2Y₁ is more heavily expressed dorsally. Activation of cP2Y₁, leading to increased proliferation in expressing cells may contribute to the dorsal curvature that
begins to appear soon after cP2Y1 transcripts are first detected. In general at stage 25 and later stages cP2Y1 expression is consistently strong distally surrounding the most recently differentiating cartilage elements and is maintained at lower levels surrounding both cartilage and tendon in more proximal regions of the limb mesenchyme. This pattern of expression suggests this purinoceptor may be marking areas of relatively undifferentiated mesenchyme where it has the potential to regulate proliferation and/or differentiation of these cells. The length of time that expression persists, coupled with the dynamic expression within limb mesenchyme certainly raises the possibility that cP2Y1 fulfills more than one function within the developing limb.

- **cP2Y1 and Glomerular Vascularisation**

The expression of cP2Y1 within the mesonephros is cephalocaudally regulated and expression is down regulated as red blood cells become visible in the capillaries of the newly formed glomeruli. This pattern suggests a role in development rather than in the function of the glomerulus. I have not studied stages which include metanephric kidney development but it would be interesting to see if transcripts are detected at the time of glomerular vascularisation in the metanephros.

- **The Pattern of Expression in the Chick CNS Suggests a Role for cP2Y1 in Regulating Neuronal Differentiation.**

In contrast to the recently isolated Xenopus P2Y8 receptor, which is expressed at very early stages of neural plate development (Bogdanov et al., 1997), the chick P2Y1 receptor does not appear to play a role in early development of the CNS. It is expressed mostly in regions of post-mitotic neurons (e.g. superficial layers of the telencephalon) at a stage when the telencephalon is beginning its morphogenetic expansion to envelop the dorsal diencephalon. Proliferative cells in the CNS are located in the ventricular zones, but with the exception of the region close to the developing septal nuclei (Fig 6E) the ventricular cells do not express cP2Y1. However, activation of a P2Y receptor has previously been shown to be involved in differentiation of rat astrocytes (Neary et al., 57)
1994; Abbraccio et al., 1995; Neary et al., 1996), This, together with the expression of cP2Y in superficial telencephalic cells, suggests cP2Y has the potential to play a role in the differentiation of chick embryo CNS cells in which it is expressed.

- **cP2Y is Expressed in Skeletal Muscle Progenitors and the Facial Primordia.**

cP2Y is also expressed in the myotome of each somite, the cells of which give rise to the dermis, all the trunk muscles and it also gives rise to muscle cells that migrate into the limb bud (Ordahl and Le-Douarin, 1992). Physiological and pharmacological studies have demonstrated P2X like responses to ATP in later stages of chick skeletal muscle development (Hume and Hönic, 1986; Hume and Thomas, 1988; Thomas and Hume, 1990a and 1990b; Thomas et al., 1991; Wells et al., 1995), but this is the first demonstration of P2Y receptor expression at much earlier stages of chick skeletal muscle development.

At the time that cP2Y is first detected in the limbs, mesonephros and somites, expression also appears transiently in the branchial arches which give rise to skeletal elements of the head. The first arch gives rise to the jaw, while the second arch develops into the bony parts of the ear. The exact fate of the cP2Y expressing cells is currently not known.

- **Summary**

During the first ten days of embryonic development of the chick, a G-Protein coupled receptor for extracellular ATP, cP2Y was found to be expressed in a developmentally regulated manner in the limb buds, mesonephros, brain, somites and facial primordia. This is the first time that expression of an ATP receptor has been described during chick embryogenesis, and its dynamic pattern of expression in multiple tissues and stages suggests that this receptor fulfills more than one function during chick development. In the following two chapters, possible functions for cP2Y in one of these areas, the developing limb, is investigated.
CHAPTER 3
Analysing the Response of Chick Limb Mesenchyme Cells to Extracellular Nucleotides By Monitoring Intracellular Ca^{2+} Changes

- Freshly dissociated limb mesenchyme cells release Ca^{2+} from intracellular stores in response to ATP, 2MeSATP, ADP and UTP.
- Expression of cP2Y\textsubscript{1} is lost from limb mesenchyme cells within 24 hours of culturing.
- Receptor expression can be rescued by transfecting limb mesenchyme cells with a cP2Y\textsubscript{1} expression construct.
- Cells transfected with cP2Y\textsubscript{1} show strong Ca^{2+} responses to 2MeSATP, ATP and UTP, but weak responses to ADP.

Introduction

- The Chick Limb is an Excellent System to Investigate a Function for cP2Y\textsubscript{1}, and by Monitoring Intracellular Calcium Several Important Questions can be Answered.

In this chapter I begin to investigate the function of cP2Y\textsubscript{1} in the developing limb. The limb was chosen for several reasons. Firstly there were a large number of expressing cells at stages 20-22. The chick limb is also very amenable to manipulation both in-ovo and in-vitro. In recent years considerable progress has been made in unravelling the molecular mechanisms that pattern and direct the development of the limb (Johnson and Tabin, 1997; Cohn and Bright, 1999). Any information obtained concerning the function of cP2Y\textsubscript{1} can therefore be considered in a wider context and related to the function of other signalling molecules that are now known to be important in limb development.

In this chapter, monitoring of intracellular free calcium concentration ([Ca^{2+}]) was used to characterise the response of stage 20-22 chick limb mesenchyme cells to a variety of nucleotides known to activate both P2Y and P2X receptors. This technique would
answer four important questions when investigating the function of cP2Y₁ in the developing limb:

1. Is the receptor functional at these early stages of limb development?
2. What concentration of nucleotides are required to elicit a response in these cells?
3. Are other P2 receptor subtypes present on these cells?
4. Is receptor function maintained in-vitro?

• Pharmacological Studies of the Recombinant cP2Y₁ Receptor Show That it Responds Mainly to ATP and 2MeSATP, and can Functionally Couple to Phospholipase C.

cP2Y₁ was cloned from an embryonic day 10 whole chick brain cDNA library (Webb et al., 1993). The entire coding region encodes a polypeptide of 362 amino acids with a calculated molecular weight of 41kDa and the hydrophobicity profile of the predicted amino acid sequence revealed the typical seven hydrophobic transmembrane domains of a G-protein coupled receptor (Webb et al., 1993). cP2Y₁ shows 39% amino acid sequence homology to the other cloned chick P2Y receptor, cP2Y₃ (Webb et al., 1996) but the two receptors have distinct pharmacologies. cP2Y₃ is activated by ADP, UTP and UDP (Webb et al., 1996), whilst electrophysiological analysis of the recombinant cP2Y₁ receptor expressed in Xenopus oocytes showed a relative order of agonist potency: 2MeSATP ≥ ATP > ADP > α,βMeATP, β,γ-MeATP, UTP (Webb et al., 1993). Suramin (100µM) and Reactive blue-2 (10µM) antagonised the responses to ATP and 2MeSATP (Webb et al., 1993). Both ATP and 2MeSATP induce 1,4,5 InsP₃ accumulation in COS-7 cells transfected with recombinant cP2Y₁, indicating functional coupling of cP2Y₁ to phospholipase C (PLC) (Simon et al., 1995). These studies, and the release of Ca²⁺ as a result of cP2Y₁ activation enables the technique of monitoring Ca²⁺ to be used to address the questions above.
Methods

- **Ratiometric Measurements of \([\text{Ca}^{2+}]\) Using Fura Red.**

In this chapter, changes in \([\text{Ca}^{2+}]\) were monitored using the fluorescent Ca\(^{2+}\) indicator Fura Red. The visible light-excitable fura-2 analogue, Fura Red undergoes a shift in its excitation spectrum upon binding Ca\(^{2+}\) (Kurebayashi et al., 1993) (Fig 3.1). In the experiments described in this chapter, fluorescence emission from Fura Red is read at a constant wavelength of 590nm, while the excitation wavelengths are switched between 490nm and 440nm. In the absence of free Ca\(^{2+}\) the emission intensity from Fura Red excited at 490nm is high, since the amplitude of the fluorescence excitation spectrum is high at this excitation wavelength. Under the same Ca\(^{2+}\) free conditions the emission intensity of Fura Red excited at 440nm is low, due to the low amplitude of the excitation spectrum at this wavelength. The emission intensity ratio of Fura Red excited at 440nm/490nm is therefore low. When Fura Red binds free Ca\(^{2+}\) the excitation spectrum shifts so that at 440nm the amplitude of the excitation spectrum is now high, while at 490nm it is low. The emission intensity ratio of Ca\(^{2+}\)-bound Fura Red excited at 440nm/490nm is therefore high. Such ratiometric measurements eliminate distortions of data caused by photobleaching, uneven dye loading, detection efficiency and cell thickness. Fura Red also has a very long-wavelength emission maximum (approximately 660nm) eliminating the possibility of interference from cell autofluorescence (Kurebayashi et al., 1993).

- **Fluorescence Detection.**

Ratiometric measurements of \([\text{Ca}^{2+}]\) using Fura Red requires the following:

1. An excitation source. In this study a Xenon lamp was used.

2. 440nm and 490nm wavelength filters to isolate excitation photons of the required wavelength from the excitation source, and a mechanism for alternating between these two filters to make ratiometric measurements. In the set up used in this chapter the two filters are mounted on a rotating wheel.
Figure 3.1. The excitation spectrum of Fura Red shifts upon binding Ca\textsuperscript{2+}, so that in the presence of low Ca\textsuperscript{2+} the amplitude of the excitation spectrum peaks at 490nm, and the amplitude of the excitation spectrum at 440nm is lower. In the presence of high Ca\textsuperscript{2+} the excitation spectrum shifts so that its peak is now at 440nm, and the amplitude of the excitation spectrum at 490nm is lower. This shift in excitation spectrum allows ratiometric (440nm/490nm) measurements to be made with Fura Red.
Figure 3.2 Apparatus for calcium imaging.
1. A Xenon lamp is used as an excitation source.
2. 490nm and 440nm wavelength filters mounted on a wheel isolate excitation photons of the required wavelengths.
3. Excitation photons are directed onto the Fura-Red loaded cells in a heated chamber on a microscope stage, via a dichroic mirror.
4. Emission photons from excited Fura-Red are directed to a 590nm longpass filter (5) which isolates emission photons from excitation photons.
6. Fluorescence from Fura red loaded cells was measured using epifluorescence and a Newcastle Photometrics Multipoint System (Newcastle upon Tyne, UK). This system can monitor fluorescence from up to 16 cells simultaneously using a CCD camera. Fluorescence from Fura red is plotted as an excitation ratio at 440 nm / 490 nm.
3. A 590nm wavelength longpass filter to isolate emission photons from excitation photons.

4. A detection system that registers emission photons and produces a recordable output, such as an electrical signal. In this chapter a CCD camera was used. Figure 3.2 shows how these various components are arranged relative to one another.

- **Cell Culture**
  Chick wing buds from stage 20-22 embryos were dissected in sterile Howard's Ringers solution. Wings buds were then briefly rinsed in DMEM medium and HBSS (both from Gibco), before the ectoderm was removed by incubating in 10 x trypsin (Gibco) for 1 hour on ice. Cells were thoroughly dissociated by trituration before plating 100μl drops at 3 x 10^6 cells/ml on 22 mm diameter acid cleaned glass coverslips in DMEM+10% foetal bovine serum + 1% antibiotic/antimicotic solution (all from Gibco). Cells were then flooded with 1ml of the above culture medium and cultured for 1 hour at 37°C, 5% CO₂

- **Dye Loading.**
  In the following experiments the cell permeant AM ester form of Fura Red was used. This form of the dye can be passively loaded into cells, where it is cleaved by intracellular esterases into a cell impermeant dye. After 1 hour cells were loaded with Fura-Red (Molecular Probes) using the -AM form of the dye, at a final concentration of 5μM in HEPES buffered DMEM+ 250μM sulfinpyrazone (ICN), for 1 hour at room temperature. Fura Red-AM was made up as 2mM stocks in dimethyl sulfoxide (DMSO) plus 5% pluronic F127 (Molecular probes). Inclusion of the anion exchange inhibitor sulfinpyrazone in the loading medium prevents compartmentalisation and extrusion of the dye (Di Virgilio et al., 1990).
• Monitoring of Intracellular Calcium.

The coverslips were transferred to a 37°C recording chamber containing HEPES buffered DMEM. Single cells or small groups of cells were selected for each experiment. Test compounds (ATP, ADP, AMP, Adenosine, UTP - all from Boehringer Mannheim, and 2MeSATP from RBI) were then added directly to the recording chamber.

Reduced Ca^{2+} measuring conditions were created by adding EGTA to a final concentration of 5mM to the HEPES buffered DMEM (the Max chelate computer program was used to calculate the effective concentration of EGTA required). Thapsigargin was purchased from Calbiochem. Reactive blue-2 was purchased from Sigma. Fluorescence from Fura Red loaded cells was measured using epifluorescence and a Newcastle Photometrics Multipoint System (Newcastle upon Tyne, UK). This system can monitor fluorescence from up to 16 cells simultaneously using a CCD camera. Fluorescence from fura red is plotted as an excitation ratio at 440nm / 490nm (Kurebayashi et al., 1993).

• RNase Protection

- Total RNA was isolated from freshly dissected stage 20-22 limb buds, and from 24 hour limb mesenchyme cultures using the RNeasy total RNA isolation kit (Qaigen). The cultures were prepared as described above, and cells were harvested after 24 hours by treatment with 10 x Trypsin solution (Gibco).

- Probe Preparation: The construct pRCCMV-cP2Y₁ (see figure 2.1) was linearised with the restriction enzyme Bsu 36I, and cleaned by treatment with proteinase K, phenol-chloroform extraction and ethanol precipitation. The following transcription reaction was then set up:
1.0μl (1μg) linearised pRCCMV-cP2Y,
0.5μl 2.5mg/ml BSA
0.5μl 0.5M DTT
1.0μl 5mM NTPs (A, U, G)
2.0μl 5 x Transcription buffer (Promega)
0.5μl Rnasin
2.5μl 32P CTP (BLU/NEG/508X, 800Ci/mmol, 250μCi)
0.5μl RNA polymerase
1.5μl H2O

The reaction mix was incubated at 37°C for at least one hour. 1μl DNase was added to the transcription mix and incubated for a further 15 minutes at 37°C. The probe was then phenol/chloroform extracted and precipitated in ethanol. Probes were resuspended in 10μl H2O. Transcription from the Sp6 promoter in pRCCMV-cP2Y yielded a cP2Y probe approximately 400 bases in length. A GAPDH (Panabiers et al., 1984) control probe of approximately 200 bases in length was also generated using the above protocol. When generating the GAPDH control probe 0.5μl of H2O was substituted with 0.5μl of 200μM cold CTP.

- Purifying the probe: Small glass plates were cleaned with detergent, ethanol and finally acetone. One plate was siliconised. The plates were taped together and 0.4mm spacers were used. The gel was then prepared:

6ml 10 x TBE
4.5ml 40% acrylamide
12.6g urea
make up to 30ml with H2O
Add 200μl 10% APS and 30μl TEMED.
mix well and pour, insert a 10 well, wide tooth comb.
Leave for 30 min to set.

An equal volume of 2 x formamide dyes (Ambion kit) was added to each probe and then heated to 72°C for 3 min. The tubes were spun briefly before loading onto the gel. The gel was run in 1 x TBE buffer for approximately 1.5 hours at 300 volts (until bromophenol blue dyes are just about to run off the bottom). After running, the plates were spilt apart and the gel (resting on one of the plates) was wrapped in Saranwrap. 3 pieces of tracker tape were placed on the gel for aligning with the autorad later. A film was exposed to the gel for 30 seconds. The gel and developed autorad were then aligned.
using the tracker tape. Holes were cut in the autorad where the major band of each probe was located. This hole was then used as a guide to cut the probe out of the gel. Each gel slice containing the probe was then placed in a clean eppendorf tube.

- Eluting the probe: 40μl 10mg/ml tRNA was added to the gel slice with 400μl of elution buffer: 0.1% SDS
  1mM EDTA
  0.5M NH₄OAc
  10mM MgOAc

This was then incubated at 50°C for 2 hours with periodic vortexing. The gel pieces were then spun down briefly, and 440μl transferred into a fresh eppendorf tube. This was then spun again briefly to remove residual gel pieces, before transferring 400μl into a fresh tube. 1ml of 100% ethanol was added and the probes were precipitated on dry ice. The probes were spun down for 20 minutes at 4°C, washed in 70% ethanol and resuspended in 25μl H₂O. 1μl was dropped onto DE81 paper and the counts per minute (cpm) was measured. Probes were diluted to 1-5cpm/μl.

- Hybridisation: The following hybridisation was set up for each probe:

  10μg RNA
  4μl 10 x PIPES buffer (0.4M PIPES pH 6.4, 4M NaCl, 10mM EDTA)
  20μl deionized formamide
  1μl 32P RNA probe
  H₂O to 40μl

A control reaction was set up for each probe with 10μg tRNA. The tubes were vortexed and incubated at 85°C for 5 minutes, followed by an overnight incubation at 50°C.

- RNase step: Rnase T1 was added to the digestion mix at a final concentration of 700U/ml.

  Digestion mix: 30μl 1M Tris pH 7.4
  60μl 0.25M EDTA pH 8.0
  180μl 5M NaCl
  2.8ml H₂O

300μl of the digestion mix was then added to each hybridisation reaction. The tubes were then incubated at 37°C for 30 min. 20μl of 10% SDS and 50μl 1mg/ml proteinase
K was added, and then incubation was continued for a further 15 min at 37°C. The RNA was then phenol chloroform extracted and precipitated on dry ice in 100% ethanol. The precipitated RNA was spun down for 20 minutes at 4°C, and the pellet washed in 70% ethanol. 1µl H₂O was added to the wet pellet, followed by 5µl of formamide dyes. Each sample was then diluted 1 in 500, and 1µl of the resulting solution was added to 5µl of formamide dyes. The samples were then heated for 5 minutes at 85°C. The samples were spun briefly before loading onto a large gel:

```
10 ml 10 x TBE
15 ml 40% acrylamide
42g urea
make up to 100ml with H₂O
600µl 10% APS and 100µl TEMED
```

The gel was run at 60 Watts until the bromophenol blue ran out of the bottom gel. The plates were then split apart, and the gel was fixed in 1 litre of 10% Methanol/10% Acetic acid. A wet sheet of Whatman paper was then placed on top of the gel (avoiding bubbles). The gel/Whatman sheet was then peeled away from the glass plate, and covered in Saranwrap. The gel was dried at 80°C for 2 hours before exposing to X-ray film with 2 intensifying screens overnight at -70°C.

- **Transfecting Limb Mesenchyme Cells With a cP2Y₁ Expression Construct.**

Cultures were set up as described above and were left for 24 hours prior to transfecting. The plasmid construct pRCCMV-cP2Y₁ (see figure 2.1 for plasmid map) was transfected into limb mesenchyme cells using Fugene 6 (Roche Biochemicals), a lipid (non-liposomal) based transfection reagent, according to the manufacturers instructions. Briefly: 5µl Fugene 6 was added to 95µl of serum free DMEM in a sterile 0.5ml eppendorf tube and left to incubate for 5 minutes at room temperature. This was then added dropwise to 2µl of 1µg/µl plasmid DNA in a second eppendorf tube. DNA-Fugene 6 complexes were then allowed to form at room temperature for 20 minutes. This mixture was added dropwise to the limb mesenchyme cultures which were then
incubated for a further 8 hours at 37°C, 5% CO₂. Cultures were then washed with fresh DMEM+10% foetal bovine serum + 1% antibiotic/antimicotic solution and left for 2 days before assaying for Ca^{2+} responses to 100μM ATP, ADP, 2MeSATP and UTP. These were found to be the optimal transfection conditions (as assayed by the percentage of cells generating a Ca^{2+} response to 100μM ATP). Variables affecting transfection efficiency included: cell plating density, volume and type of transfection reagent used, amount of DNA, and transfection reagent:DNA ratio. Some cultures were also transfected with empty vector (pRCCMV without the cP2Y₁ insert) as a negative control.

Dye loading and monitoring of [Ca^{2+}] in control (pRCCMV) and pRCCMV-cP2Y₁ transfected cultures were carried out as described above.
Results

- ATP, 2MeSATP, ADP and UTP Elicited Calcium Responses in Freshly Dissociated Stage 20-22 Limb Mesenchyme Cells.

Large increases in [Ca\(^{2+}\)] were elicited by ATP, ADP and 2MeSATP at concentrations of 100\(\mu\)M and 10\(\mu\)M. Fig 3.3 shows typical [Ca\(^{2+}\)] increases in single cells or small clusters of cells to these nucleotides. The expression pattern of cP2Y\(_1\) at stage 20 (see figure 2.3 A) suggested that not all limb mesenchyme cells expressed the receptor at this stage. This heterogeneity was also evident in the size of the [Ca\(^{2+}\)] responses. Some cells did not respond when each nucleotide was added. A small Ca\(^{2+}\) transient was also detected in response to 100\(\mu\)M UTP (Fig 3.3), a nucleotide which did not activate cP2Y\(_1\) when expressed in Xenopus oocytes. (Webb et al., 1993). Adenosine and AMP (metabolites of ATP) were inactive. These data are summarised in Table 3.1.

No chick P2X receptors have yet been cloned, but physiological studies have revealed P2X like responses in embryonic chick myotubes (Hume and Thomas, 1988; Thomas and Hume, 1990a, 1990b, and 1993) and ciliary neurons (Abe et al., 1995). The increases in [Ca\(^{2+}\)] observed in limb bud cells in response to the above agonists may therefore result from the opening of an unidentified chick P2X ion channel allowing the influx of Ca\(^{2+}\) into the cell from the culture medium. In order to investigate any influence of P2X receptor activity on the [Ca\(^{2+}\)] increases, calcium measurements were performed in reduced Ca\(^{2+}\) medium. Addition of the calcium chelator EGTA to a final concentration of 5mM was calculated to reduce the free Ca\(^{2+}\) concentration in the measuring medium to less than 40nM. The free Ca\(^{2+}\) concentration in most cells is around 100nM. Thus addition of EGTA reduced the potential of extracellular Ca\(^{2+}\) to flow into the cell along its concentration gradient. Under these conditions 100\(\mu\)M ATP, ADP, 2MeSATP and UTP still elicited [Ca\(^{2+}\)] increases (Fig 3.4). However the size of the responses was approximately halved and the proportion of cells responding was also reduced in low Ca\(^{2+}\) conditions (Table 3.2).

The reduction in size of the Ca\(^{2+}\) response may be due to a number of reasons. Firstly, P2X receptors may be present on these cells. Alternatively, rapid depletion of Ca\(^{2+}\)
Figure 3.3. Calcium responses in freshly dissociated stage 20-22 chick limb mesenchyme cells. In each figure the nucleotide added is shown at the top of the trace. These traces show responses to 100μM concentrations of each nucleotide. ATP, 2MeSATP and ADP also elicited responses at a concentration of 10μM. UTP was inactive at this concentration. The time at which each nucleotide was added is shown by the arrows. Fluorescence ratio is shown on the y axis, and time in seconds is shown on the x axis.
Figure 3.4. Calcium responses in freshly dissociated stage 20-22 chick limb mesenchyme cells in the presence of 5mM EGTA. The nucleotide added is shown at the top of each trace, and the time at which it was added is shown by the arrows. Each nucleotide was used at a concentration of 100μM. Sequestration of extracellular Ca^{2+} by EGTA reduces the size of the Ca^{2+} response.
Figure 3.5. Comparing Ca\(^{2+}\), responses of freshly dissociated stage 20-22 chick limb mesenchyme cells to Thapsigargin in Ca\(^{2+}\) containing, and reduced Ca\(^{2+}\) conditions. The trace on the left shows the response to 1µM Thapsigargin in Ca\(^{2+}\) containing medium. The size of the response to 1µM Thapsigargin was halved in the presence of 5mM EGTA (shown in the trace on the right). The time at which Thapsigargin was added is shown by the arrows.
stores in reduced Ca^{2+} medium (as occurs in Ehrlich-ascites-tumour cells, rat thymocytes and human neutrophils (Montero et al., 1990; Montero et al., 1991)) may be occurring. A final possibility is that depletion of Ca^{2+} stores by these extracellular nucleotides results in the influx of extracellular Ca^{2+}, a phenomenon known as a capacitative entry. Capacitative entry would be prevented in reduced Ca^{2+} conditions, and this may account for the reduced amplitude of the responses to these nucleotides. In order to test these possibilities the Ca^{2+} responses of limb bud cells to the drug Thapsigargin were compared in normal and low Ca^{2+} conditions. Thapsigargin releases Ca^{2+} by inhibiting endoplasmic reticular Ca^{2+}-ATPase. A reduced Ca^{2+} response to Thapsigargin in low Ca^{2+} conditions would indicate either the depletion of intracellular Ca^{2+} stores, or that capacitative entry occurs in these cells following depletion of Ca^{2+} stores. It was found that in reduced Ca^{2+} conditions, the Ca^{2+} responses of limb cells to 1μM thapsigargin was halved (Fig 3.5), and the percentage of cells responding was also reduced (Table 3.2), indicating either the depletion of intracellular Ca^{2+} stores in low Ca^{2+} containing medium, or that in medium containing higher levels of Ca^{2+} capacitative entry of extracellular Ca^{2+} occurs following treatment with either thapsigargin or extracellular nucleotides. These results suggest that the Ca^{2+} responses of freshly dissociated stage 20-22 limb mesenchyme cells to ATP, 2MeSATP, ADP and UTP are mediated by members of the P2Y receptor family, and that the rises in [Ca^{2+}] in response to these nucleotides are a result of Ca^{2+} release, and not influx of extracellular Ca^{2+} through nucleotide-gated, P2X ion channels.

- **Limb Mesenchyme Cells Cultured for 24 Hours no Longer Responded to ATP, 2MeSATP, ADP and UTP. Expression of cP2Y_{1} was also downregulated during this period.**

Chick limb bud mesenchyme cells maintained in culture for 24 hours no longer responded to these nucleotides by releasing Ca^{2+} (Fig 3.6, Table 3.3). Addition of the Ca^{2+} ionophore, Ionomycin, demonstrated that these cells were still competent to
Figure 3.6. Expression of cP2Y₁ was lost from chick limb mesenchyme cells within 24 hours of culturing. (A) Response of limb cells to 100μM ATP after 24 hours in culture. (B) Ionomycin was added to 24 hour cultures to check that cells were competent to respond. The arrows in A and B indicate when each compound was added. (C) RNase protection showed that within 24 there was almost complete downregulation of cP2Y₁ expression. A GAPDH probe was used as a loading control.
generate \([\text{Ca}^{2+}]\), increases (Fig 3.6). The loss of responsiveness to ATP, 2MeSATP, ADP and UTP may be due to either: a) post-transational repression of receptor(s) expression or b) downregulation of receptor(s) expression at the transcriptional level. RNase protection was therefore used to compare the levels of cP2Y, mRNA in freshly dissociated limb cells with cells cultured for 24 hours. It was found that there was almost complete downregulation of receptor expression during this period (Fig 3.6).

- **Cells Transfected with the cP2Y, Receptor Responded Strongly to 2MeSATP, ATP and UTP but only Weakly to ADP.**

Native expression of cP2Y, was lost after 24 hours in culture. Therefore, in order to carry out in-vitro functional studies of cP2Y, it was necessary to rescue its expression in cultured limb mesenchyme cells. This was achieved by transiently transfecting cells with the cP2Y, expression construct (pRCCMV-P2Y,), whose construction is described in chapter 2 (see also fig 2.1 for a plasmid map). Monitoring of \([\text{Ca}^{2+}]\) was then used to ensure that functional receptor was produced in transfected cells (Fig 3.7). Monitoring of \([\text{Ca}^{2+}]\) was also carried out on cells transfected with empty pRCCMV (i.e. not containing the cP2Y, cDNA) as a negative control. In these control transfected cells the average fluorescence ratio change in response to 100\(\mu\)M ATP was 0.15 (Fig 3.7 E, Table 3.5) , and only 19\% of cells measured showed a \([\text{Ca}^{2+}]\) response (Table 3.5). In contrast, 64\% of cells transfected with pRCCMV-cP2Y, responded to 100\(\mu\)M ATP (Table 3.4). The average size of the fluorescence ratio change in response to 100\(\mu\)M ATP in pRCCMV-cP2Y, transfected cells was 0.44 (Fig 3.7 A, Table 3.4), slightly higher than the value seen for the same dose of ATP in freshly dissociated cells which was 0.39. The response profile to 100\(\mu\)M ATP in pRCCMV-cP2Y, transfected cells, and freshly dissociated cells was very similar in terms of response time and duration. These data suggest that by transfection with pRCCMV-cP2Y, it was possible to rescue functional expression of cP2Y, in cultured limb mesenchyme cells. Robust responses to 100\(\mu\)M ATP could be elicited in pRCCMV-cP2Y, transfected cells 24, 48 and 72 hours
Figure 3.7. Figures A-D show typical Ca\(^{2+}\) responses in limb cells transfected with pRCCMV-cP2Y. The nucleotide added (100μM) is shown at the top of each trace, and the arrows indicate when each compound was added. E. Typical Ca\(^{2+}\) response to 100μM ATP in a cell transfected with a control construct (pRCCMV). F. Ca\(^{2+}\) responses to 100μM ATP in cells transfected with pRCCMV-cP2Y are blocked by 100μM Reactive Blue 2.
after transfection indicating transfected receptor expression is detectable soon after transfection and remains detectable for at least 3 days.

Having rescued expression of cP2Y₁ in these cells, monitoring [Ca²⁺] could be used to look at the pharmacology of cells transfected with cP2Y₁. Do these cells respond to ADP and UTP, as do freshly dissociated cells, or are the responses to these two nucleotides mediated by other receptor subtypes which like cP2Y₁ disappear with time in culture? It was found that in pRCCMV-cP2Y₁ transfected cells, 2MeSATP, as well as ATP elicited robust Ca²⁺ responses (Fig 3.7 A and B), while ADP was much less potent (Fig 3.7 D). These data are in agreement with electrophysiological studies done on the recombinant receptor expressed in Xenopus oocytes (Webb et al., 1993). The surprising result was that transfected cells responded strongly to UTP (Fig 3.7 C). The study by Webb et al., 1993 showed that UTP was inactive at the cP2Y₁ receptor. The response to 100μM ATP could be blocked in pRCCMV-cP2Y₁ transfected cells by addition of 100μM Reactive blue-2, a competitive antagonist at the cP2Y₁ receptor (Webb et al., 1993) (Fig 3.7 F, Table 3.6). The calcium responses of transfected cells are summarised in Table 3.4.

The calcium imaging data from this chapter is summarised in figure 3.8.
### Table 3.1. Calcium Responses in Freshly Dissociated Limb Mesenchyme Cells.

<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>CONCENTRATION (µM)</th>
<th>NUMBER OF CELLS MEASURED</th>
<th>CELLS RESPONDING (%)</th>
<th>MEAN FLUORESCENCE RATIO CHANGE</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>100</td>
<td>44</td>
<td>91</td>
<td>0.39</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>12</td>
<td>84</td>
<td>0.24</td>
<td>0.03</td>
</tr>
<tr>
<td>2MeSATP</td>
<td>10</td>
<td>27</td>
<td>96</td>
<td>0.57</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>9</td>
<td>100</td>
<td>0.51</td>
<td>0.05</td>
</tr>
<tr>
<td>ADP</td>
<td>10</td>
<td>48</td>
<td>69</td>
<td>0.25</td>
<td>0.02</td>
</tr>
<tr>
<td>UTP</td>
<td>100</td>
<td>27</td>
<td>96</td>
<td>0.57</td>
<td>0.05</td>
</tr>
<tr>
<td>AMP</td>
<td>100</td>
<td>10</td>
<td>100</td>
<td>0.51</td>
<td>0.05</td>
</tr>
<tr>
<td>ADENOSINE</td>
<td>100</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>THAP</td>
<td>1</td>
<td>39</td>
<td>100</td>
<td>0.43</td>
<td>0.02</td>
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</table>

### Table 3.2. Calcium Responses in Freshly Dissociated Limb Mesenchyme Cells in Calcium Free Medium.

<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>CONCENTRATION (µM)</th>
<th>NUMBER OF CELLS MEASURED</th>
<th>CELLS RESPONDING (%)</th>
<th>MEAN FLUORESCENCE RATIO CHANGE</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>100</td>
<td>48</td>
<td>69</td>
<td>0.25</td>
<td>0.02</td>
</tr>
<tr>
<td>2MeSATP</td>
<td>100</td>
<td>39</td>
<td>46</td>
<td>0.21</td>
<td>0.02</td>
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<tr>
<td>ADP</td>
<td>100</td>
<td>24</td>
<td>54</td>
<td>0.20</td>
<td>0.04</td>
</tr>
<tr>
<td>UTP</td>
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<td>31</td>
<td>19</td>
<td>0.13</td>
<td>0.01</td>
</tr>
<tr>
<td>THAP</td>
<td>1</td>
<td>28</td>
<td>35</td>
<td>0.20</td>
<td>0.04</td>
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### Table 3.3. Calcium Responses in Limb Mesenchyme Cells Cultured for 24 hours.

<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>CONCENTRATION (µM)</th>
<th>NUMBER OF CELLS MEASURED</th>
<th>CELLS RESPONDING (%)</th>
<th>MEAN FLUORESCENCE RATIO CHANGE</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>100</td>
<td>77</td>
<td>9</td>
<td>0.08</td>
<td>0.007</td>
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<tr>
<td>2MeSATP</td>
<td>100</td>
<td>32</td>
<td>9</td>
<td>0.08</td>
<td>0.027</td>
</tr>
<tr>
<td>ADP</td>
<td>100</td>
<td>21</td>
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<td>-</td>
<td>-</td>
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<tr>
<td>UTP</td>
<td>100</td>
<td>22</td>
<td>0</td>
<td>-</td>
<td>-</td>
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</tbody>
</table>

### Table 3.4 Calcium Responses in Limb Mesenchyme Cells Transfected with pRCCMV-cP2Y1

<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>CONCENTRATION (µM)</th>
<th>NUMBER OF CELLS MEASURED</th>
<th>CELLS RESPONDING (%)</th>
<th>MEAN FLUORESCENCE RATIO CHANGE</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>100</td>
<td>72</td>
<td>64</td>
<td>0.44</td>
<td>0.04</td>
</tr>
<tr>
<td>2MeSATP</td>
<td>100</td>
<td>38</td>
<td>61</td>
<td>0.42</td>
<td>0.1</td>
</tr>
<tr>
<td>ADP</td>
<td>100</td>
<td>39</td>
<td>23</td>
<td>0.14</td>
<td>0.03</td>
</tr>
<tr>
<td>UTP</td>
<td>100</td>
<td>134</td>
<td>36</td>
<td>0.55</td>
<td>0.09</td>
</tr>
</tbody>
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80
Table 3.5. Calcium Responses in Limb Mesenchyme Cells Transfected with Empty pRCCMV.

<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>CONCENTRATION (µM)</th>
<th>NUMBER OF CELLS MEASURED</th>
<th>CELLS RESPONDING (%)</th>
<th>MEAN FLUORESCENCE RATIO CHANGE</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>100µM</td>
<td>52</td>
<td>19</td>
<td>0.15</td>
<td>0.06</td>
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Table 3.6. Calcium Responses in Limb Mesenchyme Cells Transfected with pRCCMV-cP2Y, in the Presence of 100µM Reactive Blue 2.

<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>CONCENTRATION (µM)</th>
<th>NUMBER OF CELLS MEASURED</th>
<th>CELLS RESPONDING (%)</th>
<th>MEAN FLUORESCENCE RATIO CHANGE</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>100µM</td>
<td>36</td>
<td>16</td>
<td>0.14</td>
<td>0.04</td>
</tr>
</tbody>
</table>
Figure 3.8. Summary of \([\text{Ca}^{2+}]\) monitoring data. Mean fluorescence ratio change is shown on the y axis, and the experimental conditions are shown on the x axis. Only cells that responded (fluorescence ratio > 0.05) to each nucleotide used at 100\(\mu\)M were used to calculate the mean response size. The main points are summarised below.

1. Freshly dissociated limb mesenchyme cells respond to ATP, 2MeSATP, ADP and UTP.
2. Limb mesenchyme cultured for 24 hours show weak responses to ATP and 2MeSATP, and show no response to UTP and ADP.
3. The response sizes to ATP, 2MeSATP, ADP and UTP are reduced in \(\text{Ca}^{2+}\) conditions. The response to Thapsigargin is also reduced in \(\text{Ca}^{2+}\) free conditions indicating depletion of \(\text{Ca}^{2+}\) stores.
4. Limb mesenchyme cells transfected with pRCCMV-cP2Y\(_1\) respond to ATP, 2MeSATP and UTP, but respond only weakly to ADP. (Thapsigargin was not tested).
5. The response to 100\(\mu\)M ATP in cells transfected with pRCCMV-cP2Y\(_1\) is blocked by 100\(\mu\)M Reactive Blue 2 (RB-2).
Discussion

The aim of this chapter was to monitor \([Ca^{2+}]\) to answer four questions. The first of these questions (is cP2Y functional in stage 20-22 limb buds?) was addressed by looking at the response of freshly dissociated limb cells to the ligands known to activate the recombinant receptor expressed in Xenopus oocytes (Webb et al., 1993). Consistent with the presence of a functional cP2Y receptor, ATP and 2MeSATP elicited \([Ca^{2+}]\) increases in freshly dissociated limb mesenchyme cells. There was variation in the size of the responses, and some cells failed to respond. This was also consistent with the heterogeneous pattern of expression of cP2Y within limb mesenchyme cells. By looking at the responses of freshly dissociated limb cells to a variety of other nucleotides, a second question was addressed, are other P2 receptor subtypes expressed by these cells? ADP, a weak agonist at the recombinant cP2Y receptor (Webb et al., 1993) elicited strong \(Ca^{2+}\) responses, and UTP which was found to be inactive at the recombinant receptor (Webb et al., 1993) initiated weak \(Ca^{2+}\) responses in freshly dissociated limb cells. The responses to these two nucleotides raises the possibility that other P2 receptor subtypes are present on these cells that respond to ADP and/or UTP. ATP, 2MeSATP and ADP elicited \(Ca^{2+}\) responses at concentrations of 10\(\mu\)M and 100\(\mu\)M, while UTP only generated a \(Ca^{2+}\) response at 100\(\mu\)M.

No chick P2X receptors have yet been cloned, but P2X like responses have been described in ciliary neurons acutely dissociated from day 14 embryonic ciliary ganglia (Abe et al., 1995), on patched membranes of myoblasts and myotubes cultured from chick embryos (Kolb and Wakelam, 1983; Hägglad et al., 1985; Hume and Höning, 1986; Hume and Thomas, 1988; Thomas and Hume, 1990a and b, 1993; Thomas et al., 1991), and on intact chick skeletal muscle (Wells et al., 1995). In the fifth chapter of this thesis immunohistochemistry is used to show that at least two members of the P2X receptor family are expressed during chick embryogenesis (although not in the limb buds at stage 20-22). It may therefore be possible that the \(Ca^{2+}\) responses observed in freshly dissociated limb cells may also be due to P2X receptor activity. However, the...
fact that Ca\(^{2+}\) responses to ATP, 2MeSATP, ADP and UTP could still be elicited in limb cells cultured in reduced Ca\(^{2+}\) make this unlikely. (The size of the responses were reduced but it was shown that this was likely to be due to depletion of Ca\(^{2+}\) stores in reduced Ca\(^{2+}\) containing medium, or that in Ca\(^{2+}\) containing medium, capacitative entry of extracellular Ca\(^{2+}\) follows release of Ca\(^{2+}\) in limb cells).

In order to carry out in-vitro studies of cP2Y\(_1\) function, it was necessary to answer the fourth question, is receptor expression is maintained in-vitro? The [Ca\(^{2+}\)] monitoring experiments were therefore repeated on cells cultured for 24 hours. It was found that within this period the cells no longer responded to ATP, 2MeSATP, ADP and UTP. RNase protection was used to show that within 24 hours there is almost complete downregulation of cP2Y\(_1\) expression. Since these limb mesenchyme cells are cultured in the absence of limb ectoderm these findings raise the possibility that ectodermal signals are required to maintain cP2Y\(_1\) expression. Alternatively a mesenchymal signal (either diffusible or contact mediated) essential for maintaining cP2Y\(_1\) expression is diluted out under the culture conditions. The expression of cP2Y\(_1\) was rescued in cultured limb cells by transient transfection with pRCCMV-cP2Y\(_1\). In transfected cells the Ca\(^{2+}\) responses matched the pharmacological profile of the recombinant receptor expressed in Xenopus oocytes and COS-7 cells (Webb et al., 1993; Simon et al., 1995), in that 2MeSATP and ATP elicited strong responses whilst ADP was much less potent. The large response to ADP in freshly dissociated therefore suggests that another P2Y receptor subtype is mediating this response. A surprising result was that the response to UTP, a nucleotide which did not activate the recombinant receptor (Webb et al., 1993) was rescued. Possible explanations for the response to UTP are:

1. A UTP receptor may be genetically downstream of cP2Y\(_1\), and by rescuing expression of cP2Y\(_1\), expression of a UTP receptor is rescued. P2Y receptors have been shown to be regulators of gene expression. For example, treatment of astrocytes with ATP or UTP has been shown to lead to the formation of activator protein (AP)-1 complexes (Neary et. al., 1996), which are transcriptional activators. Mammalian UTP
receptors include P2Y₂, P2Y₄, and P2Y₆ but homologues of these have yet to be identified in chick.

2. An alternative explanation for the response to UTP is that UTP is acting as a phosphate donor for ADP, thus generating ATP which then activates cP2Y₁. The extracellular enzyme, nucleoside diphosphokinase (NDPK) catalyzes such transphosphorylation of nucleotides. This phenomenon has been shown to occur in 1321N1 human astrocytoma cells stably expressing the UTP selective P2Y₄ (Lazarowski et al., 1997). In the study by Lazarowski et al, (1997) addition of ATP or UDP did not elicit Ca²⁺ responses in 1321N1 astrocytoma cells expressing P2Y₄, but simultaneous addition of these nucleotides resulted in elevation of [Ca²⁺]. This effect was due to the activity of NDPK, transferring the γ-phosphate from ATP to UDP to generate the P2Y₄ ligand, UTP. In chapter 4 of this thesis it was shown that ATP is both produced, and broken down by limb mesenchyme cells in culture. The rate of ADP breakdown by these cells is also slower than the rate of ATP breakdown (Hatori et al., 1995), thus there is the potential for ADP to accumulate in the extracellular environment and to make this scenario plausible.

• Summary
In this chapter four important questions have been answered. Firstly, cP2Y₁ is functional in cells isolated from stage 20-22 limb buds, and responses to cP2Y₁ agonists can be elicited in the 10-100μM range. The large responses to ADP and UTP suggest that other P2 receptor subtypes may also be present on these cells. Expression of cP2Y₁ is lost from cultured limb cells within 24 hours, but functional expression of the receptor can be transiently rescued by transfection with the cP2Y₁ expression construct pRCCMV-cP2Y₁.
CHAPTER 4
A Role for cP2Y₁ in Regulating Cartilage Formation.

- Expression of cP2Y₁ inhibits cartilage formation in limb micromass cultures.
- The P2Y receptor antagonist Reactive blue-2, blocks the effect of cP2Y₁ expression on cartilage formation in limb micromass cultures.
- ATP is rapidly broken down in limb micromass cultures, but small quantities of cellular derived ATP can still be detected.

Introduction

The expression pattern of cP2Y₁ in the developing limb suggests that it is preferentially expressed in undifferentiated cells, and may be downregulated as cells differentiate. This pattern was particularly evident surrounding areas of cartilage formation at stages 29 and after (see chapter 2, fig 2.3). P2Y receptor signalling has also been shown to regulate differentiation of a number of cell types (see general introduction). Therefore, in this chapter a role for this receptor in regulating cartilage formation was tested using an in-vitro model of cartilage formation using early limb cells (Micromass culture), and by injecting a cP2Y₁ antagonist into the early limb.

An Outline of Vertebrate Limb Development.

- Anatomy of the Developing Limb.

The vertebrate limb is derived from lateral plate and somitic mesoderm (Chevallier et al., 1977; Christ et al., 1977). Through localised proliferation of the flank, specific regions of the lateral plate form buds at presumptive limb levels (Searls and Janners, 1971). Cells that will form limb muscle then migrate into the limb from the lateral edges of the adjacent somites. Limb nerves and vasculature also have their origins in extra-limb regions, while cartilage and tendons are derived from lateral plate mesoderm. The early limb bud consists of a core of loose mesenchymal cells encased in an ectodermal jacket. At the distal tip of the limb a specialised epithelial structure, the apical ectodermal ridge (AER) forms (Saunders, 1948). Cells directly under the AER, in what is known as the progress zone.
remain undifferentiated and proliferate rapidly. Soon after the AER forms, mesenchymal
cells in proximal limb regions condense to from cartilage elements, that prefigure the limb
skeleton (Hall and Miyake, 1992). The cartilage elements are laid down in a proximo-
distal sequence in the chick, so that the humerus forms first, then the radius and ulna,
then the carpals (wrist), and finally the digits (Fig 4.1)

- **Pattern Formation in the Developing Limb.**

Pattern formation is the process by which the well ordered, three dimensional form of
organisms is achieved. General features of animal body plans are initially laid out in broad
strokes. For example cell fates are specified along the rostro-caudal axis. Further
refinement of these broad plans then occurs leading to the formation of semi-autonomous
regions called secondary fields, an example of which is the developing limb. Pattern
formation in these secondary fields can be considered to take place in four stages. First,
cells that make up the secondary field are defined; second, signalling centres are set up
within this field that provide cells with positional information; third, cells record this
positional information in the form of specific gene expression, and finally cells
differentiate in response to additional cues according to their already encoded position.
The limb is patterned along its three axes; anterior to posterior (thumb to little finger),
dorsal to ventral (back of hand to palm), and proximal to distal (shoulder to finger). The
mechanisms of pattern formation along these three axes are outlined below.

- **Dorso-Ventral Patterning.**

Reversal of the limb ectoderm 180° about its dorso-ventral (D/V) axis results in inversion
of D/V polarity of distal limb mesoderm (MacCabe et al., 1974). Ectodermal signals must
therefore specify cell fate along the D/V axis of the limb. Figure 4.2 shows three genes
that are differentially expressed along this axis. The transcription factor *Engrailed-1 (En-
I)* is expressed in ventral ectoderm, the secreted factor *Wnt-7a* is expressed in dorsal
ectoderm, and the LIM-homeodomain protein *Lmx-1* is expressed in dorsal mesoderm
(Dealy et al., 1993; Parr et al., 1993; Riddle et al., 1995; Vogel et al., 1995). Ectopic
Figure 4.1
(Taken from Wolpert, 1998). The progress zone underlies the AER at the distal tip of the limb bud. Cells in the progress zone proliferate and acquire positional value. Positional identity along the anterior/posterior axis is specified by the polarizing region (ZPA). When cells leave the progress zone they begin to differentiate into cartilage, resulting in cartilage elements being laid down in a proximo-distal sequence.

Figure 4.2
(Taken from Wolpert, 1998). The ectoderm controls dorso-ventral patterning of the developing limb. The diagram above shows the differential expression across the dorso-ventral axis, of Wnt-7a, engrailed and Lmx-1.
expression of either Lmx-1 or Wnt-7a can dorsalize ventral mesoderm in the distal limb bud (Riddle et al., 1995; Vogel et al., 1995). Loss of function En-1 mutants also leads to similar dorsalisation (Loomis et al., 1996). Loss of Wnt-7a leads to ventralisation of the distal dorsal limb regions (Parr and McMahon, 1995). These findings have been incorporated into a model in which Wnt-7a represents a dorsal signal that results in Lmx-1 expression in dorsal mesenchyme. En-1 acts as a ventral regulator by repressing expression of Wnt-7a in ventral ectoderm. Together En-1 and Wnt-7a ensure that Lmx-1 is confined to dorsal mesenchyme, where it directs dorsal patterning. Lmx-1 is a transcription factor, and should therefore regulate the expression of other genes which themselves may specify dorsal fates. It may be that ventral pattern is the default state, and Lmx-1 modifies this pathway.

- **Anterior-Posterior Patterning.**

Heterotopic grafting of small blocks of posterior mesenchyme into ectopic anterior locations results in whole mirror-image duplications of the distal limb (Saunders and Gasseling, 1968). If the graft was of quail origin, it was clear that the duplicated structures were not of quail origin, instead the graft induced the surrounding host tissue to proliferate, change its fate and become a mirror-image of the posterior limb. The grafted region became known as the Zone of Polarising Activity (ZPA), or polarising region because of its ability to reorganize the anterior-posterior (A/P) order of structures in the limb. According to the model of ZPA function (Wolpert, 1969), ZPA cells secrete a morphogen which sets the identity of cells along the A/P axis. Cells near the ZPA would receive a high concentration of the morphogen and so, adopt posterior fates, while cells further away would see a lower concentration of morphogen and so adopt anterior fates. Several lines of evidence support this model. Firstly, the number and morphology of ectopic digits formed is a function of the number of ZPA cells transplanted (Tickle, 1981), and secondly when the endogenous ZPA signal is attenuated by reducing the number of ZPA cells, the number of endogenous digits is reduced (Smith et al., 1978). The model of ZPA function is depicted in figure 4.3. The molecule responsible for the
Figure 4.3
The zone of polarizing (ZPA) activity can specify pattern along the anterior-posterior axis. If the ZPA is a source of a graded morphogen, the digits could be specified by different concentration thresholds of that morphogen (left panel). Grafting an additional polarizing region to the anterior margin of a host limb (center panels) would result in a mirror image gradient, hence a mirror image duplication of digits. The polarizing signal can be attenuated by grafting only a small number of cells so that only an extra digit 2 develops. (Right panel). (Taken from Wolpert, 1998)
morphogenetic properties of the ZPA is Sonic hedgehog (Shh) (Riddle et al., 1993). Shh expression colocalises with ZPA activity in the chick limb bud, and mirror image duplications, identical to those seen with ZPA grafts can be obtained by implanting beads loaded with recombinant Shh protein into the anterior limb (Riddle et al., 1993). ZPA deletion experiments have shown that if the entire ZPA is deleted in an early limb bud, pattern is truncated along the A/P and proximo-distal (P/D) axes (Pagan et al., 1996), demonstrating that Shh is essential for polarising activity, and polarising activity is essential for A/P and P/D patterning of the limb.

- **The Progress Zone Model of Proximal-Distal Patterning.**

Once limb buds form, their continued growth depends on the AER. If the AER is removed, outgrowth is affected, resulting in truncated limbs (Saunders, 1948; Summerbell, 1974). The level of truncation depends on when the AER is removed. Early removals lead to proximal truncations, later removals lead to truncation of the distal elements (Fig 4.4). According to the progress zone model (Summerbell et al., 1973), cell fate along the P/D axis is specified by the time spent in the progress zone, a region of distal mesenchyme underlying the AER. Cells that exit the progress zone early adopt proximal fates, while cells that remain in the progress zone longer adopt progressively more distal fates. How these fates might be specified and recorded remains unknown. What is known however is that a single factor can substitute for the patterning activity of the AER. Application of FGF soaked beads to limb buds that have had the AER removed restores both outgrowth and patterning (Niswander et al., 1993; Fallon et al., 1994). The AER expresses FGF-8 and FGF-2 along its entire length, and FGF-4 in its posterior two thirds (Niswander and Martin, 1992; Savage et al., 1993; Crossley and Martin, 1995), and any one of these can substitute for the AER in maintaining distal outgrowth (Niswander et al., 1993; Fallon et al., 1994; Crossley et al., 1996).
Figure 4.4
The apical ectodermal ridge (AER) is required for proximo-distal development. Limbs develop in a proximo-distal sequence. Removal of the AER from an early limb bud results in proximal and distal truncations. Removal of the AER at later stages of limb development result in distal truncations only. (Taken from Wolpert, 1998).

Figure 4.5
Pattern of Hox gene expression in the chick wing bud. Hoxa genes are expressed in a nested pattern along the proximo-distal axis (top panel). The Hoxd genes are expressed in a nested pattern along the antero-posterior axis. (Taken from Wolpert, 1998)
• **Patterning Along the Three Axes is Integrated.**

When cells exit the progress zone they carry with them positional information along all three axes of the limb. Manipulations of axial development (ZPA grafts, ectoderm reversal and AER removals) gives different results depending on the age of the host limb mesenchyme (MacCabe et al., 1974; Summerbell and Lewis, 1975). Manipulations carried out early affect both the proximal and distal fates, while manipulations carried out later affect distal structures only. These experiments demonstrate that only progress zone cells are plastic with respect to their positional identity along all three axes, and that patterning along the three limb axes is temporally and spatially coordinated. A positive feedback loop between mesenchyme and ectoderm signalling coordinates patterning and outgrowth. FGF has been shown to maintain $Shh$ expression in posterior mesenchyme, and $Shh$ expressing cells can activate $Fgf-4$ expression in the AER (Laufer et al., 1994; Niswander et al., 1994). Bone morphogenetic proteins $Bmp-2$ and $Bmp-7$, and the $HoxD$ cluster genes are also expressed in posterior chick wing mesenchyme in response to Shh and FGF-4 signalling (Laufer et al., 1994; Niswander et al., 1994). Dorsalising and polarising signals are also interdependent. In $Wnt-7a$ mutants (Parr and McMahon, 1995), the limbs are shorter and lack the most posterior skeletal elements. This can be explained by the fact that $Shh$ expression is reduced in these mutants, and so the posterior digit is not specified. At the same time $FGF-4$ expression in the posterior AER is reduced. The combined reduction of $Shh$ and $FGF-4$ expression leads to posterior and distal truncations. Dorsal ectoderm is also required for maintenance of $Shh$ expression. Removal of $Wnt-7a$ expressing dorsal ectoderm also leads to reduction in the level of $Shh$ expression, an effect that can be rescued by grafting cells that express $Wnt-7a$. (Yang and Niswander, 1995).

• **Hox Genes Mediate the Instructions Encoded by the Early Patterning Signals.**

The early patterning signals provide cells in the limb with positional information. These signals must also act to change the properties of cells so, that predictable patterns of
growth and differentiation occur that give rise to a normal limb. A component of this process is the activation of key regulatory genes that mediate the instructions encoded by early patterning signals. An important group of these regulatory genes are the clustered *Hox*. The 5' members of the *HoxD* cluster, *Hoxd-9 - Hoxd-13* are initially expressed in a nested pattern around the posterior limb bud (Dolle et al., 1989) (Fig 4.5). These genes can be activated under the combined influence of Shh and FGFs, suggesting that these *Hox* genes may be a readout of positional signalling in the limb. However, the initiation of their expression may involve other factors besides FGFs and Shh since some of these genes are expressed prior to that of Shh or AER formation (Nelson et al., 1996). Later on *HoxD* expression patterns are dynamic and go through several distinct phases (Duboule, 1994; Nelson et al., 1996). The early nested pattern of *HoxD* genes is in the future forearm/lower leg. At later stages, the same genes are expressed in the presumptive hand/foot. The expression patterns of the *Hoxa 9 -Hoxa-13* genes are also dynamic and define domains along the P/D axis (Yokouchi et al., 1991) (Fig 4.5). The phenomenon of posterior prevalence (Duboule, 1994) dictates that when more than one *Hox* gene is coexpressed in the same cell the more 5' genes of the *Hox* cluster will exert a dominant effect. The dynamic patterns of *Hox* gene expression during limb development therefore lead to different *Hox* genes exerting dominant roles in different parts of the limb at different stages of development. Since different *Hox* genes are thought to have different effects on proliferation and differentiation their dynamic expression patterns lead to differential growth of the limb elements. Analysis of *Hox* gene gain or loss of function mutants has indicated that the primary role of *Hox* genes is to regulate the rate and timing of cartilage proliferation and differentiation (Dolle et al., 1993; Davis et al., 1995; Duboule, 1995; Goff and Tabin, 1997). *Hox* genes can also influence proliferation of undifferentiated mesenchyme, condensation of mesenchyme and organisation of cartilage cells within the skeletal elements (Davis et al, 1995; Duboule, 1995; Yokouchi et al., 1995; Goff and Tabin, 1997). The target genes regulated by *Hox* gene expression are unknown, but a number of local signals, including members of the TGFβ, FGF, and Hedgehog families which influence growth and patterning of individual elements are
beginning to be identified. It is likely that Hox genes regulate either the production or interpretation of these signals. Bone growth, and the role of locally acting signals in regulating the different stages of bone development is discussed below.

- Development of the Limb Skeleton.

A developmental time course of limb skeleton formation is shown in figure 4.6. At stage 24-25 (Fig. 4.6 A) the humerus and ulna become visible in the developing limb almost simultaneously. The radius at this stage is less apparent. By stage 26 posterior wrist parts begin to take up the alcian green stain. The humerus, radius and ulna are well demarcated by stage 27, and the digits also become visible (Fig 4.6 B). By stage 29 the hand plate is still better formed, and by stage 30 (Fig 4.6 C) only the terminal elements of digits 3 and 2 have yet to form. The former appears by stage 32 and the latter by stage 34. The onset of osteogenesis begins at stage 36 (Fig 4.6 E). (Summerbell, 1976).

Bone develops through two different processes. Intramembranous and endochondral bone formation. Intramembranous bones form directly from an initial mesenchyme condensation. The first morphological sign of endochondral bone formation is also the formation of a mesenchymal condensation, but the two processes of bone formation differ in that there is an obvious cartilage intermediate in endochondral but not intramembranous bone formation (for review see Erlebacher et al., 1995). The majority of the bone in the limbs develop through the endochondral mechanism (Cancedda et al., 1995). The initial mesenchymal condensation that marks the initiation of limb skeletogenesis forms through an increase in cell adhesion and/or proliferation. As the cells within this condensation begin to differentiate they begin to express Type II Collagen (Col-II) (Hyun-Duck et al., 1988). Around each cartilage condensation, a thin layer of cells forms and differentiates into the perichondrium, which inhibits chondrocyte differentiation and maturation and so helps to control growth and differentiation of the cartilage elements (Vortkamp et al., 1996; Long and Linsenmayer, 1998). As the cartilage elements grow, different zones that demarcate the progressive differentiation of the chondrocytes can be distinguished. Cells at the ends of the elements (the epiphyseal zone) are immature and undergo rapid
Figure 4.6
Time course of development of the cartilaginous limb skeleton. Developmental stage is indicated in the bottom right hand corner. Cartilage is visualised by alcian green staining. See text for explanation. h, humerus; r, radius; u, ulna; w, wrist. Digits are numbered 2, 3 and 4. Scale bars equal 1mm.
proliferation. Adjacent to the proliferative zone are the more sparsely packed prehypertrophic chondrocytes. These constitute the metaphyseal zone. Terminally differentiated, Type X Collagen (Col-X) expressing, enlarged, hypertrophic chondrocytes occupy the center of the cartilage elements, in what is known as the diaphyseal zone (Linsenmayer et al., 1991). These cells eventually undergo programmed cell death and are replaced by osteoblasts, which mature into osteocytes that secrete bone matrix, forming the mature skeleton. Chondrocyte differentiation and ossification occur in a precise sequence and pattern. A wave of chondrocyte differentiation, proliferation, hypertrophy, and ossification initiates in the center of the element and moves towards the ends. This sequence is well illustrated in the epiphyseal, or growth plate, where it provides directional growth to increase the length of the cartilage element. Cartilage elements also grow by appositional chondrogenesis. During this process, the perichondrium surrounding the cartilage element divides and differentiates, resulting in an increase in the element's width (Burkitt et al., 1993). Morphogenesis of the limb skeleton is therefore a multistage process that starts with the formation of mesenchymal condensation that forms through increased cell adhesion and proliferation. The differentiation of these cells from the proliferative to the prehypertrophic and finally the hypertrophic state must then be tightly regulated to ensure the correct formation of a cartilaginous intermediate. Matrix secretion by, and programmed cell death of the hypertrophic chondrocytes must then occur to allow invasion of blood vessels and osteoblasts. Finally, osteoblasts mature into bone forming osteocytes. Regulation of the rate of endochondral ossification is critical to bone morphogenesis, because it plays a major role in determining the shape and length of the skeletal elements.

- **Molecular Mechanisms Operate at Multiple Stages to Regulate Endochondral Ossification**

Only recently have the molecular mechanisms that regulate each stage of endochondral ossification begun to be elucidated. Mice with loss of function mutations in two members of the TGF-β superfamily, BMP-5 (Bone morphogenetic protein-5) (the short ear, se,
mutant) and GDF-5 (growth and differentiation factor 5) (the brachypodism, bp, mutant) have skeletal elements with abnormal sizes and shapes, and these can be attributed to defects in size and shape already apparent in the mesenchymal condensations of the affected skeletal elements (Kingsley et al., 1992; Storm et al., 1994). Misexpression studies suggest that GDF-5 acts at these initial stages of chondrogenesis by promoting cell adhesion (Francis-West et al., 1999). The condensation also expresses BMP receptor 1B (BMPR-1B), and mutations in this receptor also disrupt the early steps of mesenchymal condensation (Zou et al., 1997).

Indian hedgehog (Ihh), Parathyroid hormone-related peptide, and BMP signalling are incorporated into a model to negatively regulate the number of cells committing to a prehypertrophic state. Ihh is a member of the hedgehog family of secreted proteins that is expressed in prehypertrophic chondrocytes. The primary target genes of Ihh, Ptc and Gli are expressed in the perichondrium bordering the zone of Ihh expression (Tabin and McMahon, 1997; Vortkamp et al., 1996). Members of the TGF-β can act as secondary signals downstream of hedgehog signalling (Bitgood and McMahon, 1995; Roberts et al., 1995). Consistent with this, the BMP ligands Bmp2, Bmp4, Bmp5, and Bmp7 are expressed in the perichondrium surrounding the cartilage elements (Jones et al., 1991; Kingsley, 1994; Lyons et al., 1989, 1990), and Bmp6 is expressed in the hypertrophic and prehypertrophic chondrocytes themselves (Kingsley, 1994; Vortkamp et al., 1996). Bmp receptor-IA (BMPRIA) is expressed throughout the limb mesenchyme at early stages and is later found in prehypertrophic chondrocytes (Zou et al., 1997). BMPRIA is also expressed in the perichondrium at the cartilage ends (the periarticular region) (Vortkamp et al., 1996; Zou et al., 1997). Parathyroid hormone/Parathyroid hormone-related peptide receptor (PTH/PTHrP receptor) is expressed in the prehypertrophic chondrocytes, prior to the onset of Ihh expression in these cells (Vortkamp et al., 1996). The ligand for this receptor, parathyroid hormone-related peptide (PTHrP) is expressed in the periarticular region.

Misexpression of Ihh or constitutively active BMPRIA, genes normally expressed in the prehypertrophic chondrocytes, leads to a delay in maturation of the proliferating
PERIARTICULAR REGION

PROLIFERATING CELLS IN EPIPHYSEAL ZONE

PREHYPERTROPHIC CHONDROCYTES IN METAPHYSEAL ZONE

HYPERTROPHIC CHONDROCYTES IN DIAPHYSEAL ZONE

PERICHONDRIUM

Figure 4.7. Molecular regulation of chondrocyte differentiation. Schematic representation of zones of cartilage and corresponding molecular markers. See text for explanation (Adapted from Vortkamp et al., 1996; Zou et al., 1997).
chondrocytes to the prehypertrophic state (Vortkamp et al., 1996; Zou et al., 1997). Misexpression of Parathyroid hormone related peptide (PTHrP) or constitutively active PTH/PTHrP receptor, normally expressed in the periarticular region and in the prehypertrophic cells respectively, also leads to a similar chondrocyte delay (Lee et al., 1996; Weir et al., 1996; Schipani et al., 1997). Conversely, mice mutant for PTHrP or PTH/PTHrp receptor show the opposite phenotype in that chondrocyte differentiation and ossification are accelerated (Amizuka et al., 1994; Karaplis et al., 1994; Lanske et al., 1996). These studies prompted a model in which IHH signalling through PTC leads to production of BMPs in the perichondrium. These BMPs activate BMPRIA in the prehypertrophic chondrocytes and block their progression to the hypertrophic state. BMPRIA is also activated in the periarticular region (Zou et al., 1997). This results in periarticular expression of PTHrP and activation of the PTH/PTHrP receptor in prehypertrophic chondrocytes. (Lanske et al., 1996; Vortkamp et al., 1996; Zou et al., 1997). Activation of PTH/PTHrP receptor prevents expression of Ihh in prehypertrophic cells. As prehypertrophic cells commit to the hypertrophic state and loose expression of Ihh, the Ihh signal is temporarily attenuated, shutting down the signalling cascade thus allowing more cells to commit to the prehypertrophic fate. This feedback mechanism serves to negatively regulate the number of cells committing to a prehypertrophic fate. This model is depicted in figure 4.7.

Finally a recent study has implicated the Delta/Notch signalling system in negatively regulating the final stage of terminal chondrocyte differentiation. Delta-1, is expressed in hypertrophic chondrocytes, and misexpression of Dl-1, blocks the progression from the prehypertrophic to the terminally differentiated hypertrophic state (Crowe et al., 1999).

From this introduction it can be seen that remarkable progress has been made, in a very short space of time, in unravelling the molecular mechanisms by which the early limb is patterned, and differentiation and growth of cells in the limb are controlled. It was therefore very exciting to find that ATP, and one of its receptors may have a role to play
in development of the vertebrate limb. In the remainder of this chapter evidence is provided for a role for cP2Y₁ in regulating differentiation during limb development.
Methods

• **Micromass Cultures and Transfections.**

Limb mesenchyme cultures were set up as described in chapter 3, with the following modifications. Cells were plated in 80μl spots in 4 well tissue culture plates, and transfection was carried out using the transfection reagent Fugene 6 as described in chapter 3. ATP (Boehringer) was added to cultures at a final concentration of 100μM after the 8 hour transfection period, and every 24 hours thereafter. Reactive Blue-2 (Sigma) was prepared in distilled water and filter sterilised before adding to cultures at a final concentration of 100μM immediately after transfection.

• **Alcian Blue Staining of Micromass Cultures and GAG Assay.**

Cartilage was visualised in micromass cultures by staining with Alcian blue, and production of sulphated glycosaminoglycans (GAGs) in micromass cultures was determined by extracting the bound dye, and measuring the optical density of the resulting solution. Briefly, cultures were fixed in 4% paraformaldehyde in PBS, washed in PBS, and stained overnight with 1% Alcian blue in 0.1M HCl. Cultures were then washed in running tap water and unbound Alcian blue was extracted by exhaustive washing in 1% acetic acid in 70% ethanol. Bound Alcian blue was then extracted with 1ml 4M guanidine hydrochloride in 33% isopropanol overnight. The optical density of the supernatant was read on a spectrophotometer at A630nm.

• **ATP Assay.**

Limb mesenchyme cultures were set up as described in chapter 3, with the following modifications. For the ATP breakdown assay 1.2 x 10^7 cells were plated in 400μl spots in 6 well tissue culture plates. Cells were left to settle for 1 hour before flooding with 2ml of medium (DMEM+10% FBS+1% antibiotic/antimicotic). Cultures were left for 3 days before washing in tissue culture grade PBS (Sigma). Cells were then incubated in 2ml of fresh PBS for 10 minutes before addition of ATP to a final concentration of 10μM. Samples of 60μl were taken and frozen 2, 5, 10, 30, 60, 120 and 240 minutes after the
initial addition of ATP. Control experiments to assay ATP breakdown in the absence of cells were run in parallel. For these, ATP at a final concentration of 10μM was added to 2ml of PBS in tissue culture plates, and incubated under identical conditions to the above cultures. Sixty μl samples were again taken at the same time intervals. To measure ATP production by cells in micromass cultures, cultures were set up in 4 well plates as described above. Cultures were also left for 3 days before washing in PBS, and then incubated for 4 hours. Samples of 100μl were removed and spun at 2000rpm for 10 minutes to pellet any cells that may contaminate the samples. Samples of supernatant (60μl) were carefully removed and assayed for ATP content. The concentration of ATP in each sample was measured using an ATP-monitoring reagent (Luciferin-luciferase) (BioOrbit), and a LUCY1 luminometer (Anthos Labtech).

- **Reactive Blue 2 Injections.**

Eggs were incubated on their sides for two days at 37°C. The blunt end of the egg was pierced with a needle, and 1ml of albumin was sucked out using a needle and syringe. A window was then cut in the top of the egg, which was sealed with scotch tape. The eggs were then left to incubate for a further day. A 1mM concentration of Reactive blue-2, prepared in distilled water and sterile filtered was then pressure injected into stage 20-22 limb buds using a 15msec pulse at 40psi. The eggs were then resealed and returned to the incubator for a further 6 days. Control embryos either had distilled water injected into limb buds or Reactive blue-2 injected into the three somites immediately posterior to the posterior wing bud. Somite injections were performed at stages 20-22 and 23-24.

- **Wholemount Cartilage Staining.**

At embryonic day 9 injected embryos were removed their eggs and dissected free of extra-embryonic membranes. Embryos were then fixed in 5% trichloroacetic acid overnight at room temperature. They were then stained in 0.1% Alcian green in acid alcohol (70% ethanol, 1% HCl) overnight. Embryos were then washed all day in several changes of acid alcohol, and finally washed in absolute ethanol for a minimum of three hours.
embryos were cleared and stored in methyl salicylate. The length of skeletal elements were measured using an eyepiece graticule on a dissecting microscope.

Nile Blue Sulphate Staining.
Embryos were dissected free of extra-embryonic membranes in PBS, before incubating in 3μg/ml Nile blue sulphate in PBS, for 20 minutes at room temperature. Embryos were then washed in PBS, and examined and photographed immediately afterwards.
Results

- Transfection of Limb Micromass Cultures with \( \text{cP2Y}_1 \) Reduces the Amount of Cartilage Formed.

Micromass cultures are a standard in-vitro model for studying chondrogenesis, and have been used to study the mechanisms of action of a number of TGF-\( \beta \) family members for example (Jiang et al., 1993; Roark and Greer, 1994; Francis-West et al., 1999). In micromass cultures mesenchymal cells from early limb buds are plated at high density. Within 1-2 days cellular aggregates form, and by day 3 these aggregates have differentiated into cartilage (Ahrens et al., 1977). All this occurs in the absence of \( \text{cP2Y}_1 \) expression, which as chapter 3 of this thesis demonstrated, disappears from limb mesenchyme cells within 24 hours of culturing. A gain-of-function approach was therefore used to investigate possible roles for \( \text{cP2Y}_1 \) in regulating chondrogenesis.

Micromass cultures were transfected with either empty vector (pRCCMV), or vector carrying the \( \text{cP2Y}_1 \) cDNA (pRCCMV-cP2Y) prior to the onset of chondrogenesis. Figure 4.8 shows control and \( \text{cP2Y}_1 \) transfected micromass cultures fixed and stained with Alcian Blue 2, 4 and 6 days after transfection. In 6 day cultures the amount of cartilage formed in control transfected and \( \text{cP2Y}_1 \) transfected cultures was compared by measuring sulphated glycosaminoglycan (GAG) content by extracting the bound Alcian blue and assaying spectrophotometrically. In control cultures the average GAG content (A630nm) was 0.33 ± 0.04 (n=12), while in \( \text{cP2Y}_1 \) transfected cultures the mean A630nm was 0.18 ± 0.04 (n=12), a reduction of 55% compared to controls (P<0.01; Student’s t-test) (Fig 4.9). Daily addition of the \( \text{cP2Y}_1 \) ligand, ATP to a final concentration of 100\( \mu \)M to pRCCMV-cP2Y transfected cultures did not significantly increase this reduction in cartilage formation (Mean A630nm of control transfected cultures + ATP=0.36 ± 0.05, n=10, A630nm of \( \text{cP2Y}_1 \) transfected cultures + ATP = 0.18 ± 0.05, n=10) (Fig 4.9).
Figure 4.8.
Expression of cP2Y1 reduces the amount of cartilage formed in limb micromass cultures. Cartilage nodules have been stained with Alcian Blue. In row A, the micromass cultures have been transfected with pRCCMV-cP2Y1, and fixed 2, 4, and 6 days after transfection. In row B cultures have been transfected with the control construct (empty pRCCMV), and fixed 2, 4, and 6 days after transfection. Scale bar equals 1mm.
• The Effect of cP2Y, Expression on Cartilage Formation in Micromass Cultures can be Blocked by the P2Y Receptor Antagonist Reactive Blue-2.

Addition of Reactive blue-2, a cP2Y, receptor antagonist (Webb et al., 1993; see also chapter 3 of this thesis) to the culture medium at a final concentration of 100μM, blocked the effect of cP2Y, expression on cartilage formation (Fig 4.9). In control cultures treated with Reactive blue-2 the mean A630nm=0.36 ± 0.06, n=11. In cP2Y, expressing cultures treated with Reactive blue-2 the mean A630nm=0.33 ± 0.07, n=12, whereas the mean A630nm of cP2Y, transfected cultures which were not treated with Reactive blue 2 was 0.18 ± 0.04 (n=12). Statistical analysis showed that there is no significant difference in the amount of cartilage formed between cP2Y, transfected cultures treated with Reactive blue-2, and control cultures treated with Reactive blue-2 (P>0.1; Student’s t-test). There was also no significant difference in the amount of cartilage formed in untreated control cultures (mean A630nm=0.33 ± 0.04 n=12), and control cultures treated with Reactive Blue-2 (mean A630nm=0.36 ± 0.06, n=11), (P>0.1; student’s t-test), indicating that Reactive blue-2 itself does not promote cartilage formation.

• cP2Y, Acts at the Initial Stages of Chondrogenesis.

To determine the effect of cP2Y, expression on the initiation of chondrogenesis, the number of cartilage nodules formed two days after transfection was compared in control and cP2Y, transfected cultures (Fig 4.10). In control cultures the mean number of nodules formed was 243 ± 75 (n=9), while the mean number of cartilage nodules in cP2Y, transfected cultures was 20 ± 8 (n=9). This represents a 12 fold reduction in the number of cartilage nodules initially formed in cP2Y, transfected cultures (P<0.01; Student’s t-test). These results suggest that cP2Y, may prevent the formation of the cellular aggregates that mark the initiation of cartilage formation in micromass cultures, and this effect can be blocked by Reactive blue-2, a P2Y receptor antagonist.
Figure 4.9. GAGs are the principal matrix component of the skeletal elements. GAG content of 6 day micromass cultures are shown on the y axis, and experimental conditions are shown on the x axis. GAG content of control (pRCCMV transfected) cultures are shown in blue, and GAG content of pRCCMV-cP2Y1 transfected cultures are shown in purple. Cartilage content in pRCCMV- cP2Y1 transfected cultures is reduced to 50% of control cultures. Addition of ATP does not significantly alter this effect, but addition of the P2Y receptor antagonist Reactive Blue-2, blocks the effect of cP2Y1 expression on cartilage formation without affecting the level of cartilage formed in control cultures. Error bars show ±s.e.m.

Figure 4.10. The number of cartilage nodules formed in micromass cultures two days after transfection is drastically reduced in pRCCMV-cP2Y1 transfected cultures compared to control transfected cultures. Errors bars show ±s.e.m.
• ATP is Broken Down in Limb Micromass Cultures.

The addition of the cP2Y, ligand, ATP, to pRCCMV-cP2Y, transfected cultures did not further reduce the amount of cartilage formed in these cultures (Fig 4.9). This suggests that enough ATP is released from cells in micromass cultures to elicit a maximal response in cP2Y, expressing cells, and therefore addition of ATP will not make an appreciable difference. Alternatively enough ATP is released from cells to activate cP2Y, and reduce the amount of cartilage formed, but added ATP is broken down too quickly to further reduce cartilage formation. In order to test this, the rate of ATP breakdown in micromass cultures was assayed using a Luciferin-luciferase assay. Figure 4.11 shows that added ATP is broken down in micromass cultures, with a half life of approximately 60 minutes. This breakdown of ATP by cells in these cultures would also mean that production of ATP by the cells themselves would be difficult to detect. However figure 4.12 shows that after a 4 hour incubation of cells in fresh PBS the concentration of ATP is 0.83nM ± 0.05 (n=4), while the concentration of ATP in PBS alone is 0.5nM ± 0.1 (n=4). Therefore during a short incubation time, and despite the breakdown of ATP there is a very small but significant (P<0.05; student’s t-test) increase in the concentration of cellular derived ATP in the culture medium. These results suggest that in micromass cultures mechanisms are in place to break down extracellular ATP. This in itself suggests that extracellular ATP is present in these cultures, and the detection of small quantities of cellular derived ATP confirmed this. Therefore enough ATP may be produced by cells in micromass cultures to activate transgenic cP2Y, and reduce cartilage formation.

• Injection of Reactive blue-2 into Stage 20-22 Wing Buds Results in Shortening of the Skeletal Elements.

In order to assay the effect of blocking cP2Y, function in-vivo, Reactive blue-2 was injected into stage 20-22 limb buds, a stage when the largest percentage of cells seemed to express this receptor (see chapter 2). Reactive blue-2 was clearly visible in the injected tissue immediately after injection. However within 4 hours all of the blue colour in the injected tissue had disappeared (data not shown). Reactive blue-2, at a concentration of
Figure 4.11. ATP is broken down in limb micromass cultures. ATP (10μM) was added to micromass cultures and the concentration of ATP in samples taken at the time points shown on the x axis is shown by the purple bars. The blue bars show the concentration of ATP in control cultures (in the absence of cells). The half life of ATP in limb micromass cultures is approximately 1 hour, while in the absence of cells only 10% of the ATP was broken down over the 4 hour assay period. For each time point n=2 and error bars show ± s.e.m.

Figure 4.12. ATP is produced by cells in limb micromass cultures. The purple bar shows the concentration of ATP in a samples of PBS that has been incubated with cells for 4 hours (n=4). The blue bars show the baseline luminometer reading of control samples of PBS that have not been incubated with cells (n=4). Error bars show ±s.e.m.
100μM was effective at blocking the effect of cP2Y1 on cartilage formation in micromass cultures. However, injection of 100μM Reactive blue-2 into the limb had no effect. Given that Reactive blue-2 is a competitive antagonist of P2Y receptors, it is therefore likely that its injection into limb buds will only block cP2Y1 function for as long as Reactive blue-2 concentration remains high enough. A relatively high concentration of Reactive blue-2 (1mM) was therefore injected. Injected embryos were left until embryonic day 9 before fixing and wholemount staining of the cartilaginous skeleton with Alcian green. Figure 4.13 shows that injected limbs were considerably smaller than their contralateral controls. Particularly striking in injected limbs was the shortening of the skeletal elements. Figure 4.13 B shows that in some injected limbs radial growth of some of the limb cartilage elements was also reduced. Patterning of the limb skeleton was unaffected by injection of Reactive blue-2. In order to quantify the degree of shortening of the skeletal elements, the humerus, radius and ulna of injected limbs and their contralateral controls were measured. Figure 4.14 shows that on average in injected limbs the humerus is 15% shorter (P<0.001; student’s t-test), the ulna is 23% shorter (P<0.001; student’s t-test), and the radius is 21% shorter (P<0.001; student’s t-test), than the control limbs (n=15). The digits in injected limbs were also visibly shorter than control limbs, but these were not measured. These data are summarised in table 4.1. Control injections of distilled water into limb buds at the same stage did not disrupt limb development, as determined by measuring the length of the skeletal elements in control and injected limbs (n=4) (see table 4.2).

Reactive blue-2 has been used extensively as an affinity reagent for the purification and labelling of multiple enzymes that bind and/or use nucleotides as substrates. Therefore, it is possible that Reactive blue-2 disrupts development of the limb skeleton through mechanisms other than cP2Y1 antagonism. Reactive blue-2 was therefore injected into another cartilaginous bone forming region which does not express cP2Y1. Vertebral and sternal ribs are derived from the somites (Chevallier, 1975). Extirpation experiments have shown that somites 21-23 (which are immediately posterior to the wing bud) give rise to the most rostral vertebral and sternal ribs (Kato and Aoyama, 1998). Reactive blue-2 was
Figure 4.13.
Injection of Reactive blue-2 into stage 20-22 wing buds results in shortening of the limb skeletal elements. Figures A and B show embryonic day nine wings (injected and contralateral control) stained with Alcian green to show the cartilaginous skeleton. Figure B shows that in some injected limbs, radial growth is inhibited (arrowed).
Figure 4.14. Quantifying the degree of shortening of the humerus, ulna and radius as a result of injecting Reactive Blue-2 into stage 20-22 wing buds. The length of each element in un-injected contralateral controls is shown in the blue bars, and the length of elements in injected limbs are shown in purple. On average, in injected limbs the humerus is 85%, the ulna is 77% and the radius is 79% of the length of their respective controls. n=15, error bars show ±s.e.m.
### Table 4.1. Summary of data obtained from Reactive blue-2 injections into stage 20-22 wing buds.

<table>
<thead>
<tr>
<th>EMBRYOS INJECTED</th>
<th>EMBRYOS SURVIVING TO E9</th>
<th>MEAN LENGTH OF LIMB SKELETAL ELEMENTS (mm)</th>
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<td></td>
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<td>CONTROL</td>
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### Table 4.2. Control injections of water into stage 20-22 limb buds.

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<th>EMBRYOS SURVIVING TO E9</th>
<th>MEAN LENGTH OF LIMB SKELETAL ELEMENTS (mm)</th>
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<td></td>
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<td>CONTROL</td>
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### Table 4.3. Reactive blue-2 injections into somites 21-23 of stage 20-22 embryos does not affect rib morphogenesis.

<table>
<thead>
<tr>
<th>EMBRYOS INJECTED</th>
<th>EMBRYOS SURVIVING TO E9</th>
<th>MEAN LENGTH OF RIB (mm)</th>
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<td>7th vertebral</td>
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<td>7th sternal</td>
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</tbody>
</table>

### Table 4.4. Reactive blue-2 injections into somites 21-23 of stage 23-24 embryos does not affect rib morphogenesis.

<table>
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<tr>
<th>EMBRYOS INJECTED</th>
<th>EMBRYOS SURVIVING TO E9</th>
<th>MEAN LENGTH OF RIB (mm)</th>
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injected into right hand somites 21-23 at stages 20-22, the same stage embryos that received injections to the wing buds. However, the ribs become cartilaginous at about day embryonic 7-7.5, approximately a day later than wing bud chondrogenesis (Bellairs and Osmond, 1998). Injections into the same somites were therefore also performed a day later, at stages 23-24 of development. Injected embryos were again fixed at day 9, and the cartilaginous skeleton visualised by wholemount Alcian green staining. There were no visible differences in rib morphology between injected and contralateral sides, and measurement of vertebral and sternal ribs showed that injection of Reactive blue-2 did not significantly (P>0.05; students t-test) affect length of any of the ribs on the injected side. These data are summarised in tables 4.3 and 4.4. These data suggest that: a) Reactive blue-2 does not remain in the tissue into which it is injected, but is washed out within a matter of hours, b) Reactive blue-2 injection into the early limb results in shortening of skeletal elements, but does not affect patterning, and c) Reactive blue-2 injections into rib forming somites does not affect rib formation, suggesting that Reactive blue-2 is not a general inhibitor of cartilage formation.

**Injection of Reactive blue-2 into stage 20-22 Limb Buds Causes Cell Death.**

Since transfection of limb mesenchyme cells with cP2Y₁ reduces the amount of cartilage formed in micromass cultures, one would predict that by blocking cP2Y₁ in-vivo, cartilage formation would be promoted. Instead, injection of a cP2Y₁ antagonist, Reactive blue-2 resulted in shortening of all the limb skeletal elements. This phenotype suggests that Reactive blue-2 is either blocking proliferation of cells, or causing cell death. Killing mesenchyme cells at the distal tip (the progress zone) of a stage 20-21 limb buds, has been shown to result in reduced proximal structures (Wolpert et al., 1979). In order to investigate the possibility that Reactive blue-2 is causing cell death, injected limbs were stained with Nile blue sulphate, 6 and 24 hours following injection. Nile blue sulphate is taken up by dead and dying cells, but is excluded from living ones. It was found that within 6 hours a significant number of dead cells could be detected throughout the
Figure 4.15.
Injection of Reactive-blue 2 causes cell death in the early limb bud. In the figure shown above, dead cells within the limb are intensely stained with Nile blue sulphate. Dead cells appear as dark blue spots. The diffuse blue colour is background staining. (A) A limb stained with Nile blue sulphate 6 hours following injection of Reactive blue 2. Dead cells are scattered throughout the mesenchyme. (B) The uninjected contralateral control limb shows little death in the mesenchyme. (C) In some injected limbs (shown on the right), an increase in cell death in the AER was observed (arrowed). The uninjected limb is shown on the left. Scale bar equals 100μM, and stage is indicated in the bottom right hand corner.
mesenchyme in Reactive blue-2 injected limbs (n=4) but not in uninjected contralateral control limbs, or in limbs injected with PBS (n=3) (Fig 4.15). Some Reactive blue-2 injected limbs also showed an increase in cell death in the apical ectodermal ridge (AER), a region which was not injected with Reactive blue-2 (Fig 4.15). At 24 hours following injection, the level of cell death in the mesenchyme of Reactive blue-2 injected limbs was less pronounced than that seen at 6 hours, but the level of death in the AER was increased compared to uninjected contralateral controls (n=4).

- **Prolonged Exposure of Cells in Micromass Cultures to Low Doses of Reactive blue-2 does not Cause Cell Death, but Brief Exposure to High Concentrations of Reactive blue-2 Increases the Level of Cell Death in Micromass Cultures.**

The observation that injection of 1mM Reactive blue-2 into limb buds increased the level of cell death, raised concerns that Reactive blue-2 may also cause cell death in micromass cultures. In order to investigate this possibility micromass cultures were treated for 24 hours with Reactive blue-2, at a final concentration of 100µM (n=4). (This was the concentration of Reactive blue-2 used to block the effect of cP2Y, expression on cartilage formation). Treated cultures, and control cultures which were not treated with Reactive blue-2 (n=4), were then stained with Nile blue sulphate to look at the level of cell death. Very few darkly stained cells were visible in both sets of cultures, indicating that the level of cell death was low. There was also no visible difference in the level of cell death between treated and untreated cultures. A possible reason for the increased cell death in the limb is that a higher concentration of Reactive blue-2 was injected into the limb (1mM), than was used in micromass cultures. However the concentration of Reactive blue-2 injected into the limb would be diluted immediately upon injection, and would be further reduced as Reactive blue-2 is washed out of the tissue. The possibility that a brief exposure to a high concentration of Reactive blue-2 is sufficient to cause cell death was explored by treating micromass cultures with Reactive blue-2 at a final concentration of 1mM for a period of one hour. Following this period, staining treated cultures (n=4) with
Nile blue sulphate revealed a clearly visible increase in the level of cell death compared to untreated control cultures (n=4). These results suggest that at a concentration of 100μM, Reactive blue-2 does not cause cell death in micromass cultures, but is sufficient to block the effect of cP2Y1 on cartilage formation. However, a brief exposure to higher doses of Reactive blue-2 does cause cell death in micromass cultures, and this may explain the increased cell death that results from injection of 1mM Reactive blue-2 into the limb bud.
Discussion

• Results Suggest that the Loss of Extracellular ATP Signalling via the cP2Y₁ Receptor is an Important Step in Cartilage Formation in Micromass Cultures.

The expression pattern of cP2Y₁ in the developing limb suggests that it may be involved in regulating development of cartilage. At later stages of limb development cP2Y₁ was strongly expressed surrounding the most recently differentiating regions of cartilage, but was clearly absent from the cartilage condensations themselves (see chapter 2, figure 2.3). A role for cP2Y₁ in regulating chondrogenesis was therefore tested using micromass cultures - a standard in-vitro model of cartilage formation. The fact that expression of cP2Y₁ was lost from cultured limb mesenchyme cells (see chapter 3), was exploited by using a gain-of-function approach to test a role for cP2Y₁ in regulating cartilage formation. Micromass cultures were transfected with an expression vector carrying the cP2Y₁ cDNA (pRCCMV-cP2Y₁). In chapter 3 of this thesis it was shown that within 24 hours functional receptor is detectable in approximately 60% of cells in transfected cultures, and remains detectable for at least 3 days. A reduction in the amount of cartilage formed in cP2Y₁ transfected cultures was clearly visible 2,4 and 6 days after transfection, which suggests that loss of cP2Y₁ expression in micromass cultures may be an important step in the process of chondrogenesis. At day 6 the difference in the amount of cartilage formed between cP2Y₁ and control transfected cultures was less dramatic than the difference seen at days 2 and 4. The most likely explanation for this is that the level of expression of transgenic cP2Y₁ reduced over time. Such a reduction could be due to vector degradation and dilution of vector copy number through cell division.

• Expression of cP2Y₁ Prevents Formation of Prechondrogenic Condensations in Micromass Cultures.

The process of endochondral bone formation is a multi-stage process, beginning with mesenchymal condensation and proceeding through tightly regulated stages of
differentiation from rapidly proliferating chondrocytes, prehypertrophic chondrocytes, post-mitotic, terminally differentiated hypertrophic chondrocytes, and finally ossification.

In order to shed light on the mechanism by which cP2Y₁ inhibits the formation of cartilage in micromass cultures, the initiation of chondrogenesis was examined by counting the number of cartilage nodules formed in early (2 days after transfection) micromass cultures. It was found that the number of cartilage nodules initially formed was drastically reduced in cP2Y₁-transfected cultures. Comparison of cP2Y₁ transfected and control cultures under phase contrast showed that the condensation of undifferentiated mesenchyme cells had failed to occur (data not shown). These data suggest that cP2Y₁ acts at the very start of the chondrogenic process, by negatively regulating the formation of the prechondrogenic condensation. This fits with the pattern of expression of cP2Y₁ during limb development, which is expressed strongly in loosely packed mesenchyme but is clearly absent from condensing cartilage itself.

- ATP, the Ligand for cP2Y₁ is Produced by Limb Mesenchyme Cells, and these Cells have Mechanisms in Place to Break down Extracellular ATP.

Transfecting micromass cultures with cP2Y₁ negatively regulated the formation of cartilage, but addition of ATP, the ligand for cP2Y₁ to these cultures did not further reduce the amount of cartilage formed. This suggested that enough ATP is produced by cultured limb cells to activate transgenic cP2Y₁ and/or exogenously added ATP is broken down too quickly to further reduce cartilage formation. Using a luciferase ATP assay, it was shown that ATP added to micromass cultures was indeed broken down, with a half-life of approximately one hour. Despite this breakdown of extracellular ATP, very small quantities (0.33 nM) of cellular derived ATP could be detected in the culture medium. The concentration of ATP detected was very low, but given that ATP is broken down by these cells, and that cellular derived ATP is diluted in the culture medium, it is plausible that in the microenvironment of the cell, the concentration of cellular derived ATP is high enough to activate transgenic cP2Y₁. The very fact that limb mesenchyme cells possess such
efficient mechanisms for breaking down extracellular ATP suggests that they release or are exposed to this nucleotide. Other, more in-depth studies using high-performance liquid chromatography (HPLC) have shown that differentiating, and terminally differentiated, post-mitotic hypertrophic chick chondrocytes also secrete significant quantities of adenine nucleotides into the culture medium (Hatori et al., 1995). The study by Hatori et al., 1995 showed that concentrations of 400-800nM ADP were detectable in chondrocyte conditioned medium. ATP was detected at much lower concentrations, but it was shown that 70% of exogenously added ATP was degraded within 15 minutes in chondrocyte cultures. The rate of ADP breakdown by these cells was found to be considerably slower, with a half-life of 2-3 hours. These data suggest that in-vitro chick chondrocytes are a source of extracellular nucleotides. In the developing chick limb, expression of cP2Y_1 was strongest surrounding differentiating chondrocytes, raising the interesting possibility that differentiating cartilage signals, by release of ATP to surrounding cP2Y_1 expressing cells to negatively regulate their recruitment into the chondrogenic pathway.

- **Addition of Reactive Blue-2 to Transfected Cultures Blocks the Effect of cP2Y_1 Expression on Cartilage Formation.**

In chapter 3 of this thesis it was shown that 100μM Reactive blue-2 was effective at blocking the Ca^{2+} response of cP2Y_1 transfected cells to 100μM ATP. If cP2Y_1, activated by ATP released from cells in transfected cultures prevents cartilage formation, then addition of Reactive blue-2 to these cultures should block this effect. This was indeed shown to be the case (Fig 4.9). However Reactive blue-2 is a general antagonist for P2Y receptors, and is not specific for cP2Y_1. It is also possible that Reactive blue-2 interacts with cellular mechanisms unrelated to P2 receptor signalling (for example, it has been used as an affinity reagent in chromatography studies for the purification of enzymes that use nucleotides as a substrates or cofactors). It may be therefore, that the effect of Reactive blue-2 on cartilage formation is a consequence not only of cP2Y_1 antagonism. This is unlikely for several reasons. Firstly, in chapter 3 of this thesis it was shown that
within 24 hours of culturing, the Ca^{2+} responses of limb mesenchyme cells to the P2Y receptor agonists, ATP, ADP and UTP were lost. It is unlikely therefore that in cP2Y1 transfected cultures, Reactive blue-2 exerts its effect on cartilage formation by blocking other P2Y receptor subtypes. Secondly addition of Reactive blue-2 to control transfected cultures did not significantly alter the level of cartilage formation compared to untreated control cultures, suggesting that Reactive blue-2 itself does not promote cartilage formation. Reactive blue-2 treated cultures also appeared morphologically indistinct from untreated cultures.

- **The Phenotype of Reactive blue-2 Injected Limbs may be due to Cell Death.**

Since expression of cP2Y1 in micromass cultures prevented cartilage formation, it was predicted that blocking cP2Y1 function in-vivo with Reactive blue-2 would promote cartilage formation. However, Reactive blue-2 injected into stage 20-22 limb buds resulted in shortening of the humerus, ulna and radius and in some cases the digits. The phenotype of injected limbs suggests an effect on either proliferation or cell death. An increase in cell death was indeed observed in injected limbs. Following injection of Reactive blue-2, the blue dye was clearly visible in the limb tissue, but within 4 hours the blue colouration had disappeared. If Reactive blue-2 is killing cells during this five hour period, then only cells present in the limb during this time, and the skeletal elements that they give rise to should be affected. At stages 20-22, the humerus has been specified, and specification of the ulna and radius is complete by stage 21 (Summerbell, 1974), therefore only these structures should be affected by Reactive blue-2 induced cell death. However the digits were also shorter in injected limbs, and specification of the digits is not complete until stage 28 (Summerbell, 1974), long after Reactive blue-2 has disappeared from the limb. The observation that increased cell death in the AER was still apparent 24 hours after injection (stage 25-26) could explain why digits were also affected. According to the progress zone of proximo-distal patterning (Summerbell et al., 1973), attenuation of the AER signal to cells in the progress zone (in this instance through death of cells in
the AER) would result in either loss or shortening of structures being specified at the time that the signal is attenuated.

Reactive blue-2 was not injected into the AER, but was injected into the underlying mesenchyme. A possible explanation for increased death in the AER, is that Reactive blue-2 is attenuating the mesenchymal signal that is required for AER maintenance. A positive feedback loop between mesenchyme and ectoderm signalling coordinates patterning and outgrowth. FGF has been shown to maintain $Shh$ expression in posterior mesenchyme, and $Shh$ expressing cells can activate $Fgf-4$ expression in the AER (Laufer et al., 1994; Niswander et al., 1994). Reactive blue-2 may attenuate this signal by killing the cells that produce it. However, cell death as a result of Reactive blue-2 injection was not extensive, so it is tempting to speculate that cP2Y$_1$ may have a role to play in relaying the signal between the polarising region and the AER, and by blocking cP2Y$_1$ with Reactive blue-2, growth of the early developing limb is inhibited. However, given that cell death does occur following injection of Reactive blue-2, and that it is impossible to demonstrate the specificity of Reactive blue-2 for cP2Y$_1$ antagonism in-vivo, other, more reliable methods (which are discussed in chapter 6) would have to be employed to investigate the function of cP2Y$_1$ in-vivo.

- **Hypothesis.**

Significant progress has been made in understanding the molecular mechanisms that regulate the initial formation of the prechondrogenic condensation. Mice with loss of function mutations in two members of the TGF-β superfamily, BMP-5 (Bone morphogenetic protein-5) (the short ear, se, mutant) and GDF-5 (growth and differentiation factor 5) (the brachypodism, bp, mutant) have shortened skeletal elements with abnormal sizes and shapes, and these can be attributed to defects in size and shape already apparent in the mesenchymal condensations of the affected skeletal elements (Kingsley et al., 1992; Storm et al., 1994). Treatment of micromass cultures with recombinant GDF-5 protein increases cartilage formation by acting to promote the formation of cartilage condensations through increasing cell-cell adhesion, and
misexpression of GDF-5 in the chick limb increases the length of the skeletal elements (Francis West et al., 1999). The pattern of expression of \textit{GDF-5} in the developing chick limb mirrors the pattern of expression of cP2Y\textsubscript{1}. \textit{GDF-5} is expressed in the condensing cartilage, perichondrium and developing joints (Francis West et al., 1999), areas from which cP2Y\textsubscript{1} is conspicuously absent. These data suggest that a balance may exist between members of the BMP and GDF families expressed in the prechondrogenic condensation that serve to positively regulate the formation of the condensation, and cP2Y\textsubscript{1} expressed in the surrounding mesenchymal cells which prevents their premature recruitment into the chondrogenic pathway. It would be very interesting to compare directly the temporal and spatial patterns of expression of cP2Y\textsubscript{1} and \textit{GDF-5} during development of the chick limb. By controlling the size and timing of formation of the prechondrogenic condensation ATP, signalling through cP2Y\textsubscript{1} may have an important role to play in determining the dimensions of the final skeletal elements.

- **Summary.**

In this chapter evidence is provided that cP2Y\textsubscript{1} could regulate the development of the limb skeleton. Data obtained from micromass cultures suggests that cP2Y\textsubscript{1} may negatively regulate the formation of cartilage by preventing the recruitment of cells into the prechondrogenic condensations that prefigure the limb skeleton. In fulfilling this role, cP2Y\textsubscript{1} may have an important role to play in regulating morphogenesis of the limb skeletal elements.
Expression of Two ATP-Gated Ion Channels, P2X<sub>5</sub> and P2X<sub>6</sub>
During Chick Embryogenesis.

Introduction

- Physiological and Pharmacological Studies Have Suggested that Extracellular ATP may be an Important Signalling Molecule During Development of Chick Skeletal Muscle.

Although embryonic expression of specific P2X receptor subtypes has not been demonstrated, a number of physiological and pharmacological studies have shown P2X-like responses in embryonic tissues. Responses to ATP have been described in ciliary neurons acutely dissociated from day 14 embryonic ciliary ganglia (Abe et al., 1995). A transmitter-like action of ATP on patched membranes of myoblasts and myotubes cultured from 12-day chick embryos was first demonstrated by Kolb and Wakelam (1983), and ATP-induced cation influx was later demonstrated in myotubes prepared from 11-day chick embryos (Haggblad et al., 1985). ATP has a potent depolarising action on myotubes derived from pectoral muscle cultured from day 11 chick embryos (Hume and Höög, 1986) and its physiological and pharmacological properties have been described in a series of papers (Hume and Thomas, 1988; Thomas and Hume, 1990a and b, 1993; Thomas et al., 1991). The sensitivity to extracellular ATP has been tested at various stages of development of different muscles (Wells et al., 1995). At embryonic day 6; stage 30 (Hamburger and Hamilton, 1951), ATP elicits contraction in all muscles tested, but by day 17 (stage 43) none of the muscles contract in response to ATP.

These data strongly suggest that in developing chick skeletal muscle P2X receptors have a role to play during embryonic development. In order to investigate this further I looked for P2X receptor immunoreactivity in the developing chick embryo using polyclonal antibodies raised against the rat P2X receptor peptides.
Methods

• Embryos.
Fertilised White Leghorn chicken eggs were incubated at 37°C and staged according to the Hamburger and Hamilton series (Hamburger and Hamilton, 1951). Embryos were dehydrated in 20% sucrose in PBS prior to freezing. Early stage embryos (stage 14 to 20) were placed on a block of gelatine to facilitate sectioning and frozen in isopentane cooled in liquid nitrogen. Later stage embryos were fixed onto a cork block with OCT compound. These were also frozen in liquid nitrogen cooled isopentane. Cryostat sections were cut at 12μm and collected on gelatinised slides.

• Antibodies and Western Blotting
Polyclonal rabbit antisera to all rat P2X receptors (subtypes 1-7) were obtained from Roche Bioscience (Palo Alto, CA). In a preliminary investigation, P2X^5 and P2X^6 antibodies showed strong immunoreactivity in developing skeletal muscle. The immunogens used were peptides corresponding to 15 receptor type specific amino acids in the C-terminal region of the receptors. The peptide sequences used are: P2X^5 (RENAIVNKVQSQILH), residues 437-451, and P2X^6 (EAGFYWRTKYEEARA), residues 357-371. The antiserum to chicken myosin (fast twitch glycolytic) (a-PM) was obtained after injection of the purified immunogen into rabbits. The specificity of this antiserum is described in detail by Groschel-Stewart et al., 1997. Immunoglobulins G (IgG) were isolated from the immune sera according to the method of Harboe and Ingild, 1973. Specificity of the P2X-antibodies was verified by immunoblotting with chick embryo crude extracts (Stages 30-32). (Western blots of the myosin antibodies are shown in Groschel-Stewart et al., 1997). Generation of crude extracts: 5 embryos between the stages of 30 and 32 were frozen in liquid nitrogen and ground to a fine powder with a mortar and pestle. Three volumes (4ml) of extraction buffer (2ml 8M urea/1% SDS and 2 ml 2xsample buffer for SDS-PAGE) was added and the mixture transferred to an Eppendorf tube which was placed in a boiling water bath for 5 minutes. 40μl of 1M DTE
solution was added and the sample was centrifuged for 15 minutes at 13000 rpm at 4°C. The supernatant was stored at -20°C until use. The Western blots were performed after SDS-PAGE on 8-16% gradient gels (Bio Rad).

- Immunohistochemistry

The avidin-biotin (ABC) technique was employed according to the protocol described by Llewellyn-Smith et al., 1992. The slide mounted sections were fixed in 4% paraformaldehyde and 0.2% of a saturated picric acid solution in PBS for 2 minutes. To inactivate endogenous peroxidases the sections were then treated with 50% methanol containing 0.4% hydrogen peroxide for 10 minutes. Non-specific binding was blocked by incubating for 20 minutes in 3% normal horse serum in PBS containing 0.05% merthiolate. Sections were incubated overnight at room temperature in 5μg/ml of either P2X5 or P2X6 antibody diluted in 3% normal horse serum in PBS. The secondary antibody was a biotinylated donkey anti-rabbit IgG (Jackson Immunoresearch) used at 1:500 for 30 minutes followed by the extraavidin peroxidase conjugate (Sigma) at 1:1000 for 30 minutes. At some stages of development the tyramide amplification step was used according to the recommendation of the supplier (TSA indirect kit, NEN Life Science Products). In these cases the concentration of the primary antibodies was reduced to 1 μg/ml. Stained sections were photographed using the Edge R-400 high definition light microscope (Edge Scientific Instruments Co) and Kodak TMX 100 film.

To further ensure specificity of the P2X antibodies in chick tissues a twofold absorption of the antibodies was performed with their respective peptides prior to performing immunohistochemistry. Briefly: 1.5μl of anti-P2X5 or P2X6 at 1mg/ml were incubated overnight at 4°C with 24μl of the respective peptide at 5μg/ml. A further 24μl of peptide at 5μg/ml was added the following morning and the incubation extended for a further 6 hours. 250μl of 3% normal horse serum was then added to give 300μl of a 5μg/ml concentration of the P2X5 or P2X6 antibodies. This solution was centrifuged for 15 minutes at 13000 rpm at 4°C. The supernatant was used for immunohistochemistry on chick embryo sections.
Results

• Checking the Specificity of the P2X$_5$ and P2X$_6$ Antibodies in Chick Tissues.

Polyclonal rabbit antisera to the rat P2X$_5$ and rat P2X$_6$ receptors were generated by Roche Bioscience (Palo Alto, USA), by immunising rabbits with a 15 amino acid receptor-specific peptide corresponding to the C-terminal region of each receptor. In order to establish as far as possible the antibody specificity in chick embryo tissues, immunoblotting was performed with stage 30-32 chick embryo crude extracts. A single P2X$_5$ reactive band at 70 kDa and two P2X$_6$ reactive bands in the 60-70 kDa range were detected (Fig. 5.1). These bands were in the expected weight range, as determined by immunoblotting with crude rat tissue extract (Gröschel-Stewart et al., 1999), and membrane fractions of cell lines expressing recombinant P2X$_5$ or P2X$_6$ receptors (Oglesby et al., 1999). Although these results suggest that these antibodies are specific for a single protein they cannot rule out the possibility that these antibodies are detecting chick proteins unrelated to P2X$_5$ and P2X$_6$ but which are of similar molecular weights to these two receptors. Specificity of the P2X$_5$ and P2X$_6$ antibodies on chick embryo tissue sections was therefore checked by pre-absorption of the antibodies with the peptides used to generate them. This abolished nearly all immunoreactivity to both antibodies on chick embryo sections (Fig. 5.2). (The weak staining remaining on the sections shown in figure 5.2, may be due to incomplete pre-absorption of the P2X$_5$ and P2X$_6$ antibodies with their peptides. Weak background staining was also observed in control experiments in which the primary antibody was omitted). These data combined with the consistent and tissue-specific staining described below suggest that these antibodies are labelling chick P2X receptors.

• P2X$_5$ and P2X$_6$ Immunoreactivity in Developing Chick Skeletal Muscle.

Staining with P2X$_5$ and P2X$_6$ antibodies was first seen in the somites of a stage 14 embryo (Fig. 5.3 B and C). Expression at this stage could only be visualised using the
Figure 5.1
Western blotting of stage 30-32 chick embryo crude extracts. The blot was probed with polyclonal antibodies to rat P2X5 and P2X6 peptides. A single P2X5 reactive band of approximately 70 kDa and two P2X6 reactive bands in the 60-70 kDa range were detected. Molecular weight markers (kDa) are shown in the left hand lane.

Figure 5.2
Immunoreactivity to P2X5 and P2X6 in chick skeletal muscle is blocked by preabsorbing the antibodies with the peptides used to generate them. The photos in the top panel show immunoreactivity to P2X5 and P2X6 in developing chick skeletal muscle, and the photos in the bottom panel show the result of immunohistochemistry on adjacent sections with P2X5 and P2X6 antibodies preabsorbed with their respective peptides. Stage is indicated in the bottom left hand corner, and the antibody used is shown in the top left hand corner of each figure. Scale bar equals 100μm.
Figure 5.3.
Immunohistochemistry on early chick embryo sections showing P2X5 and P2X6 immunoreactivity in developing skeletal muscle. Developmental stage is shown in the bottom left hand corner of each photo, and the antibody used is shown in the top right hand corner. Scale bar equals 100 µm.
(A) Myosin (a-PM) staining of the myotomes in a longitudinal section through the spinal cord (sc) of a stage 14 embryo. dm, dermamyotome.
(B) P2X5 staining in the dermamyotome of an adjacent section to the one stained for myosin. (C) Staining for P2X6 is also seen in the dermamyotome at stage 14. (D) P2X5 staining is seen in the vertebral muscles (vm), and in the ventral roots, vr. (E) P2X6 immunoreactivity in vertebral muscle.
Figure 5.4
P2X5 and P2X6 Immunoreactivity in skeletal muscle at later stages of development. Stage is indicated in the bottom left hand corner, and the antibody used is shown in the top right hand corner. Scale bar equals 100μM. (A and B) Strong expression of both P2X5 and P2X6 in muscle surrounding the cartilage condensations (c) in the developing wing. (C) Myosin Staining (a-PM) of the rhomboidis superficialis muscle (rs), and the latissimus dorsi muscle (Id); na, neural arch. (D) P2X5 staining in the rs muscle at this stage is still strong, while staining in the underlying ld muscle is beginning to disappear. (E) P2X6 staining in the rs and ld muscles.
tyramide amplification step. Immunohistochemistry with an antibody to chicken myosin (a-PM) on sections from the same embryo showed that the strongest staining seen in figures 5.3 B and C was confined to the dermamyotome of each somite (Fig. 5.3A). This skeletal muscle marker was used throughout this study in order to identify areas of developing skeletal muscle. Less strong staining was visible throughout the remaining tissue in sections 5.3 B and C. P2X₅ and P2X₆ immunoreactivity in all developing chick skeletal muscle strengthened from this stage onward with particularly strong expression seen in the inter-vertebral muscles (Fig. 5.3 D and E) and in muscle surrounding the developing skeletal elements in the limbs (Fig. 5.4 A and B). Expression of both these receptors began to disappear at around stage 39 (Fig. 5.4 D and E). At different stages of development there were temporal and spatial differences in patterns of P2X₅ and P2X₆ expression between different muscle groups. The expression of these receptors was developmentally regulated and expression and disappearance of these receptors occurred earlier in some muscle groups than in others. This is seen in figures 5.4 D and E. The rhomboidis superficialis (rs) muscle still expressed P2X₅ and P2X₆ while the underlying latissimus dorsi (ld) muscle had begun to lose expression of both these receptors.

- **P2X₅ Immunoreactivity in the Developing Nervous System.**

P2X₅ was first detected in the developing nervous system at stage 22 in the ventral roots (Fig. 5.3 D). Figure 5.5 A also shows strong expression of P2X₅ in sensory axons in the dorsal roots and in motor axons in ventral roots at stage 28. Expression of P2X₅ was also detected in the dorsal funiculus which contains the central axons of dorsal root ganglia neurons, and also in ventral spinal cord. Diffuse, less intense staining was visible in the mantle layer, but the ventricular zone did not express P2X₅. At stage 29 P2X₅ immunoreactivity was detected in a subset of small cells in the dorsal root ganglion. The remaining cells in the drg were also P2X₅ positive, but were stained less strongly (Fig. 5.5 B). A similar pattern of staining was visible in the mantle layer at stage 29, with some cells strongly stained for P2X₅, against a background of lightly stained cells. P2X₅ was also observed in what appeared to be nerve fibres innervating the vertebral muscles, and
Figure 5.5.
P2X5 expression in the nervous system. Developmental stage is indicated in the bottom left hand corner. Scale bar equals 100μm. (A) Strong staining for P2X5 was seen in the dorsal funiculus (df), the dorsal root (dr), ventral root (vr), and in ventral spinal cord. Diffuse, less intense staining was also visible in the mantle layer (m). No staining was observed in the ventricular zone. (B) A subset of cells in the dorsal root ganglion (drg) strongly express P2X5, and less intense staining was observed in the remainder of the drg. A similar pattern of staining was seen in the mantle layer. (C) P2X5 expression in vertebral muscles (vm), and in what appear to be nerves innervating them (indicated by large arrow). (sc, spinal cord; ld, latissimus dorsi muscle).
in dorsal root ganglion at stage 30. (Fig. 5.5 C). \( P2X_6 \) was not detected in the developing nervous system at any stage.
Discussion

The observation that ATP has a potent depolarising action on embryonic chick muscle (Hume and Höning, 1986), and that the sensitivity to extracellular ATP seems to be developmentally regulated (Wells et al., 1995) suggests that ATP may be an important signalling molecule during development of chick skeletal muscle. The finding that a single class of ATP-activated ion channel conducts both cations and anions in the myotube (Thomas and Hume, 1990a) and that the P2 receptors involved show marked desensitization (Thomas and Hume, 1990b), suggests that the receptor(s) involved are members of the P2X ligand gated ion channel family. In this study we show using immunohistochemistry that two members of the P2X family are indeed expressed in all developing skeletal muscles of the chick embryo.

• Antibody Specificity

Specificity of P2X5 and P2X6 antibodies on chick tissues was tested by Western blotting with chick embryo crude protein extracts. A single P2X5-reactive band of approximately 70kDa was detected, and two P2X6 reactive bands in the 60-70 kDa range were detected. These were in the expected weight range (Gröschel-Stewart et al., 1999; Oglesby et al., 1999), but the two bands detected by P2X6 raises the possibility that this antibody is recognising other P2X receptor subtypes, or proteins unrelated to P2X6. This is unlikely for several reasons. Firstly, the antibodies were raised against receptor subtype specific peptides making it unlikely that the P2X6 antibody is binding to other P2X receptor subtypes. Secondly, P2X6 consistently labelled only skeletal muscle in this study, so if the P2X6 antibody is labelling more than one protein, these proteins would have to be colocalised in this tissue. Finally, pre-absorption of both P2X5 and P2X6 antibodies with their respective peptides abolished nearly all immunoreactivity to these antibodies in chick tissues. The two bands detected by the P2X6 antibody on the Western blot could be due to alternative splicing of P2X6 mRNA generating proteins of different molecular weights. An increasing number of splice variants are being detected for other P2X receptor
subtypes (Simon et al., 1997; Brändle et al., 1997; Lê et al., 1997). Post translational modification of the P2X₅ protein, by glycosylation for example, would also result in the detection of proteins with different molecular weights.

- **A Number of Functionally Distinct P2X Ion Channels Could Potentially Exist in Developing Chick Skeletal Muscle.**

Immunoreactivity to P2X⁵ and P2X⁶ was first detected in the dermamyotome of stage 14 chick embryos and expression of P2X⁵ and P2X⁶ within skeletal muscle strengthened from this stage onward. Within developing skeletal muscle there was considerable temporal and spatial overlap in expression of these receptors, raising the possibility that a number of functionally distinct P2X receptors are present in developing skeletal muscle. Homomeric P2X ion channels made up of either P2X⁵ or P2X⁶ subunits alone could exist, or, in areas where these receptors are co-expressed, heteropolymerization of P2X⁵ and P2X⁶ subunits could occur to form a third type of ion channel. Heteropolymerization of other P2X receptor subtypes (although not of P2X⁷ and P2X₈) has been shown to occur; co-expression of P2X₂ and P2X₃ is required to produce the responses to ATP of adult sensory neurons (Lewis et al., 1995), and in rat dorsal root and nodose ganglia P2X₂ and P2X₃ immunoreactivity were found to be highly colocalised (Vulchanova et al., 1997). It has also been shown that P2X₅ can coassemble with P2X₁ to form a novel channel (Torres et al., 1998). A recent study examined the ability of all members of the P2X receptor family to interact using a co-immunoprecipitation assay. It was found, that with the exception of P2X₆, all P2X subunits can form homo-oligomeric complexes, and when co-assembly between pairs of subunits were examined, all were able to form hetero-oligomeric complexes with the exception of P2X₇ (Torres et al., 1999). These result suggest that in developing chick skeletal muscle at least two functionally distinct ATP gated ion channels may exist, one homo-oligomeric channel made up of P2X₅ subunits, and a hetero-oligomeric assembly of P2X₅ and P2X₆ subunits.
Patterns of Expression Suggest that P2X₅ and P2X₆ Mediate the Developmentally Regulated Responses of Chick Skeletal Muscle to Extracellular ATP.

The expression pattern of the receptors described here bear striking similarities to the developmental pattern of sensitivity of skeletal muscle to ATP described by Wells et al. 1995. That study showed that all skeletal muscle from embryonic day 6 (stage 30) onward contracted in response to extracellular ATP and that by stage 40 the proportion of muscles responding dropped quickly to zero. However, in culture, mononucleate myotubes and recently fused myotubes (which are not contractile) are responsive to ATP (Hume and Thomas, 1988), so as the expression pattern of P2X₅ and P2X₆ suggests, ATP responsiveness is likely to be present in developing muscles much earlier than stage 30. The study by Wells et al., 1995 also showed reappearance of ATP responsiveness following surgical denervation of the latissimus dorsi muscles of chicks 1-2 days after hatching, suggesting that expression of the ATP receptor(s) involved may be regulated by innervation or muscle function. This would also explain why in vitro, recently fused myoblasts which are not innervated still respond to ATP (Hume and Thomas, 1988). It would be interesting to see if expression of P2X₅ and P2X₆ returns following denervation of skeletal muscle in post-hatch chicks. Although their patterns of expression make it plausible to assume that P2X₅ and P2X₆ receptors mediate the responses of developing chick skeletal muscle to ATP, the absence of P2X subtype specific agonists and antagonists make it difficult to say unequivocally whether this is the case. The time course of the disappearance of P2X₅ and P2X₆ expression, and the appearance and disappearance of ATP responsiveness described by Wells et al, 1995, have striking similarities to the pattern of expression of the embryonic form of the ACh receptor, which is known to be regulated by motor neurons. In mammals and frogs, the embryonic form of the ACh receptor disappears during development, and reappears upon denervation (Katz and Miledi, 1972; Sakmann and Brenner, 1978; Fischbach and Scheutze, 1980; Kullberg et al., 1981). The close parallels between the pattern of ATP responsiveness and P2X₅ and P2X₆ expression, and the expression of embryonic ACh
receptors suggest that the mechanisms that regulate embryonic ACh expression may also regulate the expression of P2X<sub>5</sub> and P2X<sub>6</sub>. It would be interesting to carry out a more detailed study into P2X<sub>5</sub> and P2X<sub>6</sub> expression to investigate the possibility that P2X<sub>5</sub> and P2X<sub>6</sub> cluster at the neuromuscular junction, following their down-regulation in the rest of the muscle. ATP acts as a postjunctional modulator of ACh release and action in mature neuromuscular synapses (for review see Henning, 1997). P2X<sub>5</sub> and P2X<sub>6</sub>, clustered at the neuromuscular junction could potentially mediate this role.

• Sources of Extracellular ATP, and Possible Functions for P2X<sub>5</sub> and P2X<sub>6</sub> in Developing Chick Skeletal Muscle.

Possible sources of extracellular ATP required to activate P2X<sub>5</sub> and P2X<sub>6</sub> are motor neurons and the muscles innervated by them. ATP is known to be co-stored in synaptic vesicles within pre-synaptic motor nerve terminals and to be co-released with acetylcholine (Silinsky and Hubbard, 1973; Zimmerman, 1978). Muscle cells are also known to secrete substantial amounts of ATP in response to electrical activity (Lindgren and Smith, 1986), and in the chick, synaptic responses can be elicited as early as embryonic day 6 (Landmesser and Morris, 1975). The possibility that extracellular ATP co-released with ACh, is important for the development of neuromuscular synapses in Xenopus embryos has also been raised (Fu and Poo, 1991; Fu, 1995). Therefore, in addition to acting as a pre- and postjunctional modulator of ACh release and action in mature neuromuscular synapses (for review see Henning, 1997), P2X<sub>5</sub> and P2X<sub>6</sub> may mediate the transmitter-like action of ATP in developing chick skeletal muscle (Kolb and Wakelam, 1983) and may also play a role in the development of chick neuromuscular synapses.

• P2X<sub>5</sub> in the Nervous System

P2X<sub>5</sub> was also found to be expressed on sensory and motor axons in dorsal and ventral roots respectively. A subset of small cells, possibly glia, neural progenitors or drg axons in the dorsal root ganglion also expressed P2X<sub>5</sub>. P2X<sub>5</sub> immunoreactivity was strong in
dorsal and ventral spinal cord. This pattern of P2X5 immunoreactivity in the nervous system of the chick embryo correlates well with the expression pattern of P2X5 receptor mRNA in adult rat, where expression was found in motoneurons of the ventral horn and in the dorsal root ganglion (Collo et. al., 1996).

• Summary

In this chapter, embryonic expression of specific P2X receptor subtypes, P2X5 and P2X6, is demonstrated for the first time. These receptors were expressed in a developmentally regulated manner in chick skeletal muscle. Their pattern of expression makes them strong candidates for mediating the responses of myoblasts, myotubes and developing chick skeletal muscle to extracellular ATP (Hume and Thomas 1988; Wells et al., 1995), but the precise roles for these receptors in skeletal muscle development, and the functional and/or developmental role for P2X5 in the nervous system remain to be investigated.
CHAPTER 6
**General Discussion**

The aim of this thesis was to test the idea that extracellular nucleotides, signalling through P2 receptors are involved in regulating developmental processes. This idea came from the observation that in a number of cells and cell lines, extracellular nucleotides, acting through these receptors can regulate proliferation, differentiation, and death, processes that are fundamental during embryonic development. Elevations in \([\text{Ca}^{2+}]\) have also been demonstrated in a number of embryonic cell types in response to extracellular nucleotides. Although these studies indicate that P2 receptors are expressed during embryogenesis of a number of different organisms, it does not automatically follow that the role of these receptors is to regulate developmental processes. They may fulfill a housekeeping role, for example they may regulate a fundamental metabolic process that occurs in most cell types. The observation that expression of a P2 receptor is developmentally regulated would lend support to the idea that this type of signalling is important developmentally, and a demonstration that this receptor directs the development of the tissue in which it is expressed would strongly support the hypothesis that extracellular nucleotides, signalling through P2 receptors are used to regulate developmental processes. In this thesis, the chick embryo was used as a model to address these gaps in our knowledge.

- **cP2Y₁, a G Protein-Coupled Receptor for Extracellular ATP, is Expressed in Developmentally Regulated Patterns In a Number of Tissues During Chick Embryogenesis.**

In the first chapter of this thesis it was shown, using in-situ hybridisation, that a member of the P2Y receptor family, cP2Y₁ was expressed in developmentally regulated patterns in the embryonic chick limb, mesonephros, somites, branchial arches and CNS. This is the first description of P2 receptor subtype expression during development of the chick, and only the second time that developmentally
regulated expression of a specific P2 receptor has been described during the
development of an organism. In the following two chapters the a role for cP2Y₁ in
the developing limb was investigated.

- **Monitoring Intracellular Calcium was used to Answer Some Important Questions Regarding cP2Y₁ Function in the Developing Limb.**

In recent years remarkable progress has been made in elucidating the molecular
mechanisms that control development of the vertebrate limb (Cohn and Bright,
1999; Johnson and Tabin, 1997). This, and the ease with which the chick limb can
be manipulated in-ovo and in-vitro made it an attractive system in which to
investigate a function for cP2Y₁. The cP2Y₁ receptor had been cloned, and
pharmacologically characterised elsewhere (Webb et al., 1993; Simon et al.,
1995), therefore it was not the aim of this thesis to further characterise the
pharmacology and transduction mechanisms of this receptor. Instead, monitoring
of intracellular calcium was used as a tool to answer some questions that would be
important when trying to investigate the function of cP2Y₁ in limb development.
Using this technique it was shown that freshly dissociated early limb cells release
Ca²⁺ in response to 2MeSATP, ATP, and ADP at concentrations of 10µM and
100µM, and to UTP at a concentration of 100µM. The recombinant cP2Y₁
receptor expressed in Xenopus oocytes and COS-7 cells was found to respond
primarily to ATP and 2MeSATP (Webb et al., 1993; Simon et al., 1995). The
responses to UTP and ADP therefore suggest that other P2 receptor subtypes are
expressed on these cells. The response to all these nucleotides was lost within 24
hours of culturing, and expression of cP2Y₁ was drastically downregulated over
the same period in culture. The response to ATP, 2MeSATP, and UTP, but not to
ADP could be rescued by transfecting limb cells with an expression vector carrying
the cP2Y₁ cDNA. The fact that the response to ADP was not rescued in transfected
cells suggest that the response to this nucleotide in freshly dissociated cells is
indeed mediated by another P2 receptor subtype. The identity of this receptor is not known, but a second chick P2Y receptor (cP2Y\textsubscript{3}) has been cloned that responds to ADP (Webb et al., 1996). However Northern blotting, in-situ hybridisation, and RNase protection failed to detect expression of cP2Y\textsubscript{3} receptor transcripts in any tissues at these stages of chick development (data not shown). As yet, other ADP selective P2 receptors have yet to be identified in any species. However, cP2Y\textsubscript{1} has a different agonist potency profile from the mammalian and turkey P2Y\textsubscript{1} receptors, in that cP2Y\textsubscript{1} is preferentially activated by ATP, while the mammalian and turkey P2Y\textsubscript{1} receptors are preferentially activated by ADP (Filtz et al., 1994; Palmer et al., 1998; Hechler et al., 1998). Therefore, a chicken homologue of a cloned P2 receptor may indeed exist in limb cells, but species differences may make this homologue an ADP selective receptor. Alternatively the ADP receptor expressed in limb cells may represent a novel subtype. One possible function of this ADP receptor may be to prolong/increase the response initially elicited by ATP. ATP acting through cP2Y\textsubscript{1} would initiate a response, and then ADP (the direct metabolite of ATP) would then initiate an additional response through a separate receptor.

Monitoring intracellular calcium was used to show which concentration of nucleotides were required to elicit a response in these cells, that other P2 receptor subtypes are expressed on early limb cells in addition to cP2Y\textsubscript{1}, and that expression of cP2Y\textsubscript{1} is lost from limb cells in culture. It also proved to be a very useful way of showing that expression of functional cP2Y\textsubscript{1} could be rescued in these cells using a cP2Y\textsubscript{1} expression vector.

**Expression of cP2Y\textsubscript{1} Reduces Cartilage Formation in Micromass Cultures.**

The loss of cP2Y\textsubscript{1} from limb cells in culture was exploited, using a gain-of-function approach, to show that cP2Y\textsubscript{1} negatively regulates the formation of cartilage. In micromass cultures, limb mesenchyme cells plated at high density
condense to form mesenchymal aggregates, the cells of which then differentiate to form cartilage. This process happens in the absence of cP2Y1 expression, which as shown in chapter 3, is lost from these cells within 24 hours. Transfection of micromass cultures with cP2Y1 reduced the level of cartilage formation in these cultures. However the most striking observation in transfected cultures was that the number of mesenchymal aggregates formed in early cultures was drastically reduced. This suggests that cP2Y1 may act at the start of the chondrogenic pathway to negatively regulate the recruitment of cells into the mesenchymal condensations. This would certainly fit with the expression pattern of cP2Y1 at later stages of limb development (stages 29 and onward), when it is strongly expressed in the undifferentiated, loosely packed mesenchyme but is clearly absent from the areas of condensing cartilage themselves. In this thesis it was also shown that cells in micromass cultures possess mechanisms for the breakdown of extracellular ATP, but despite this small quantities of cellular derived ATP could still be detected in the culture medium. Other studies have also shown that differentiating, and terminally differentiated, post-mitotic hypertrophic chick chondrocytes also secrete significant quantities of adenine nucleotides into the culture medium (Hatori et al., 1995). Therefore, in the developing limb, cells undergoing the process of chondrogenesis could signal to surrounding, cP2Y1 expressing cells to negatively regulate their recruitment into the chondrogenic pathway. Such a mechanism could play an important part in controlling the size and shape of the limb skeletal elements.

Analysis of Hox gene gain or loss of function mutants has indicated that the primary role of Hox genes is to regulate the rate and timing of cartilage proliferation and differentiation in the limb (Dolle et al., 1993; Davis et al., 1995; Duboule, 1995; Goff and Tabin, 1997). Hox genes can also influence proliferation of undifferentiated mesenchyme, condensation of mesenchyme and organisation of cartilage cells within the skeletal elements (Davis et al, 1995; Duboule, 1995; Yokouchi et al., 1995; Goff and Tabin, 1997). The target genes regulated by Hox
genes are unknown, but since cP2Y₁ may also play a role in regulating chondrogenesis, its expression may be regulated by the Hox genes. However there is no simple correlation between patterns of Hox gene expression (Nelson et al., 1996) and cP2Y₁ expression in the limb, indicating that direct regulation of cP2Y₁ expression by a particular Hox gene is unlikely.

- cP2Y₁ may Perform Multiple Roles During Limb Development.

Expression of cP2Y₁ was absent from areas of differentiating cartilage, and differentiation and morphogenesis of the limb skeleton is easily observed using micromass cultures, and wholемount skeletal preparations. However, it may be misleading to concentrate solely on the effect of cP2Y₁ on cartilage formation. At later stages of limb development expression of cP2Y₁ was also downregulated in areas of joint and tendon formation. cP2Y₁ may therefore play a role in regulating the differentiation of a number of cell types, and not just cartilage. cP2Y₁ is also expressed strongly, and in broad domains at early stages of limb development. This expression pattern could suggest that cP2Y₁ simply plays a role in maintaining cells in an undifferentiated state. Extracellular nucleotides have also been shown to regulate proliferation of a number of different cell types (Huang et al., 1989; Gonzales et al., 1990; Wang et al., 1994; Neary et al., 1994a and 1994b, Rathbone et al., 1992; Abbraccio et al., 1994 and 1995; Ciccarelli et al., 1994; Wang et al., 1992; Erling et al., 1995; Satterwhite et al., 1999). Therefore by maintaining cells in an undifferentiated state cP2Y₁ may also have a role to play in regulating proliferation of early limb cells. However, cells within the mesenchymal condensations that prefigure the cartilaginous skeleton don’t express cP2Y₁, but do proliferate (Francis-West et al., 1999). Therefore, cP2Y₁ may not simply be a marker of proliferative versus non-proliferative cells. The effect of cP2Y₁ expression may be more subtle in that it regulates rate of proliferation. The effects of extracellular nucleotides have been shown to synergise with polypeptide growth factors, members of the FGF family for example (Neary et al., 1994a).
The role of cP2Y, could therefore change according to the expression of various polypeptide growth factors that are known to be important during limb development. Therefore it would be good to investigate the mitogenic potential of ATP signalling through cP2Y, in limb cells, and to investigate possible synergy between this type of signalling and polypeptide growth factors.

• The Autocrine Nature of P2 Receptor Signalling Could Reinforce Developmental Decisions and Coordinate Cells to make these Decisions at the Same Time.

ATP is ubiquitous in cells, and ATP has been shown to stimulate its own release (Osipchuk and Cahalan, 1992). Therefore in groups of cP2Y, (and P2X, and P2X) expressing cells, this autocrine method of signalling may be used to encourage these groups of expressing cells to make the same developmental decisions, and the ability of ATP to stimulate its own release via P2 receptors could also serve to reinforce the signal within and between expressing cells. Such a mechanism may result in the propagation of waves of Ca$^{2+}$ release within a P2 receptor expressing tissue, and this could potentially coordinate the behaviour of a large population of cells.

• The P2X, and P2X Antisera can be used as Tools to Investigate the Function and Control of Expression of these Receptors During Skeletal Muscle Development.

The previous experimental chapters had focused on the expression and function a P2Y receptor during chick embryogenesis. In the final experimental chapter the possibility that members of the P2X, ligand-gated ion channel family of receptors are also important developmentally was explored. Polyclonal antibodies to rat P2X receptor peptides were used to screen for expression of P2X receptors during chick development. Developmentally regulated immunoreactivity to the P2X and

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P2X₆ antibodies was observed in developing chick skeletal muscle, and P2X₅ immunoreactivity was also detected in the nervous system. As far as possible, specificity of these antibodies in chick tissues was checked using Western blotting, and performing immunohistochemistry on chick tissue with antibody pre-absorbed with the peptides used to generate them. The observation that P2X receptors are expressed in developing chick skeletal muscle is not entirely surprising, since the response of this tissue to extracellular ATP has been well characterised in a number of physiological and pharmacological studies (Kolb and Wakelam, 1983; Haggblad et al., 1985; Hume and Hönig, 1986; Hume and Thomas 1988; Thomas and Hume, 1990a and 1990b; Thomas et al., 1991; Thomas and Hume, 1993; Wells et al., 1995). However, this thesis has identified the expression of specific P2X receptor subtypes that may account for the responses of developing chick skeletal muscle to ATP, and these antibodies can now be used as probes to investigate the mechanisms that control receptor expression, and give insights into possible receptor function. For example, these antibodies can be used to see if P2X₅ and P2X₆ expression returns in denervated muscle of post-hatch chicks, and to explore the possibility that the mechanisms used to control ACh receptor expression during skeletal muscle development also regulate P2X₅ and P2X₆ expression.

Fu, 1995 has suggested that at developing neuromuscular synapses, ATP exerts positive regulatory actions on neuromuscular transmission by pre-synaptic potentiation of spontaneous ACh release, and post-synaptic potentiation of ACh responses. Spontaneous ACh release at developing neuromuscular synapses may play an important role in muscle maturation, since excitation of the post-synaptic muscle cell as a result of this spontaneous release of ACh produces a global influence on muscle development, as shown by the accelerated appearance of myofibril striation in innervated muscle cells (Kidokoro and Saito, 1988). Synaptic activity is also known to play a crucial role in many aspects of development of the neuromuscular synapse, including elimination of polyneuronal elimination
(Greensmith et al., 1998), and regulation of ACh receptor synthesis (Goldman et al., 1988). Post-synaptic, activity-dependent production of retrograde signals may also modulate presynaptic structure and function (Dan and Poo, 1994). In the chick a transmitter-like action of ATP in developing skeletal muscle has been demonstrated (Kolb and Wakelam, 1983), and muscle cells are also known to secrete substantial amounts of ATP in response to electrical activity (Lindgren and Smith, 1986). Therefore ATP released from pre-synaptic nerve terminals and activating P2X₅ and P2X₆ in developing skeletal muscle may play an important role in muscle maturation, and specialisation of the post-synaptic membrane. In response to this electrical activity, skeletal muscle may release ATP that binds to P2X₅ that appeared to be expressed on nerve fibres innervating developing skeletal muscle. This retrograde signal may be important for development of the pre-synaptic cell.

• Future Directions.
This thesis has provided evidence that extracellular nucleotides, signalling through P2 receptors may indeed play important roles in regulating developmental processes. However many questions stem from this work, such as what is the mechanism by which cP2Y₁ prevents the formation of the mesenchymal aggregates that mark the initiation of the chondrogenic process? One possibility is that cP2Y₁ may prevent mesenchymal aggregation by reducing cell-cell adhesion. This could be tested by observing the effect of cP2Y₁ expression on cell aggregate formation and size in limb cell suspension cultures. This technique has been used to show that the mechanism by which GDF-5 promotes limb cartilage formation may be by promoting cell-cell adhesion (Francis-West et al., 1999). Treatment of limb cell suspension cultures with GDF-5 increased the number and average size of cell aggregates. Ultimately one would like to address the function of cP2Y₁ in-vivo. One approach would be to mis-express cP2Y₁ in the developing chick limb using a retroviral vector. This approach has been widely used to investigate the function of
many genes expressed during chick limb development. If the hypothesis concerning a role for cP2Y₁ in negatively regulating the formation of mesenchymal aggregates is correct, one would expect that by misexpressing cP2Y₁ in the limb, cartilage formation would be delayed. This could be tested by looking at the effect of cP2Y₁ mis-expression on expression of cartilage markers such as Type II collagen (expressed by cells in the mesenchymal condensations as they begin to differentiate), and Type X collagen (a marker of terminally differentiated hypertrophic chondrocytes). This approach could be complemented by blocking expression of cP2Y₁ in the limb. In the absence of selective antagonists for cP2Y₁, antisense oligonucleotides could be used.

Also, what is the function of cP2Y₁ in the early limb, when there is not such a clear demarcation between cartilage condensations and cP2Y₁ expressing cells, and what controls cP2Y₁ expression at these stages? The observation that cP2Y₁ expression is lost from mesenchyme cells in culture may be due to the fact that these cells are cultured in the absence of limb ectoderm. It would be interesting to observe the effect of removing dorsal ectoderm, or the apical ectodermal ridge (important signalling centres that pattern the limb along the dorso-ventral, and proximo-distal axes respectively) on expression of cP2Y₁ within the limb. Dorsal ectoderm expresses Wnt-7a, which is thought to signal to the underlying mesoderm to induce expression of the LIM-homeodomain protein Lmx-1, which is believed to direct dorsal patterning (Riddle et al., 1995; Vogel et al., 1995). Given the strong dorsal expression of cP2Y₁ in the early limb it would be tempting to speculate that its expression may be regulated by these dorsalising signals. Removal of the AER also results in the loss of mesenchymal expression of many genes, Sonic hedgehog (Shh), in the polarising region for example (Laufer et al., 1994; Niswander et al., 1994). Bone morphogenetic proteins Bmp-2 and Bmp-7, and the HoxD cluster genes are also expressed in posterior chick wing mesenchyme in response to Shh and FGF-4 signalling (Laufer et al., 1994; Niswander et al., 1994). A simple experiment to test the influence of ectoderm on
mesenchymal cP2Y₁ expression, would be to culture limb mesenchyme in the presence of limb ectoderm, and see if cP2Y₁ expression/Ca²⁺ responses to ATP are maintained in-vitro.

Can calcium imaging of intact, cP2Y₁ expressing tissues be used to visualise any ATP induced Ca²⁺ waves? What is the function of cP2Y₁ in the mesonephros, branchial arches, CNS, and somites? What is the function of P2X₅ and P2X₆ during chick embryogenesis? Ultimately one would like to answer these questions, and also extend these sorts of studies to other model organisms such as the zebrafish, frog and mouse. The identification of developmentally regulated P2 receptor expression in these organisms would certainly lend support to the idea that this type of signalling is important developmentally, and would open up the possibility of using powerful molecular genetic techniques (knockouts and transgenics for example), that are not possible in the chick, to address the function of this type of signalling during embryonic development.
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