DEVELOPMENTAL DYNAMICS 222:494–505 (2001)

The Extracellular ATP Receptor, cP2Y$_1$, Inhibits Cartilage Formation in Micromass Cultures of Chick Limb Mesenchyme

MARTIN P. MEYER,[1] KARL SWANN,[1] GEOFFREY BURNSTOCK,[2] AND JONATHAN D.W. CLARKE[1]*
[1]*Department of Anatomy and Developmental Biology, University College London, London, United Kingdom*
[2]*Autonomic Neuroscience Institute, Royal Free Hospital School of Medicine, London, United Kingdom*

ABSTRACT **We have investigated the function of the G protein-coupled receptor for extracellular ATP, chick P2Y$_1$ (cP2Y$_1$) during development of the chick limb. cP2Y$_1$ is strongly expressed in undifferentiated limb mesenchyme cells but appears to be lost from cells as they differentiate, raising the possibility that the function of this receptor may be to inhibit cell differentiation. This pattern of expression was particularly striking surrounding areas of cartilage formation. We tested whether cP2Y$_1$ was able to regulate cartilage formation by using an in-vitro micromass model of chondrogenesis. Because limb cells in micromass culture lose expression of cP2Y$_1$, we have used a gain-of-function approach to demonstrate that cP2Y$_1$ expression can inhibit cartilage differentiation. We also demonstrate that early limb mesenchyme cells release ATP into the extracellular medium and have mechanisms to breakdown extracellular ATP. These results suggest that extracellular ATP, signaling through cP2Y$_1$, can modulate the differentiation of limb mesenchyme cells in vitro, and the expression pattern of cP2Y$_1$ suggests that this type of signaling could play a similar role in ovo.**

Key words: ATP; chick embryo; P2Y receptors; limb development; chondrogenesis

INTRODUCTION

Extracellular ATP has been shown to increase intracellular Ca^{2+} (Ca^{2+}_i) in a number of embryonic cell types (for review see Burnstock, 1996), and increases in Ca^{2+}_i can regulate cell proliferation, migration, and differentiation (Berridge, 1993; Lauder, 1993). Thus, extracellular ATP has the potential to regulate important processes in embryonic development. We have previously shown that a G protein-coupled receptor for extracellular ATP, cP2Y$_1$, shows striking patterns of expression in the limb and other tissue systems during chick embryogenesis (Meyer et al., 1999). In this article, we investigate the function of cP2Y$_1$ and provide evidence that ATP signaling through this receptor may play a role in regulating the differentiation of cells in the embryonic limb bud.

Receptors for extracellular nucleotides, including ATP, ADP, and UTP, are divided into two families, P2X and P2Y, based on molecular structure, transduction mechanisms, and pharmacological properties (Abbracchio and Burnstock, 1994; Burnstock and King, 1996). P2X receptors are members of the ligand-gated ion channel superfamily, whereas P2Y receptors constitute a family of G protein-coupled receptors for extracellular nucleotides. Activation of the P2Y receptor cP2Y$_1$ by extracellular ATP leads to mobilization of intracellular calcium (Ca^{2+}_i) and activation of protein kinase C (PKC) (Webb et al., 1993; Simon et al., 1995). Mobilization of Ca^{2+}_i, and the cascade of intracellular protein phosphorylation initiated by activation of PKC, are important regulators of cell growth and differentiation (Berridge, 1993; Lauder, 1993), and there is now considerable evidence supporting a role for P2Y receptor signaling in regulating long-term changes in cell proliferation and differentiation (reviewed in Abbracchio, 1996; Neary and Burnstock, 1996). The mitogenic effects of ATP have also been shown to synergise with polypeptide growth factors, including the fibroblast growth factors (FGFs), which are known to be important in limb development (Neary et al., 1994a).

In the developing chick limb, P2Y$_1$ is expressed in a pattern that suggests a potential role in regulating cell differentiation. By using an in vitro micromass assay of cartilage formation, we provide evidence that ATP, signaling through cP2Y$_1$, can inhibit the differentiation of precartilage condensations, and could, therefore, have a role in controlling development of the early limb bud.

RESULTS

cP2Y$_1$ Shows a Dynamic Pattern of Expression in Chick Limb Buds and Is Expressed Preferentially in Undifferentiated Mesenchyme Cells

The pattern of expression of cP2Y$_1$ has been described in detail elsewhere (Meyer et al., 1999), but we will briefly review the expression pattern in the limb

*Correspondence to: Jonathan D.W. Clarke, Department of Anatomy and Developmental Biology, University College London, Gower Street, London WC1E 6BT, UK.
Received 17 January 2001; Accepted 7 August 2001

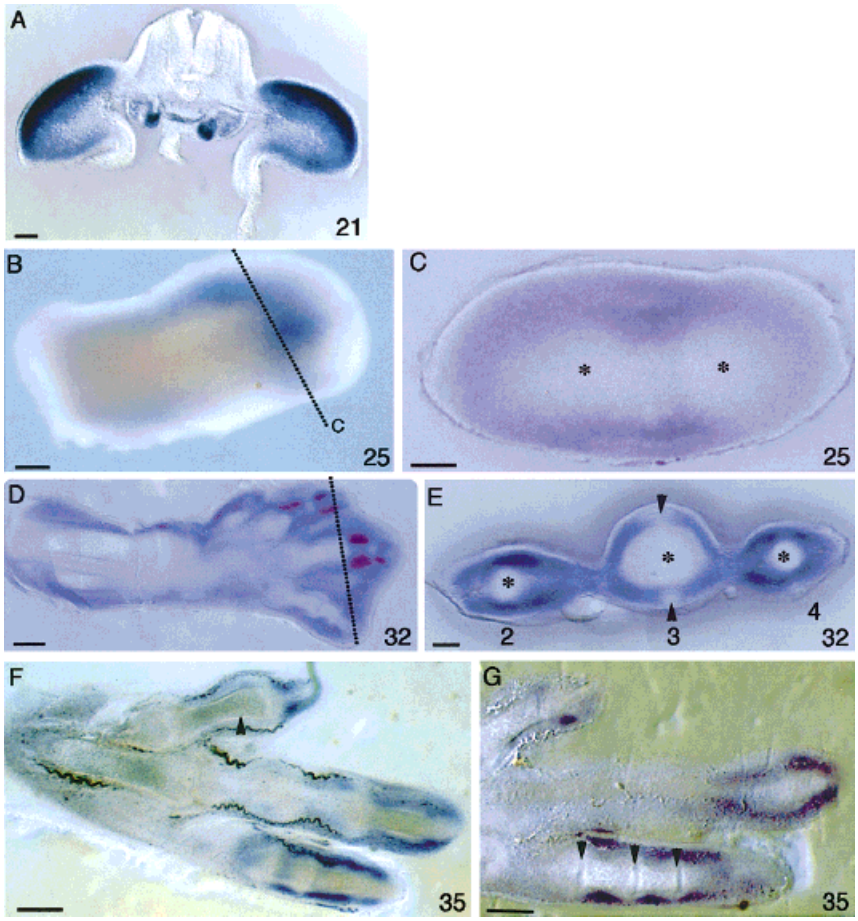

Fig. 1. Expression of cP2Y$_1$ during limb development visualized by whole-mount in situ hybridization. In A, C, and E, dorsal surface is uppermost. In B, D, F, and G, anterior surface is uppermost. A–C show expression in the early wing bud, and D–G show expression in the leg. Stage is indicated in the bottom right-hand corner. **A:** Transverse section through wing buds. Note dorsoventral difference in cP2Y$_1$ expression. **B:** Dorsal view of stage 25 wing showing a more restricted pattern of expression of cP2Y$_1$. Dotted line indicates region from which C was taken. **C:** Section through a stage 25 wing showing expression in mesenchyme surrounding cartilage condensations (asterisks). **D:** Longitudinal section through a stage 31 leg. Expression is strong distally surrounding areas of most recently differentiating cartilage and is weaker proximally. **E:** Transverse section through digits 2, 3, and 4 showing gaps in cP2Y$_1$ expression surrounding areas of cartilage (asterisks), and tendon (arrowheads) formation. Strong, broad domains of cP2Y$_1$ expression surrounding areas of cartilage formation in more distal, undifferentiated regions of digits 2 and 4. There are also no gaps in expression where tendons will later form. However, in the same section, a more proximal region through digit 3 is shown. Here expression is less strong, and gaps in areas of tendon formation are clearly visible. This finding suggests that cP2Y$_1$ expression is lost from cells as differentiation proceeds. Expression of cP2Y$_1$ in the limb is restricted to the digits at stage 35. **F,G:** Gaps in cP2Y$_1$ expression corresponding to areas of joint (arrowheads in G), tendon, and cartilage are clearly visible, but expression of cP2Y$_1$ is strong in surrounding tissues. Expression from the perichondrium is also absent (arrowhead in F). Scale bars = 100 μM in A–G.

buds here. This receptor is expressed strongly and dynamically in mesodermal cells of the limb buds, which form the focus of this study. Expression in the posterior mesodermal cells of the limb begins at stage 19 and intensifies and spreads by stage 21 to include all but the core mesoderm and the anterior third of the bud (Fig. 1A). At stage 25, expression is largely restricted to cells just proximal to the progress zone mesenchyme, which lies immediately subjacent to the apical ectodermal ridge (Fig. 1B). Sections through this region show strong expression bordering the condensing cartilaginous elements, but the regions of cartilage formation themselves do not express cP2Y$_1$ (Fig. 1C). At stage 32, expression is more intense distally than proximally (Fig. 1D). By stage 35, cP2Y$_1$ expression is maintained in undifferentiated mesenchyme and is seen to be excluded not only from cartilage elements but also from associated perichondria, developing tendons, and sites of joint formation (Fig. 1F, G).

Monitoring Intracellular Free Calcium Levels Demonstrates That ATP Receptors Are Functional in Early Limb Bud Cells but Lost by 24 Hours in Vitro

We wanted to ensure that cP2Y$_1$ was functional at these early stages of limb development and if so, what concentrations of nucleotides were required to elicit a response in these cells. We also wanted to explore the

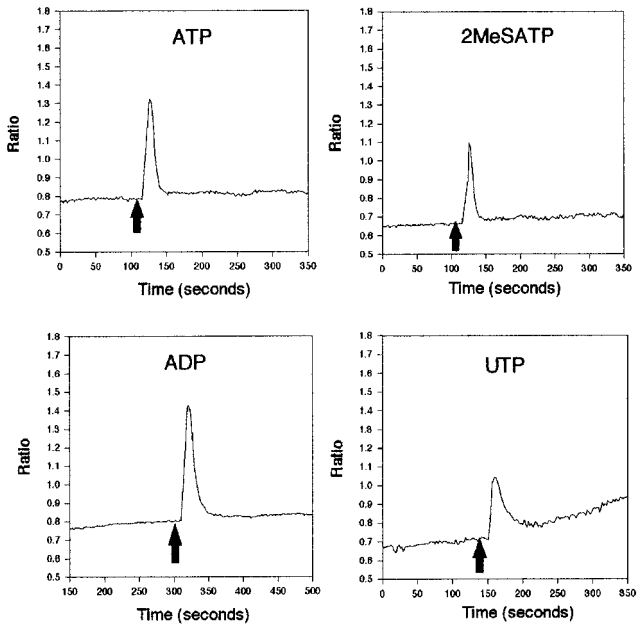

Fig. 2. Calcium responses in freshly dissociated stage 20–22 chick limb mesenchyme cells. In each figure, the nucleotide added is shown at the top of the trace. These traces show responses to 100 μM concentrations of each nucleotide. The time at which each nucleotide was added is shown by the arrows. Fluorescence ratio is shown on the y axis, and time in seconds is shown on the x axis.

possibility that other P2 receptor subtypes may be present on these cells. To answer these questions, we monitored the intracellular free calcium concentration ($[Ca^{2+}]_i$) of stage 21–23 chick limb mesenchyme cells to characterize their response to a variety of nucleotides known to activate both P2Y and P2X receptors.

In freshly dissociated limb cells, large increases in $[Ca^{2+}]_i$ were elicited by ATP, ADP, and 2MeSATP (a synthetic analogue of ATP that is a potent agonist of the recombinant $cP2Y_1$ receptor expressed in *Xenopus* oocytes, Webb et al., 1993; Simon et al., 1995) at concentrations of 100 μM and 10 μM. Figure 2 shows typical $[Ca^{2+}]_i$ increases in single cells or small clusters of cells to these nucleotides. A small $Ca^{2+}{}_i$ transient was also detected in response to 100 μM UTP (Fig. 2), a nucleotide which does not activate $cP2Y_1$ when expressed in *Xenopus* oocytes. (Webb et al., 1993). Adenosine and AMP (both catabolites of ATP which are inactive at the recombinant $cP2Y_1$ receptor; Webb et al., 1993; Simon et al., 1995) did not elicit $Ca^{2+}{}_i$ release in limb cells. The expression pattern of $cP2Y_1$ at stage 21 suggests that not all limb mesenchyme cells express the receptor at this stage, and this heterogeneity was reflected in the $[Ca^{2+}]_i$ response data as some cells did not respond to added nucleotides. These data are summarized in Table 1. The paucity of selective agonists and antagonists for P2 receptors make it difficult to assign all of these responses to $cP2Y_1$ activation, because other P2 receptor subtypes are also known to respond to these nucleotides (Burnstock and King, 1996). Indeed, the large response to ADP, and the response to UTP are uncharacteristic of $cP2Y_1$ activation (Webb et al., 1993; Simon et al., 1995), suggesting that other P2 receptor subtypes must also be expressed in the early limb.

Monitoring $[Ca^{2+}]_i$ also showed that the majority of chick limb mesenchyme cells maintained in culture for 24 hr no longer responded to any of these nucleotides by releasing $Ca^{2+}{}_i$ (Fig. 3, Table 2), and RNase protection showed that there was almost complete downregulation of $cP2Y_1$ expression during this period (Fig. 3). This loss of functional ATP receptors gave us the opportunity to investigate a role for $cP2Y_1$, by using a gain-of-function approach, against a background that was effectively null for other P2 receptor subtypes.

Transfection Successfully Restores Functional ATP Receptors to Limb Mesenchyme Cells in Vitro

Native expression of $cP2Y_1$ was lost after 24 hr in culture. Therefore, to carry out in vitro functional studies of $cP2Y_1$, we rescued its expression in cultured limb mesenchyme cells. This was achieved by transfecting cells with a $cP2Y_1$ expression construct, pRCCMV-$cP2Y_1$ (Table 3). Monitoring $[Ca^{2+}]_i$ was then used to ensure that functional receptor was produced in transfected cells (Fig. 4). In control cultures (transfected with pRCCMV, but not containing the $cP2Y_1$ cDNA) the average fluorescence ratio change in response to 100 μM ATP was 0.15 (Fig. 4E; Table 4), and only 19% of cells showed a $Ca^{2+}{}_i$ response (Table 4). In contrast, 64% of cells in cultures transfected with pRCCMV-$cP2Y_1$ responded to 100 μM ATP (Table 3). The average size of the fluorescence ratio change in response to 100 μM ATP in pRCCMV-$cP2Y_1$ transfected cells was 0.44 (Fig. 4A; Table 3), 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 μM ATP in pRCCMV-$cP2Y_1$ transfected cells and freshly dissociated cells was very similar in terms of response time and duration. These data suggest that transfection with pRCCMV-$cP2Y_1$ rescues functional expression of $cP2Y_1$ in cultured limb mesenchyme cells to a level that is very similar to endogenous expression in freshly dissociated cells. Robust responses to 100 μM ATP could be elicited in pRCCMV-$cP2Y_1$ transfected cells 24, 48, and 72 hr after transfection, indicating that receptor expression is detectable soon after transfection and remains detectable for at least 3 days.

Having rescued expression of $cP2Y_1$ in these cells, we looked at the ligand profile of cells transfected with $cP2Y_1$. It was found that in pRCCMV-$cP2Y_1$ transfected cells, 2MeSATP as well as ATP elicited robust $Ca^{2+}{}_i$ responses (Fig. 4B), whereas ADP was much less potent (Fig. 4D). These data are in agreement with electrophysiological studies performed on the recombinant receptor expressed in *Xenopus* oocytes (Webb et al., 1993; Simon et al., 1995) and suggest that in freshly

TABLE 1. Calcium Responses in Freshly Dissociated Limb Mesenchyme Cells

Compound	Concentration (μM)	Number of cells measured	Cells responding (%)	Mean fluorescence ratio change	SEM
ATP	100	44	91	0.39	0.02
	10	12	84	0.24	0.03
2MeSATP	100	21	90	0.38	0.03
	10	15	86	0.23	0.05
ADP	100	27	96	0.57	0.08
	10	9	100	0.51	0.05
UTP	100	17	88	0.27	0.06
	10	8	12	0.13	—
AMP	100	7	0	0	—
ADENOSINE	100	7	0	0	—
THAP	1	39	100	0.43	0.02

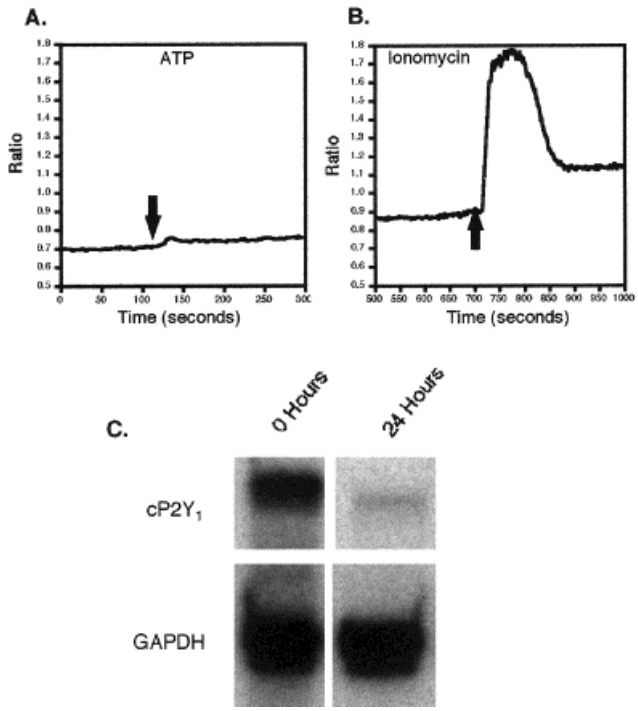

Fig. 3. Expression of cP2Y$_1$ was lost from chick limb mesenchyme cells within 24 hr of culturing. **A:** Response of limb cells to 100 μM ATP after 24 hr in culture. **B:** Ionomycin was added to 24-hr cultures to check that a $[Ca^{2+}]_i$ response could be detected in these cells. **C:** RNase protection showed that, within 24, there was almost complete down-regulation of cP2Y$_1$ expression. A GAPDH probe was used as a loading control.

dissociated limb cells, the large response to ADP is mediated by a different receptor subtype, which like cP2Y$_1$ disappears from limb cells cultured for 24 hr. Surprisingly, in transfected cells the response to UTP was also rescued (Fig. 4C). Other studies have shown that UTP is inactive at the cP2Y$_1$ receptor (Webb et al., 1993; Simon et al., 1995). The response to 100 μM ATP could be blocked in pRCCMV-cP2Y$_1$ transfected cells by addition of 100 μM Reactive Blue-2, a competitive antagonist at the cP2Y$_1$ receptor (Webb et al., 1993) (Fig. 4F; Table 5).

Transfection of Limb Micromass Cultures with cP2Y$_1$ Reduces the Amount of Cartilage Formed

In the limb bud, expression of cP2Y$_1$ appears to be strongest in the least differentiated cells and absent from areas of differentiation and this finding is particularly striking in the most distal areas of the limb bud and developing digits. This expression suggests the possibility that cP2Y$_1$ expression could play a role in maintaining cells in an undifferentiated state. To test whether cP2Y$_1$ could regulate differentiation of limb cells we decided to use an in vitro micromass model of cartilage differentiation. Micromass cultures of mesenchymal cells from the early limb bud are a standard in vitro model for studying chondrogenesis and have been used to analyze the mechanisms of action of a number of TGF-β family members (Jiang et al., 1993; Roark and Greer, 1994; Francis-West et al., 1999). In micromass cultures, the cellular aggregates that prefigure the cartilage nodules begin to form 12–24 hr after plating (Ahrens et al., 1977). Therefore, the initiation of chondrogenesis in these cultures correlates with the loss of cP2Y$_1$ expression (as shown above using RNase protection, and by monitoring $[Ca^{2+}]_i$, Fig. 3).

In the previous section, we demonstrated that we could transfect cultured limb mesenchyme cells with a transfection efficiency of 64% and we have used this gain-of-function approach to investigate whether the cP2Y$_1$ receptor is able to regulate chondrogenesis in micromass cultures. Micromass cultures were transfected with either empty vector (pRCCMV) as a control or with vector carrying the cP2Y$_1$ cDNA (pRCCMV-cP2Y$_1$). The development of condensations of chondrogenic mesenchyme was monitored by using phase contrast optics for up to 6 days. Two days after transfection, phase optics revealed numerous condensations of chondrogenic mesenchyme in control cultures. However, in cP2Y$_1$ transfected cultures, far fewer condensations appeared to have formed. To better quantify the differentiation of precartilage condensations, 2-day cultures were stained with Alcian blue (Fig. 5A) and the number of stained nodules was counted (Fig. 5B). Alcian blue binds to sulphated glycosaminoglycans (GAGs), which are one of the princi-

pal extracellular matrix components of skeletal cartilage. In control transfected cultures, the mean number of Alcian blue–positive nodules formed was 243 ± 75 (n = 9), whereas the mean number of stained nodules in $cP2Y_1$-transfected cultures was only 20 ± 8 (n = 9). This finding represents a 12-fold reduction in the number of cartilage nodules initially formed in $cP2Y_1$-transfected cultures ($P < 0.01$; Student's *t*-test). The number of Alcian blue–positive nodules increased in $cP2Y_1$-transfected and control cultures between days 2 and 6 (Fig. 5). In 6-day cultures, the amount of cartilage formed in control-transfected and $cP2Y_1$-transfected cultures was compared quantitatively by extracting the bound Alcian blue and assaying spectrophotometrically. From control cultures the average absorbance (A630nm) was 0.33 ± 0.04 (n = 12), whereas in $cP2Y_1$-transfected cultures, the average A630nm was 0.18 ± 0.04 (n = 12), a reduction of 55% compared with controls ($P < 0.01$; Student's *t*-test) (Fig. 6). These data demonstrate that expression of $cP2Y_1$ significantly inhibits cartilage differentiation in micromass cultures. The gradual increase in Alcian blue staining in $cP2Y_1$-transfected cultures over the 6-day period may reflect a gradual loss of $cP2Y_1$ receptors due to the transient nature of the transfection.

ATP Is Released and Degraded by Limb Cells in Culture

Daily addition of ATP, to a final concentration of 100 μM to pRCCMV-$cP2Y_1$–transfected cultures did not further reduce cartilage differentiation (mean A630nm of control transfected cultures + ATP = 0.36 ± 0.05, n = 10, A630nm $cP2Y_1$-transfected cultures + ATP = 0.18 ± 0.05, n = 10, and A630nm $cP2Y_1$-transfected cultures with no ATP added = 0.18 ± 0.05, n = 12, Fig. 6). This suggests that saturating levels of endogenous ATP are released by cells in micromass culture. Alternatively, sufficient ATP may be released from cells to activate $cP2Y_1$ and reduce the amount of cartilage formed, but exogenous ATP is broken down too quickly to further reduce cartilage formation. To test these ideas, we have measured ATP release and its breakdown by cultured limb bud cells by using a Luciferin-luciferase assay. ATP at a concentration of 0.83 nM ± 0.05 (n = 4) can be detected after a 4-hr incubation of cells in fresh PBS (Fig. 7), whereas the background reading obtained for cell-free PBS is 0.5 nM ± 0.1 (n = 4). Thus, during a short incubation time, there is a small but significant ($P < 0.05$; Student's *t*-test) increase in the concentration of cellular-derived ATP in the culture medium. ATP breakdown in micromass cultures was also assayed. Figure 8 shows that added ATP is broken down in micromass cultures, with a half-life of approximately 60 min. However, over the same 4-hr assay period, only 10% of ATP was broken down in control, cell-free cultures. These data show that cells in micromass culture have mechanisms to degrade extracellular ATP and that enough ATP may be produced by cells in these cultures to activate transgenic $cP2Y_1$.

A Competitive Antagonist of $cP2Y_1$ Blocks the Inhibition of Cartilage Formation in Culture

If extracellular ATP binding to the $cP2Y_1$ receptor inhibits the formation of cartilage, then pharmacologic blockade of $cP2Y_1$ function should restore the level of cartilage formation to those seen in control cultures. Addition of 100 μM Reactive Blue-2 (a $cP2Y_1$ receptor antagonist that blocks the $Ca^{2+}{}_i$ response to ATP in transfected limb cells, Fig. 4F) blocked the inhibitory effect of $cP2Y_1$ expression on cartilage formation (Fig. 6). Statistical analysis showed that there is no significant difference in the amount of cartilage formed between Reactive Blue-2–treated $cP2Y_1$-transfected cultures (A630nm of 0.33 ± 0.07, n = 12), and control transfected cultures treated with Reactive Blue-2

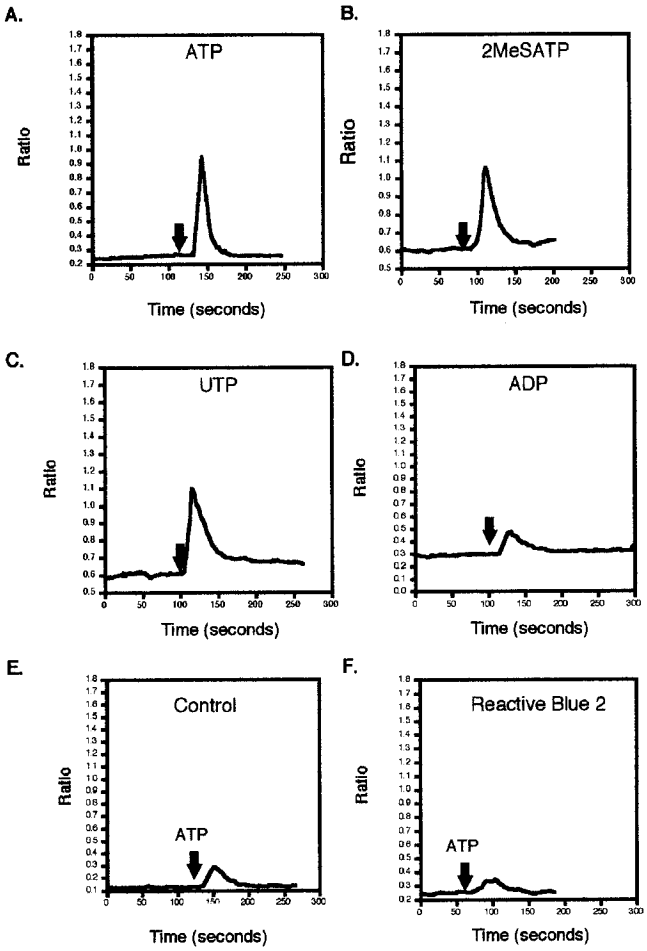

Fig. 4. **A–D:** Typical $Ca^{2+}{}_i$ responses in limb cells transfected with pRCCMV-$cP2Y_1$. The nucleotide added (100 μM) is shown at the top of each trace. **E:** Typical $Ca^{2+}{}_i$ response to 100 μM ATP in a cell transfected with a control construct (pRCCMV). **F:** $Ca^{2+}{}_i$ responses to 100 μM ATP in cells transfected with pRCCMV-$cP2Y_1$ are blocked by 100 μM Reactive Blue-2.

TABLE 2. Calcium Responses in Limb Mesenchyme Cells Cultured for 24 Hours

Compound	Concentration (μM)	Number of cells measured	Cells responding (%)	Mean fluorescence ratio change	SEM
ATP	100	77	9	0.08	0.007
2MeSATP	100	32	9	0.08	0.027
ADP	100	21	0	—	—
UTP	100	22	0	—	—

TABLE 3. Calcium Responses in Limb Mesenchyme Cells Transfected with pRCCMV-cP2Y$_1$

Compound	Concentration (μM)	Number of cells measured	Cells responding (%)	Mean fluorescence ratio change	SEM
ATP	100	72	64	0.44	0.04
2MeSATP	100	38	61	0.42	0.1
ADP	100	39	23	0.14	0.03
UTP	100	134	36	0.55	0.09

TABLE 4. Calcium Responses in Limb Mesenchyme Cells Transfected with Empty pRCCMV

Compound	Concentration (μM)	Number of cells measured	Cells responding (%)	Mean fluorescence ratio change	SEM
ATP	100μM	52	19	0.15	0.06

TABLE 5. Calcium Responses in Limb Mesenchyme Cells Transfected with pRCCMV-cP2Y$_1$ in the Presence of 100 μM Reactive Blue-2

Compound	Concentration	Number of cells measured	Cells responding (%)	Mean fluorescence ratio change	SEM
ATP	100μM	36	16	0.14	0.04

(mean A630nm was 0.36 ± 0.06, n = 11, $P > 0.1$; Student's *t*-test). The number and size of Alcian blue–positive condensations in Reactive Blue-2–treated cultures was also morphologically indistinguishable from untreated cultures. The effect of cP2Y$_1$ expression on cartilage formation can, therefore, be blocked by the receptor antagonist Reactive Blue-2. 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. The antagonism of cP2Y$_1$-transfected cells by Reactive Blue-2 also demonstrates that transfection with cP2Y$_1$ is itself not toxic to cells.

We have attempted to block cP2Y$_1$ function in vivo by injecting 100 μM Reactive Blue-2 into the limb bud. At this dose, there was no obvious effect on limb development. However, we found that injected Reactive Blue-2, which was clearly visible in the limb immediately after injection was washed out within 1 hr. Given that Reactive Blue-2 is a competitive antagonist of cP2Y$_1$, it can be inferred that cP2Y$_1$ would be blocked in vivo for a short time only. We found that at higher concentrations Reactive Blue-2 was toxic to limb cells (data not shown); therefore, we were unable to pursue the use of Reactive Blue-2 in vivo.

DISCUSSION

Extracellular ATP Signalling in the Chick Limb Bud

We have previously shown that cP2Y$_1$, a G protein-coupled receptor for extracellular ATP, is expressed in the embryonic chick limb bud, where its expression is strongest in the distal mesenchyme surrounding the condensing cartilage elements, tendons, and joint specialisations. Here, we confirm that this expression generates functional receptors that can transduce extracellular nucleotide signals and increase $Ca^{2+}{}_i$. Furthermore, we show that cells from the limb bud mesenchyme both release the cP2Y$_1$ ligand, ATP, and have mechanisms to break it down. Finally, we show that the cP2Y$_1$ receptor is lost from cells in micromass cultures as they condense to form chondrogenic aggregates, and rescuing expression of cP2Y$_1$ by transient transfection inhibits the differentiation of these condensations. These results demonstrate that limb bud mesenchyme cells have all the machinery to use extracellular nucleotides as signalling molecules, and the in vitro data together with the expression pattern suggest that the loss of the cP2Y$_1$ receptor may be an important step in the differentiation of cells within the developing limb.

We have chosen to study the effects of cP2Y$_1$ on cartilage differentiation as this is easily monitored in

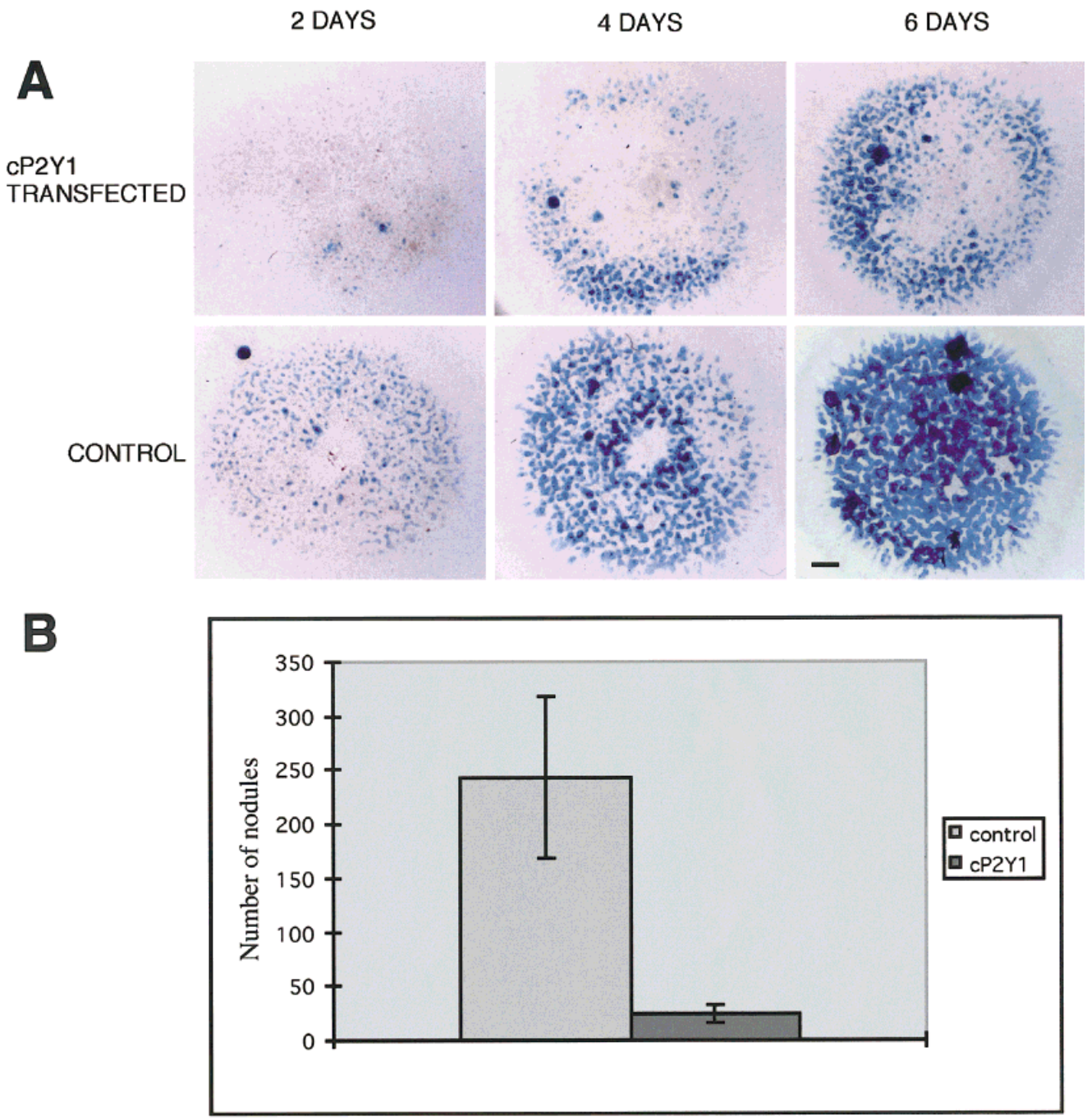

Fig. 5. **A:** Expression of $cP2Y_1$ reduces the amount of cartilage formed in limb micromass cultures. Cartilage nodules have been stained with Alcian blue. In the top row, micromass cultures have been transfected with pRCCMV-$cP2Y_1$ and fixed 2, 4, and 6 days after transfection. In the bottom row, cultures have been transfected with the control construct (pRCCMV) and fixed 2, 4, and 6 days after transfection. **B:** The number of cartilage nodules formed in micromass cultures 2 days after transfection is drastically reduced in pRCCMV-cP2Y transfected cultures (dark bar) compared with control transfected cultures (lighter bar). Error bars show ± SEM.

micromass cultures. The signaling events important for the initial commitment of mesenchymal cells to a chondrogenic lineage are known to begin within the first 24 hr of micromass culture. For example, the transcription factor *Sox9,* which is required for cartilage formation (Bi et al., 1999), is up-regulated as early as 2 hr after plating and its expression peaks between 20 and 65 hr of culture (Kulyk et al., 2000). The aggregation of mesenchyme into precartilage condensations also begins within the first 24 hr of culture. Because

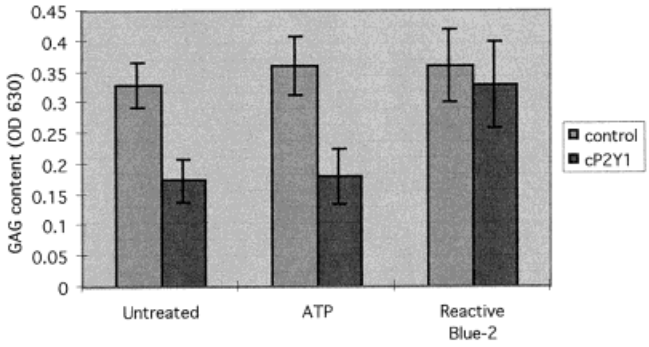

Fig. 6. Glycosaminoglycan (GAG) content of 6-day micromass cultures are shown on the y axis, and experimental conditions are shown on the x axis. GAG content of control (pRCCMV-transfected) cultures are shown in the lighter bars, and GAG content of pRCCMV-cP2Y$_1$-transfected cultures are shown in the darker bars. Cartilage content in pRCCMV-cP2Y$_1$-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 cP2Y$_1$ expression on cartilage formation without affecting the level of cartilage formed in control cultures. Error bars show ± SEM.

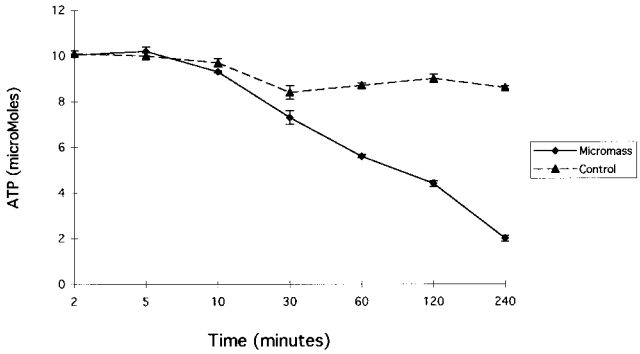

Fig. 8. ATP is broken down rapidly in limb micromass cultures. ATP (10 μM) was added to micromass cultures, and the concentration of ATP was measured over a 4-hr period (diamonds, solid line). ATP was also measured in control medium in the absence of cells (triangles, dashed line). The half-life of ATP in limb micromass cultures is approximately 1 hr, whereas in the absence of cells, only 10% of the ATP was broken down over the 4-hr assay period. Note that the time axis is nonlinear. For each time point, n = 2, and error bars show ± SEM.

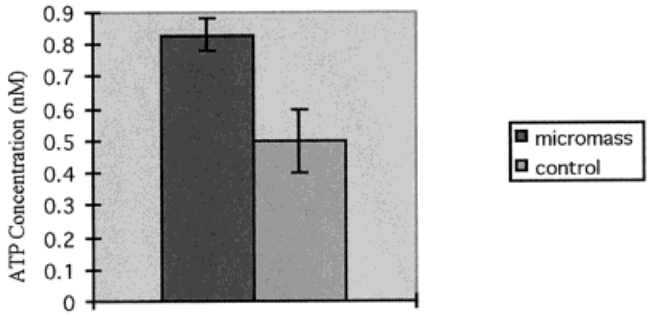

Fig. 7. ATP is produced by cells in limb micromass cultures. The darker bar shows the concentration of ATP in a samples of phosphate buffered saline (PBS) that have been incubated with cells for 4 hr (n = 4). The lighter bar shows the baseline luminometer reading obtained from control samples of PBS that have not been incubated with cells (n = 4). Error bars show ± SEM.

our transfection protocol does not begin until 24 hr after initial plating, we are unable to say whether the cP2Y$_1$ receptor inhibits these initial events but the reduction in Alcian blue staining indicates that cP2Y$_1$ expression can at least inhibit the differentiation steps that lead to sulphated glycosaminoglycan production.

There is now considerable evidence suggesting a role for P2Y receptor signaling in regulating changes in cell differentiation and proliferation (reviewed in Abbracchio, 1996; Neary and Burnstock, 1996), and the possibility that extracellular ATP signalling may be important during embryonic development has been suggested following previous work by using adult tissues, primary cells in culture and cell lines (e.g., Huang et al., 1989; Ciccarelli et al., 1994; Neary et al., 1994a,b). Physiologic and pharmacologic experiments have demonstrated that the G protein-coupled P2Y receptors are linked to signalling mechanisms that have been implicated in mitogenesis and differentiation, such as Ca^{2+} mobilisation, polyphosphoinositide metabolism, and protein phosphorylation (Dubyak and El-Moatassim, 1993). For example, ATP has been shown to activate mitogen activated protein (MAP) kinases by means of P2Y$_1$ and P2Y$_2$ receptors in rat astrocytes (Neary and Zhu, 1994). The MAP kinase cascade is a key element of signal transduction pathways involved in cellular proliferation and differentiation (Davis, 1993). In some systems, extracellular ATP signalling has been shown to synergise with polypeptide growth factors such as epithelial growth factor (EGF) and FGF (Huang et al., 1989; Neary et al., 1994a). Therefore the role of cP2Y$_1$ in the limb may depend on the expression of various polypeptide growth factors that are known to be important during limb development.

Although we have studied the effects of cP2Y$_1$ only on cartilage differentiation, its expression pattern in the limb suggests its potential role may be much broader than this. Expression of cP2Y$_1$ is down-regulated in areas of joint and tendon formation as well as cartilage development, and cP2Y$_1$ is strongly expressed in broad domains at early stages of limb development before most cells have embarked on a differentiation pathway. Together, these early and later expression patterns could suggest that cP2Y$_1$ plays a role in the function of undifferentiated cells or in maintaining the undifferentiated state. Furthermore, in addition to presumptive chondrocytes, micromass cultures also contain a population of myoblasts that differentiate into muscle cells. We have not monitored the effect of cP2Y1 transfection on this cell type, although the expression pattern in the early limb bud suggests that there is at least some overlap of cP2Y$_1$ expression and the marker of migrating myoblasts *Pax3* (Amthor et al., 1998).

Additional P2 Receptor Subtypes Are Expressed in the Developing Chick Limb

Our results demonstrate that purinergic signalling by means of the cP2Y1 receptor can inhibit the differentiation of limb mesenchyme cells at least under the conditions of micromass culture. However, the role of purinergic signalling in the intact embryonic limb remains untested and could involve the participation of other purinergic receptors whose expression has yet to be described but are probably present. ATP and 2MeSATP are found to be potent agonists and ADP a weak agonist in the recombinant $cP2Y_1$ receptor expressed in *Xenopus* oocytes and COS-7 cells (Webb et al., 1993; Simon et al., 1995). However, although the pharmacology of cultured limb cells transfected with $cP2Y_1$ matches this agonist profile, the strong response we observe to ADP in freshly dissociated limb cells does not, and this is likely mediated by another P2Y receptor subtype, that like $cP2Y_1$ disappears from limb cells when placed in culture. One possible function of this ADP receptor may be to prolong or increase the response initially elicited by ATP. ATP acting through $cP2Y_1$ would initiate a response, and then ADP (the direct catabolite of ATP) may then initiate an additional response through a separate receptor. A surprising result was that UTP (which was found not to activate the recombinant $cP2Y_1$ receptor expressed in heterologous systems [Webb et al., 1993; Simon et al., 1995]) was able to elicit responses in limb cells transfected with $cP2Y_1$. The ligand binding properties of $cP2Y_1$ could be different in limb cells and *Xenopus* oocytes and COS-7 cells, but currently, there is no evidence to suggest that the ligand binding properties of this receptor family changes from cell to cell. Alternatively, a UTP receptor may be genetically downstream of $cP2Y_1$, and by rescuing expression of $cP2Y_1$, expression of a UTP receptor is also subsequently rescued. In astrocytes, P2Y receptors have been shown to regulate the formation of activator protein (AP-1) complexes (Neary et al., 1996), which are transcriptional regulators. Further studies are needed to establish whether $cP2Y_1$ or a separate receptor are responsible for the UTP responses in limb cells. If the expression of a UTP receptor is genetically downstream of $cP2Y_1$, this finding would raise the possibility that the effect of $cP2Y_1$ expression on cartilage formation may be an indirect one.

ATP, the Ligand for $cP2Y_1$ Is Produced by Limb Mesenchyme Cells, and These Cells Have Mechanisms to Breakdown Extracellular ATP

In addition to demonstrating that $cP2Y_1$ expression inhibits the formation of cartilage in micromass cultures, we show that early limb cells in culture release small amounts of extracellular ATP and have mechanisms that effectively breakdown extracellular ATP. The concentration of released ATP was very low (0.33 nM), 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 or native $cP2Y_1$. That limb mesenchyme cells possess mechanisms for breaking down extracellular ATP suggests that they normally do release or are exposed to this nucleotide. Our work has examined cells at a much earlier stage of development than previous studies on chick embryo chondrocytes. By using high-performance liquid chromatography, Hatori et al. (1995) have shown that chondrocytes taken from embryonic day 19 chick embryos also secrete significant quantities of adenine nucleotides into the culture medium. Their study showed that concentrations of 400–800 nM ADP were detectable in chondrocyte conditioned medium but that ATP was detected at much lower concentrations. However, 70% of exogenously added ATP was degraded within 15 min in chondrocyte cultures, whereas the rate of ADP breakdown by these cells was found to be considerably slower, with a half-life of 2–3 hr. Together with our data, this finding suggests that extracellular nucleotides are used as extracellular signalling molecules over a considerable period of chick embryonic development. We are unable to identify which particular cell types release ATP in our limb mesenchyme cultures, which will contain a variety of cell types (e.g., presumptive cartilage and muscle) as well as undifferentiated cells.

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

We demonstrate that Reactive Blue-2 can reverse the inhibition of cartilage formation by $cP2Y_1$ in vitro and that Reactive Blue-2 at a concentration of 100 μM was effective at blocking the $Ca^{2+}{}_i$ response of $cP2Y_1$ transfected cells to 100 μM ATP. Reactive Blue-2 is, however, a general antagonist for P2Y receptors and not specific for $cP2Y_1$. It is also possible that Reactive Blue-2 interacts with cellular mechanisms unrelated to P2 receptor signaling as it has the potential to bind to other molecules with an ATP binding site and has, for example, been used as an affinity reagent in chromatography studies for the purification of enzymes that use nucleotides as substrates or cofactors. Although it is, thus, possible that the effect of Reactive Blue-2 on cartilage differentiation could be a result of action not related to $cP2Y_1$ antagonism, we believe this is not the case for several reasons. First, we have shown that within 24 hr of culturing, the $Ca^{2+}{}_i$ responses of limb mesenchyme cells to the P2Y receptor agonists, ATP, ADP, and UTP were all lost, thus, suggesting that all P2Y receptors are lost. It is unlikely, therefore, that in $cP2Y_1$-transfected cultures, Reactive Blue-2 exerts its effect on cartilage formation by blocking other P2Y receptor subtypes. Second, Reactive Blue-2 does not alter the differentiation of cartilage in control cultures that do not express $cP2Y_1$, suggesting that Reactive

Blue-2 itself does not promote cartilage formation. Reactive Blue-2–treated cultures also appeared morphologically indistinct from untreated cultures. Together, these observations suggest that Reactive Blue-2 only exerts its effects in our cultures by means of the cP2Y$_1$ receptor.

In conclusion, we have shown that a receptor for extracellular ATP, cP2Y$_1$, is expressed in a prolonged and dynamic manner during limb development. cP2Y$_1$ is preferentially expressed in undifferentiated cells and we have shown that expression of this receptor in vitro can inhibit the differentiation of cartilage from limb bud mesenchyme. These results suggest that purinergic signalling through the cP2Y$_1$ receptor could play a significant role in the regulation of cell differentiation in the developing limb bud.

EXPERIMENTAL PROCEDURES

Embryos

Eggs were obtained from Needle Farm, Essex, UK, incubated at 37°C and staged according to the Hamburger and Hamilton (HH) series (Hamburger and Hamilton, 1951).

Generation of Riboprobes and Whole-Mount In Situ Hybridization

A 1.4-kb cP2Y$_1$ cDNA fragment was subcloned into the *Hin*dIII site of pRCCMV (Invitrogen) to generate the construct pRCCMV-cP2Y$_1$. Generation of riboprobes and in situ hybridisation was carried out as described in Meyer et al., 1999.

Cell Culture

Chick wing buds from stage 20–22 embryos were dissected in sterile Howard's Ringer's solution. Wings buds were then briefly rinsed in Dulbecco's modified Eagle's medium (DMEM) followed by Hank's balanced salt solution (HBSS) (both from Gibco), before the ectoderm was removed by incubating in 0.5% trypsin (Gibco) for 1 hr on ice. For monitoring intracellular calcium, cells were thoroughly dissociated by trituration before plating 100-μl drops at 3×10^6 cells/ml on 22-mm diameter acid cleaned glass coverslips in DMEM + 10% fetal bovine serum (FBS) + 1% antibiotic/antimicotic solution (all from Gibco). Cells were then flooded with 1 ml of the above culture medium and cultured for 1 hr at 37°C in a 5% CO_2 incubator.

Micromass cultures were set up as described above, with the following modifications: cells were plated in 80-μl spots in 4-well tissue culture plates, and transfection was carried out by using the transfection reagent Fugene 6 as described below. ATP (Boehringer) was added to cultures at a final concentration of 100 μM after the 8-hr transfection period and every 24 hr thereafter. Reactive Blue-2 (Sigma) was prepared in distilled water and filter sterilized before adding to cultures at a final concentration of 100 μM immediately after transfection.

Monitoring Intracellular Calcium

After 1 hr of culture, cells were loaded with Fura-Red (Molecular Probes) by using the -AM form of the dye, at a final concentration of 5 μM in HEPES buffered DMEM + 250 μM sulfinpyrazone (ICN), for 1 hr at room temperature. Fura red-AM was made up as 2 mM stocks in dimethyl sulfoxide (DMSO) plus 5% pluronic F127 (Molecular Probes). Inclusion of the anion exchange inhibitor, sulfinpyrazone, in the loading medium prevents compartmentalization and extrusion of the dye (Di Virgilio et al., 1990). 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. 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 by using a CCD camera. Fluorescence from Fura Red is plotted as an excitation ratio at 440 nm/490 nm (Kurebayashi et al., 1993).

RNase Protection

Total RNA was isolated from freshly dissected stage 20–22 limb buds, and from 24-hr limb mesenchyme cultures by using the RNeasy total RNA isolation kit (Qaigen). The cultures were prepared as described above, and cells were harvested after 24 hr by treatment with 0.5% trypsin solution (Gibco). RNase protection assays were performed by using the Ambion RPA II kit according to the manufacturer's instructions. Samples were analyzed with an antisense *cP2Y$_1$* probe, and a GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (Panabieres et al., 1984) antisense probe was used as a loading control. Control hybridizations with tRNA were always negative and are not shown in the figures.

Transfection

Cultures were set up as described above and were left for 24 hr before transfecting. The plasmid construct pRCCMV-cP2Y$_1$ was transfected into limb mesenchyme cells by using Fugene 6 (Roche Biochemicals), a lipid (nonliposomal) -based transfection reagent, according to the manufacturers instructions. Briefly, 5 μl of Fugene 6 was added to 95 μl of serum-free DMEM in a sterile 0.5-ml Eppendorf tube and left to incubate for 5 min at room temperature. This was then added dropwise to 2 μl of 1 μg/μl plasmid DNA in a second Eppendorf tube. DNA-Fugene 6 complexes were then allowed to form at room temperature for 20 min. This mixture was added dropwise to the limb mesenchyme cultures, which were then incubated for a further 8 hr at 37°C, 5% CO_2. Cultures were then washed with

fresh DMEM + 10% FBS + 1% antibiotic/antimicotic solution and left for 1–3 days before assaying for $Ca^{2+}{}_i$ responses to 100 μM ATP, ADP, 2MeSATP, and UTP. These were found to be the optimal transfection conditions (as assayed by the percentage of cells generating a $Ca^{2+}{}_i$ response to 100 μM ATP). Some cultures were also transfected with empty vector (pRCCMV without the $cP2Y_1$ cDNA insert) as a negative control. Dye loading and monitoring [$Ca^{2+}{}_i$] in control (pRCCMV) and pRCCMV-$cP2Y_1$ transfected cultures were carried out as described above.

Alcian Blue Staining of Micromass Cultures and GAG Assay

Cartilage was visualized 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 (Francis-West et al., 1999). 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 1 ml 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 above, with the following modifications. For the ATP breakdown assay, 1.2×10^7 cells were plated in 400-μl spots in 6-well tissue culture plates. Cells were left to settle for 1 hr before flooding with 2 ml 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 2 ml of fresh PBS for 10 min before addition of ATP to a final concentration of 10 μM. Samples of 60 μl 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 10 μM was added to 2 ml of PBS in tissue culture plates and incubated under identical conditions to the above cultures. Samples of 60 μl were again taken at the same time intervals. To measure ATP production by cells in micromass cultures, micromass cultures were set up in 4-well plates as described in a previous section. Cultures were left for 3 days before washing and incubating for 4 hr in PBS. Samples of 100 μl were removed and spun at 2,000 rpm for 10 min to pellet any cells that may contaminate the samples. Samples of supernatant (60 μl) were carefully removed and assayed for ATP content. The concentration of ATP in each sample was measured using an ATP-monitoring reagent (Luciferin-luciferase) (Bio Orbit), and a LUCY1 luminometer (Anthos Labtech) (for detailed protocol see Bodin and Burnstock, 1996).

ACKNOWLEDGMENTS

We thank Les Dale, Jon Gilthorpe, Brian King, and Mary Rahmen for their help with this work; and Les Dale and Paul Martin for advice during preparation of this manuscript.

REFERENCES

Abbracchio MP. 1996. P1 and P2 receptors in cell growth and differentiation. Drug Dev Res 39:393–406.

Abbracchio MP, Burnstock G. 1994. Purinoceptors: are there families of P2X and P2Y purinoceptors? Pharmacol Ther 64:445–475.

Ahrens PB, Solursh M, Reiter RS. 1977. Stage-related capacity for limb chondrogenesis in cell culture. Dev Biol 60:69–82.

Amthor H, Christ B, Patel K. 1998. The importance of timing differentiation during limb muscle development. Curr Biol 8:642–652.

Berridge MJ. 1993. Inositol trisphosphate and calcium signalling. Nature 361:315–325.

Burnstock G. 1996. Purinoceptors: ontogeny and phylogeny. Drug Dev Res 39:204–242.

Bi W, Deng JM, Zhang Z, Behringer RR, de Crombrugghe B. 1999. Sox9 is required for cartilage formation. Nat Genet 22:85–89.

Bodin P, Burnstock G. 1996. ATP-stimulated release of ATP by human endothelial cells. J Cardiovasc Pharmacol 27:872–875.

Ciccarelli R, Di Iorio P, Ballerini P, Ambrosini G, Giuliani P, Tiboni GM, Caciagli F. 1994. Effects of exogenous ATP and related analogues on the proliferation rate of dissociated primary cultures of rat astrocytes. J Neurosci Res 39:556–566.

Davis RJ. 1993. The mitogen-activated protein kinase signal transduction pathway. J Biol Chem 268:14553–14556.

Di Virgilio F, Steinberg TH, Silverstein SC. 1990. Inhibition of Fura-2 sequestration and secretion with organic anion transport blockers. Cell Calcium 11:57–62.

Francis-West PH, Abdelfattah A, Chen P, Allen C, Parish J, Ladher R, Allen S, MacPherson S, Luyten FP, Archer CW. 1999. Mechanisms of GDF-5 action during skeletal development. Development 126: 1305–1315.

Hamburger V, Hamilton HL. 1951. A series of normal stages in the development of the chick embryo. J Morphol 88:49–92.

Hatori M, Teixeira CC, Debolt K, Pacifici M, Shapiro IM. 1995. Adenine nucleotide metabolism by chondrocytes in vitro: role of ATP in chondrocyte maturation and matrix mineralization. J Cell Physiol 165:468–474.

Huang N, Wang DJ, Heppel LA. 1989. Extracellular ATP is a mitogen for 3T3, 3T6, and A431 cells and acts synergistically with other growth factors. Proc Natl Acad Sci USA 86:7904–7908.

Jiang TX, Yi JR, Ying SY, Chuong CM. 1993. Activin enhances chondrogenesis of limb bud cells: stimulation of precartilaginous mesenchymal condensations and expression of NCAM. Dev Biol 155: 545–557.

Kulyk WM, Franklin JL, Hoffman LM. 2000. Sox9 expression during chondrogenesis in micromass cultures of embryonic limb mesenchyme. Exp Cell Res 255:327–332.

Kurebayashi N, Harkins AB, Baylor SM. 1993. Use of fura red as an intracellular calcium indicator in frog skeletal muscle fibers. Biophys J 64:1934–1960.

Lauder JM. 1993. Neurotransmitters as growth regulatory signals: role of receptors and second messengers. Trends Neurosci 16:233–240.

Meyer MP, Clarke JDW, Patel K, Townsend-Nicholson A, Burnstock G. 1999. Selective expression of a purinoceptor $cP2Y_1$ suggests a role for nucleotide signalling in development of the chick embryo. Dev Dyn 214:152–158.

Neary JT, Burnstock G. 1996. Purinoceptors in the regulation of cell growth and differentiation. Drug Dev Res 39:407–412.

Neary JT, Whittemore SR, Zhu Q, Norenberg MD. 1994a. Synergistic activation of DNA synthesis in astrocytes by fibroblast growth factors and extracellular ATP. J Neurochem 63:490–494.

Neary JT, Baker L, Jorgensen SL, Norenberg MD. 1994b. Extracellular ATP induces stellation and increases glial fibrillary acidic protein content and DNA synthesis in primary astrocyte cultures. Acta Neuropathol (Berl) 87:8–13.

Neary JT, Zhu Q, Kang Y, Dash PK. 1996. Extracellular ATP induces formation of AP-1 complexes in astrocytes via P2 purinoceptors. Neuroreport 7:2893–2896.

Panabieres F, Piechaczyk M, Rainer B, Dani C, Fort P, Riaad S, Marty L, Imbach JL, Jeanteur P, Blanchard JM. 1984. Complete nucleotide sequence of the messenger RNA coding for chicken muscle glyceraldehyde-3-phosphate dehydrogenase. Biochem Biophys Res Commun 118:767–773.

Roark EF, Greer K. 1994. Transforming growth factor-beta and bone morphogenetic protein-2 act by distinct mechanisms to promote chick limb cartilage differentiation in vitro. Dev Dyn 200:103–116.

Simon J, Webb TE, King BF, Burnstock G, Barnard EA. 1995. Characterisation of a recombinant P2Y purinoceptor. Eur J Pharmacol 291:281–289.

Webb TE, Simon J, Krishek BJ, Bateson AN, Smart TG, King BF, Burnstock G, Barnard EA. 1993. Cloning and functional expression of a brain G-protein-coupled ATP receptor. FEBS Lett 324:219–225.