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ATP analogues with modified phosphate chains and their selectivity for rat $P2X_2$ and $P2X_{2/3}$ receptors

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1 Heteromeric $P2X_{2/3}$ receptors are much more sensitive than homomeric $P2X_2$ receptors to $\alpha\beta$-methylene-ATP, and this ATP analogue is widely used to discriminate the two receptors on sensory neurons and other cells.

2 We sought to determine the structural basis for this selectivity by synthesising ADP and ATP analogues in which the $\alpha\beta$ and/or $\beta\gamma$ oxygen atoms were replaced by other moieties (including $-CH_2-$, $-CHF-$, $-CHCl-$, $-CHBr-$, $-CF_2-$, $-CCl_2-$, $-CBr_2-$, $-CHSO_3-$, $-CHPO_3-$, $-CFPO_3-$, $-CClPO_3-$, $-CH_2-CH_2-$, $-C\equiv C-$, $-NH-$, $-CHCOOH-$).

3 We tested their actions as agonists or antagonists by whole-cell recording from human embryonic kidney cells expressing $P2X_2$ subunits alone (homomeric $P2X_2$ receptors), or cells expressing both $P2X_2$ and $P2X_3$ subunits, in which the current through heteromeric $P2X_{2/3}$ receptors was isolated.

4 ADP analogues had no agonist or antagonist effect at either $P2X_2$ or $P2X_{2/3}$ receptors. All the ATP analogues tested were without agonist or antagonist activity at homomeric $P2X_2$ receptors, except $\beta\gamma$-difluoromethylene-ATP, which was a weak agonist.

5 At $P2X_{2/3}$ receptors, $\beta\gamma$-imido-ATP, $\beta\gamma$-methylene-ATP, and $\beta\gamma$-acetylene-ATP were weak agonists, whereas $\alpha\beta,\beta\gamma$- and $\beta\gamma,\gamma\delta$-bismethylene-AP_4 were potent full agonists. $\beta\gamma$-Carboxymethylene-ATP and $\beta\gamma$-chlorophosphonomethylene-ATP were weak antagonists at $P2X_{2/3}$ receptors (IC_{50} about 10 μM).

6 The results indicate (a) that the homomeric $P2X_2$ receptor presents very stringent structural requirements with respect to its activation by ATP; (b) that the heteromeric $P2X_{2/3}$ receptor is much more tolerant of $\alpha\beta$ and $\beta\gamma$ substitution; and (c) that a $P2X_{2/3}$-selective antagonist can be obtained by introduction of additional negativity at the $\beta\gamma$-methylene.
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Abbreviations: AP_4, adenosine 5′-tetraphosphate; PPADS, pyridoxal-5-phosphate-6-azo-2′,4′-disulphonic acid derivative; TNP-ATP, 2′,3′-*O*-(2,4,6-trinitrophenol)-ATP; $\alpha\beta$meATP, $\alpha\beta$-methylene-ATP

Introduction

The $P2X_3$ receptor subunit is of particular interest because its expression is largely restricted to primary afferent neurones (Chen *et al*., 1995; Lewis *et al*., 1995). Immunohistochemistry with antibodies directed against a C-terminal epitope showed that the subunit was expressed by a subset of cells in the nodose and dorsal root ganglia, as well as on their central and peripheral terminals (Vulchanova *et al*., 1997; 1998; Bradbury *et al*., 1998; see the review by Dunn *et al*., 2001). This subset has small diameter cell bodies and also expresses the receptor for the lectin B4. On the basis of this distribution, and earlier evidence that ATP could elicit pain (Bleehen & Keele, 1977), the $P2X_3$ subunit was considered likely to play a key role in the signalling of pain sensation (reviewed by Dunn *et al*., 2001; see North, 2002). The breeding of mice with a disrupted $P2X_3$ subunit gene has substantiated the role of the receptor in some forms of pain, but also indicated a wider role in primary afferent signalling (Cockayne *et al*., 2000; Souslova *et al*., 2000). In particular, the $P2X_3$ subunit is critical for the primary afferent signals arising from bladder distension, where ATP released from urothelium appears to be the initiating stimulus (Ferguson *et al*., 1997; Vlaskovska *et al*., 2001).

P2X receptors form as multimers of several, perhaps three, subunits (reviewed by North, 2002). Although the composition of the receptors in native tissues is not known with any certainty, there is ample evidence that a heteromeric channel containing $P2X_2$ and $P2X_3$ subunits is abundant in primary afferent neurons. This comes from electrophysiological experiments, in which the effects of ATP are compared with those of its analogue $\alpha\beta$-methylene-ATP ($\alpha\beta$meATP) (Figure 1). In most small/medium-sized primary afferent neurons from dorsal root ganglia and trigeminal ganglia, and all from the nodose ganglia, both ATP and $\alpha\beta$meATP elicit an inward

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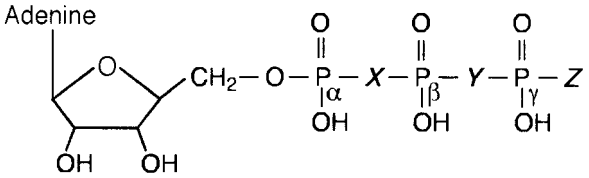

Figure 1 Nomenclature used for the ATP analogues (see Table 1). ATP: $X=Y=Z=O$. $\alpha\beta$meATP: $X=-CH_2-$, $Y=Z=O$.

current that shows little desensitisation during a few seconds (Cook *et al*., 1997; Rae *et al*., 1998; Thomas *et al*., 1998; Virginio *et al*., 1998b; Grubb & Evans, 1999; Li *et al*., 1999; Ueno *et al*., 1999; Burgard *et al*., 2000; Labrakakis *et al*., 2000; Liu *et al*., 2001). This corresponds to the properties observed when $P2X_2$ and $P2X_3$ subunits are coexpressed, and not to the properties of the currents when either $P2X_2$ or $P2X_3$ subunits are expressed separately (Lewis *et al*., 1995). The supporting observations are the findings that many small dorsal root ganglion cells, and all nodose ganglion cells, express immunoreactivity for both $P2X_2$ and $P2X_3$ subunits, and the finding that $P2X_2$ and $P2X_3$ subunits will coimmunoprecipitate in heterologous expression systems (Radford *et al*., 1997; Torres *et al*., 1999). Moreover, the finding that the current is sustained at the heteromeric $P2X_{2/3}$ receptor, but desensitises rapidly (100 ms) at the homomeric $P2X_3$ receptor, leads one to argue that the signal to the heteromeric $P2X_{2/3}$ receptor is likely to be more important in functional terms.

Receptors containing $P2X_3$ subunits can also be differentiated from other forms with the antagonist 2′,3′-*O*-(2,4,6-trinitrophenol)-ATP (TNP-ATP) (Virginio *et al*., 1998b; Burgard *et al*., 2000; North & Surprenant, 2000). This compound has nanomolar affinity as a blocker of $P2X_3$ and $P2X_{2/3}$ receptors, but blocks homomeric $P2X_2$ receptors only at concentrations some 1000 times higher. TNP-ATP has been used to demonstrate that individual sensory neurons in the nodose ganglion express more than one kind of P2X receptor (Thomas *et al*., 1998). When $\alpha\beta$meATP is used as agonist, the TNP-ATP inhibition curve is monophasic, and the concentration causing half-maximal inhibition (IC_{50}) is the same as that for heterologously expressed $P2X_{2/3}$ heteromers. But when ATP is used as the agonist, the inhibition curve is biphasic; the two IC_{50} values correspond well to those for heteromeric $P2X_{2/3}$ channels and for homomeric $P2X_2$ channels. TNP-ATP has also been used to provide further evidence for involvement of a $P2X_3$ subunit-containing receptor in visceral pain sensation. The abdominal constriction induced in mice by intraperitoneal acetic acid was inhibited by TNP-ATP; on a molar basis, TNP-ATP was comparable to morphine (Honore *et al*., 2002). However, TNP-ATP has certain drawbacks. First, it blocks $P2X_1$ receptors almost as effectively as $P2X_3$ or $P2X_{2/3}$ receptors. Secondly, it seems not to work well in some intact tissues, perhaps because of rapid degradation (Lewis *et al*., 1998). On the other hand, the metabolically stable antagonist of $P2X_3$-containing subunits recently reported by Jarvis *et al*. (2002) (A-317491: *S*-5-[(3-phenoxy-benzyl)-(1,2,3,4-tetra-hydro-naphthalen-1-yl)-carbamoyl-benzene-1,2,4-tricarboxylic acid) is effective in several models of chronic pain. Both these classes of $P2X_3$ receptor blockers retain unmodified phosphate chains.

Although ATP activates all P2X receptor subunits, $\alpha\beta$ meATP is effective only at those containing a $P2X_1$ or $P2X_3$ subunit. This discrimination on the basis of a single –O– to $-CH_2-$ substitution suggested that it may be possible to develop ATP analogues with modified phosphate chains that might be useful to separate activity at the $P2X_2$ and $P2X_{2/3}$ subunits. In the present study, we have synthesised several such analogues and compared their activity as agonists and antagonists at heterologously expressed homomeric $P2X_2$ and heteromeric $P2X_{2/3}$ receptors.

Methods

Synthesis of ATP analogues

$\alpha\beta$-substituted analogues of ADP were generally prepared using the method of Poulter (Davisson *et al*., 1987). They were phosphorylated to give $\alpha\beta$-substituted analogues of ATP using either *p*-nitrobenzyl phosphoromorpholidate (Blackburn & Langston, 1991) or equivalent methods. $\alpha\beta,\beta\gamma$-Bis-substituted analogues of ATP were also prepared using the Poulter methodology with appropriate bis-substituted analogues of tripolyphosphate (Davisson *et al*., 1987). $\beta\gamma$-substituted ATP analogues were prepared by condensation of AMP with the appropriate analogues of pyrophosphate using either diphenyl phosphorochloridate or AMP morpholidate (Blackburn *et al*., 1984; 1985; Liu *et al*., 1999). $\alpha\beta,\beta\gamma$-Bis-substituted analogues of adenosine 5′-tetraphosphate (AP_4) were prepared from the corresponding ATP analogues by phosphorylation with either *p*-nitrobenzyl phosphoromorpholidate or inorganic phosphate and carbonyl di-imidazole (Blackburn, unpublished results). $\beta\gamma,\gamma\delta$-Bis-substituted analogues of AP_4 were prepared by condensation of AMP morpholidate with the corresponding analogues of tripolyphosphate (Blackburn, unpublished results). Adenosine 5′-phosphoromorpholidate was also condensed with phosphonoacetic acid and with β-phosphono-α-alanine to provide adenylyl phosphonoacetic acid and adenylyl β-phosphono-α-alanine, respectively (Blackburn, unpublished results). The final products were purified by ion-exchange medium pressure liquid chromatography. Nucleotides were isolated as their triethylammonium salts and converted into their sodium salts for use by ion exchange. Purity was uniformly in excess of 98%, as gauged by proton and phosphorus nuclear magnetic resonance and by electrospray mass spectrometry. The identity of all compounds was established by high-resolution mass spectrometry and by phosphorus and proton nuclear magnetic resonance. ATP, $\alpha\beta$meATP, $\beta\gamma$-me-D-ATP and $\beta\gamma$-me-L-ATP were purchased from Sigma.

Cell culture and electrophysiological recording

HEK293 cells stably expressing the rat $P2X_2$ and rat $P2X_{2/3}$ receptors were used. Generation of stable cell lines has been previously described (Evans *et al*., 1995; Kawashima *et al*., 1998). Cells were plated onto 13 mm glass coverslips and maintained in Dulbecco's modified Eagle's medium, supplemented with 10% heat-inactivated fetal calf serum and 2 mM L-glutamine at 37°C in a humidified 5% CO_2 incubator. Culture media, sera and all cell culture reagents were obtained from Life Technologies (Paisley, U.K.)

Whole-cell recordings were performed at room temperature 24–72 h after the passage of stable cell lines, using an EPC9 patch-clamp amplifier (HEKA Elektronik, Lambrecht,

Germany). Experiments were performed at a holding potential of −60 mV. Patch electrodes had resistances of 4–7 MΩ and were filled with (mM): 145 NaCl or NaF, 10 HEPES, 10 EGTA. The extracellular solution was (mM): 147 NaCl, 2 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES and 13 glucose. Osmolarity and pH values of both solutions were maintained at 300–315 mOsm l^{-1} and 7.3, respectively. ATP and its analogues were applied by an array of glass inlet pipes using the RSC 200 (Biologic Science Instruments, Grenoble, France). ATP was used as the control agonist in all experiments on $P2X_2$ receptors (EC_{90} 30 μM); αβmeATP was used as the control agonist in all experiments on $P2X_{2/3}$ receptors (EC_{50} 5 μM). Agonists were applied for 3 s duration at 2 min interval, until evoked currents were stable (±5%); then an ATP analogue was applied to test for its agonist or antagonist activity. In the first case, the ATP analogue (at 100 μM) was applied for 3 s duration and the current evoked was measured. The peak current amplitude was expressed as a percentage of the amplitude obtained under the control agonist. Cumulative concentration–response curves were constructed by applying sequentially increasing concentrations at 2 min intervals. All results are expressed as the percentage response to a maximal concentration of control agonist, which was applied 2 min after the last ATP analogue concentration. The protocol for testing for antagonist activity was as follows. When the agonist-induced current amplitude was stable, the ATP analogue was applied for 30 s prior to the agonist application (for screening) or only during the agonist application (for more detailed dose–response studies). The peak current amplitude was expressed as a percentage of the amplitude obtained under control conditions. Antagonist concentration–inhibition curves were obtained using a fixed agonist concentration (αβmeATP 5 μM) and increasing antagonist concentrations.

Data analysis

Agonist concentration–current curves were fitted with $E = ([A]^n/([A]^n + EC_{50}{}^n))$, where E is the current as a fraction of the current evoked by a maximal concentration of the control agonist, EC_{50} is the concentration of agonist required for a half-maximal response and n is the Hill coefficient. Inhibition curves were fitted with $I/I_0 = 1/(1 + (IC_{50}/[B])^{-n})$, where I and I_0 are peak currents in the presence and absence of the antagonist (concentration $[B]$), IC_{50} is the concentration of antagonist required to block the control current by 50% and n is the Hill coefficient. The data in the figures are presented as mean ± s.e.m. from individual cells and the pooled data were fitted using Kaleidagraph (Synergy Software, Reading, PA, U.S.A.).

Results

In all, 22 ATP and 12 ADP derivatives with modified triphosphate and diphosphate chains were synthesised and tested. The oxygen bridge in the αβ, βγ and/or γδ position (Figure 1) was replaced with halomethylene, acetylene, imido groups and branched methylene groups bearing a negatively charged function, or more bulky substituents (Table 1).

ADP analogues

None of the ADP derivatives (compounds **23**–**34**, tested at 100 μM; Table 1) had any agonist activity either at the $P2X_2$ or $P2X_{2/3}$ receptors ($n = 2$–3 each). Likewise, none of these compounds reduced the current elicited by ATP at $P2X_2$ receptors, or by αβmeATP at $P2X_{2/3}$ receptors.

ATP analogues

At $P2X_2$ receptors, βγ-difluoromethyleneATP (**10**) was the only compound tested that showed any agonist activity. It was a weak agonist, with the maximal response at 300 μM being about 50% of the maximal ATP effect. This compound (**10**)($EC_{50} = 72 \pm 2.6$ μM, $n = 5$) was also about 10-fold less potent than ATP ($EC_{50} = 8.5 \pm 0.25$ μM, $n = 4$).

In contrast, eight ATP analogues showed agonist properties at the heteromeric $P2X_{2/3}$ receptor. These were αβ-difluoromethylene-ATP (**1**), βγ-methylene-D-ATP (**4**), βγ-imido-ATP (**5**), βγ-acetylene-ATP (**6**), βγ-difluoromethylene-ATP (**10**), αβ,βγ-bisdichloromethylene-ATP (**19**), αβ,βγ-bismethylene-AP_4 (**20**) and βγ, γδ-bismethylene-AP_4 (**21**); the last two can also be regarded as adenosine tetraphosphate analogues. The only triphosphate modification that retained full agonist activity was the replacement of both hydrogen atoms by chlorine at both the αβ and βγ bridges (αβ,βγ-bisdichloromethylene-ATP); however, this compound was still about 10-fold less potent than αβmeATP (EC_{50}: 22 ± 2.7 μM, $n = 8$). The two methylene-substituted tetraphosphate analogues were also full agonists, and αβ,βγ-bismethylene-AP_4 was the only compound examined that was more potent than αβmeATP (Figure 2). Compound **20** ($EC_{50} = 1.8 \pm 0.3$ μM, $n = 4$) gave a larger peak current than αβmeATP, whereas compound **21** ($EC_{50} = 11.8 \pm 0.7$ μM, $n = 4$) gave a peak current of amplitude similar to that of ATP (Figure 3). The other four compounds gave lower maximal currents, and were less potent than αβmeATP. The EC50's (in μM, number of observations in parentheses) were αβmeATP: 3.3 ± 0.4 (4); **4:** 176 ± 45 (6); **5:** 36.4 ± 4.6 (5) (Figure 3); **6:** 31.2 ± 1.4 (7) (Figure 3) and **10:** 41.6 ± 1.6 (6).

Cells transfected with both $P2X_2$ and $P2X_3$ plasmids express both homomeric $P2X_2$ and heteromeric $P2X_{2/3}$ receptors. As βγ-difluoromethylene-ATP (**10**) also had agonist activity at homomeric $P2X_2$ receptors, it is not possible to say whether it has an additional action at heteromeric $P2X_{2/3}$ receptors. The response to ATP of homomeric $P2X_2$ receptors is almost fully blocked by increasing the extracellular calcium concentration to 10 mM (Virginio *et al*., 1998a). We found that the current evoked by βγ-difluoromethylene-ATP (**10**) was similarly blocked by 10 mM calcium at cells expressing homomeric $P2X_2$ receptors (results not shown), but it was not affected by 10 mM calcium in the doubly transfected cells ($P2X_2$ and $P2X_3$); this implies that βγ-difluoromethylene-ATP (**10**) is an agonist also at the heteromeric $P2X_{2/3}$ receptor (Figure 4).

At $P2X_{2/3}$ receptors, βγ-carboxymethylene-ATP (**13**) and βγ-chlorophosphonomethylene-ATP (**17**) caused concentration-dependent inhibitions of the αβmeATP-evoked currents (Figure 5). Compound **17** ($IC_{50} = 14.4 \pm 12.8$ μM, $n = 2$–4) was about equipotent with **13** ($IC_{50} = 16.7 \pm 1.2$ μM, $n = 4$–6). None of the ATP derivatives displayed any antagonist activity on cells expressing $P2X_2$ receptors.

Table 1 Structures of ATP and ADP analogues used

Compound	*X*	*Y*	*Z*	*Name*	*$P2X_2$*	*$P2X_{2/3}$*
ATP	O	O	OH	ATP	Agonist	Agonist
αβmeATP	CH_2	O	OH	αβ-Methylene-ATP	None	Agonist ($EC_{50} \approx 3\,\mu M$)
1	CF_2	O	OH	αβ-Difluoromethylene-ATP	None	Weak agonist ($\approx 100\,\mu M$)[3]
2	CBr_2	O	OH	αβ-Dibromomethylene-ATP	None	Very weak agonist[2]
3	O	CH_2	OH	βγ-Methylene-L-ATP	None	Very weak agonist[2]
4	O	CH_2	OH	βγ-Methylene-D-ATP	None	Weak agonist ($\approx 200\,\mu M$)[3]
5	O	NH	OH	βγ-Imido-ATP	None	Weak agonist ($\approx 40\,\mu M$)[3]
6	O	C≡C	OH	βγ-Acetylene-ATP	None	Weak agonist ($\approx 30\,\mu M$)[3]
7	O	CH_2CH_2	OH	βγ-Ethylene-ATP	None	None
8	O	CHBr	OH	βγ-Bromomethylene-ATP	None	Very weak agonist[2]
9	O	CHCl	OH	βγ-Chloromethylene-ATP	None	Very weak agonist[2]
10	O	CF_2	OH	βγ-Difluoromethylene-ATP	Weak agonist	Weak agonist ($\approx 40\,\mu M$)[3]
11	O	CBr_2	OH	βγ-Dibromomethhylene-ATP	None	None
12	O	CCl_2	OH	βγ-Dichloromethylene-ATP	None	None
13	O	CHCOOH	OH	βγ-Carboxymethylene-ATP	None	Antagonist
14	O	$CHSO_3H$	OH	βγ-Sulphonomethylene-ATP	None	None
15	O	$CHPO_3H_2$	OH	βγ-Phosphonomethylene-ATP	None	Very weak agonist[2]
16	O	$CFPO_3H_2$	OH	βγ-Flurophosphonomethylene-ATP	None	Very weak agonist[2]
17	O	$CClPO_3H_2$	OH	βγ-Chlorophosphonomethylene-ATP	None	Antagonist
18	CH_2	CH_2	OH	αβ,βγ-Bismethylene-ATP	None	Very weak agonist[2]
19	CCl_2	CCl_2	OH	αβ,βγ-Bisdichloromethylene-ATP	None	Agonist
20	CH_2	CH_2	OPO_3H_2	αβ,βγ-Bismethylene-AP_4[1]	None	Agonist
21	O	CH_2	$CH_2PO_3H_2$	βγ,γδ-Bismethylene-AP_4[1]	None	Agonist
22	O	CCl_2	$CCl_2PO_3H_2$	βγ,γδ-Bisdichloromethylene-AP_4[1]	No	None
ADP	O	OH		ADP	None	None
23	CHBr	OH		αβ-Dibromomethylene-ADP	None	None
24	CBr_2	OH		αβ-Dibromomethylene-ADP	None	None
25	CF_2	OH		αβ-Difluoromethylene-ADP	None	None
26	CH_2CH_2	OH		αβ-Ethylene-ADP	None	None
27	CH=CH	OH		αβ-Etheno-(E)-ADP	None	None
28	C≡C	OH		αβ-Acetylene-ADP	None	None
29	$CHSO_3H$	OH		αβ-Sulphonomethylene-ADP	None	None
30	$CHPO_3H_2$	OH		αβ-Phosphonomethylene-ADP	None	None
31	CHCOOH	OH		αβ-Carboxymethylene-ADP	None	None
32	CHCOOBz	OH		αβ-Benzyloxycarbonylmethylene-ADP	None	None
33	O	CH_2COOH		CarboxymethylphosphonoAMP	None	None
34	O	$OCH_2CH(NH_2)COOH$		P^2-(O^3-L-serinyl)-ADP	None	None

[1]AP_4 = adenosine tetraphosphate. [2]Very weak agonist: analogue at 100 μM evokes a current that is <10% that caused by αβmeATP (5 μM). [3]Approximately EC_{50} for partial agonists.

Discussion

The results with ATP, ADP and αβmeATP confirm a body of work on these compounds at $P2X_2$ and $P2X_{2/3}$ receptors (North, 2002). At reasonable concentrations (<100 μM), we can say that ATP activates both, ADP activates neither, and αβmeATP is selective for $P2X_{2/3}$ receptors. In other words, $P2X_3$ subunits are tolerant of methylene in place of oxygen in the αβ position, whereas $P2X_2$ subunits are not. The complete lack of activity of any ADP analogue at either receptor (Table 1) confirms the essential requirement for a triphosphate as distinct from a diphosphate chain.

With respect to the homomeric $P2X_2$ receptor, it is remarkable that virtually every modification resulted in complete loss of activity. The only exception was the addition of two fluorine atoms onto the βγ carbon, which gave a compound (**10**) with weak partial agonist action; introduction of larger chlorine and bromine at this position was not tolerated. This finding indicates a key requirement for compactness and/or electronegativity at this position. Presumably, the βγ oxygen atom itself plays a critical role through hydrogen bonding to residues in the binding pocket of the $P2X_2$ subunit. This is also the case for the case of the αβ oxygen, where substitution by methylene results in a complete loss of activity.

Although the $P2X_{2/3}$ receptor was more tolerant than the $P2X_2$ receptor of replacement of the phosphate chain oxygen atoms, no compounds were found with agonist activity as large as αβmeATP (a direct comparison with ATP itself is difficult because ATP might activate both homomeric $P2X_2$ and heteromeric $P2X_{2/3}$ on the same cells). At the αβ position, activity was reduced by introduction of fluorine atoms, and completely abolished by the larger halogen bromine. At the βγ position, the most effective analogues (but still about 10 times higher EC_{50} than αβmeATP) were those that only modestly increased the size at this position (**5**: −NH−; **6**: −C≡C−; or **7**: $-CF_2-$), whereas larger substitutions caused complete loss of activity (**8**: −CHCl−; **9**: CHBr−; **11**: CBr_2-; **12**: $-CCl_2-$). The stereochemistry of the ribose moiety is obviously critical for activity; the D-isomer of βγmeATP was weakly able to induce currents in cells expressing the $P2X_{2/3}$ receptor (EC_{50} about 200 μM; Figure 2), whereas its L-enantiomer was essentially without activity (Table 1).

Replacement of both oxygen atoms by methylene reveals interactive effects. αβ-Methylene-ATP is fully active as an agonist, βγ-methylene-ATP (**4**) is about 100× less effective, and the doubly substituted αβ,βγ-bismethylene-ATP (**18**) is essentially without activity. However, simple extension of the phosphate chain restores activity to αβ,βγ-bismethylene-ATP

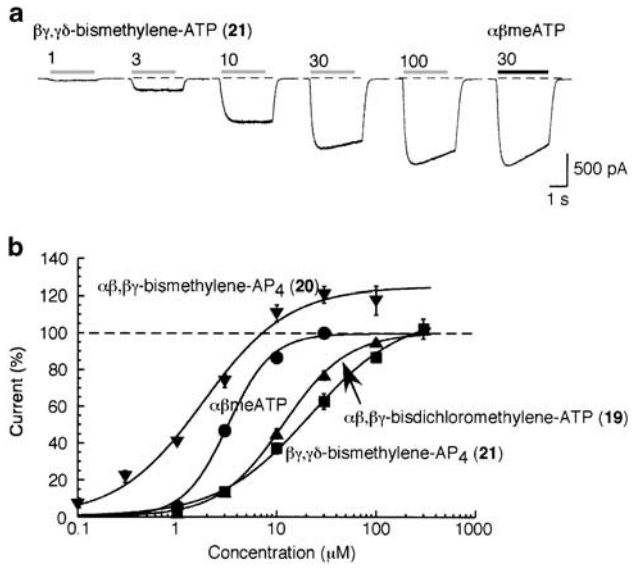

Figure 2 Full agonists at $PX_{2/3}$ receptors. Currents evoked by $\alpha\beta,\beta\gamma$-bisdichloromethylene-ATP (**19**), $\alpha\beta,\beta\gamma$-bismethylene-AP_4 (**20**) and $\beta\gamma,\gamma\delta$-bismethylene-AP_4 (**21**) at $P2X_{2/3}$ receptors. (a) Representative current traces; period of agonist application indicated by bars above traces. (b) Concentration–response curves. Currents are plotted as the per cent of that evoked by 30 μM $\alpha\beta$meATP.

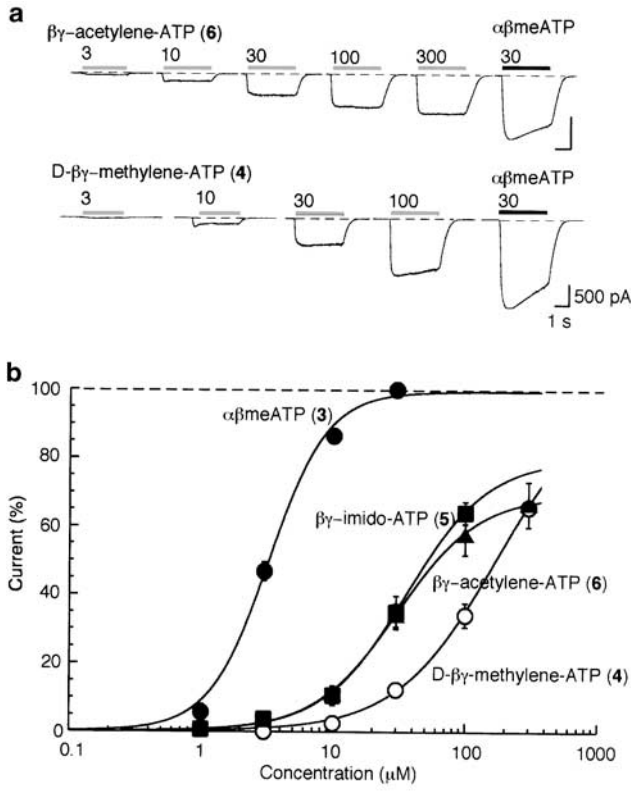

Figure 3 Weak agonists at $P2X_{2/3}$ receptors. Currents evoked by D-$\beta\gamma$-methylene-ATP (**4**), $\beta\gamma$-imido-ATP (**5**) and $\beta\gamma$-acetylene-ATP (**6**) at $P2X_{2/3}$ receptors. (a) Representative current traces; period of agonist application indicated by bars above traces. (b) Concentration–response curves. Currents are plotted as the per cent of that evoked by 30 μM $\alpha\beta$meATP.

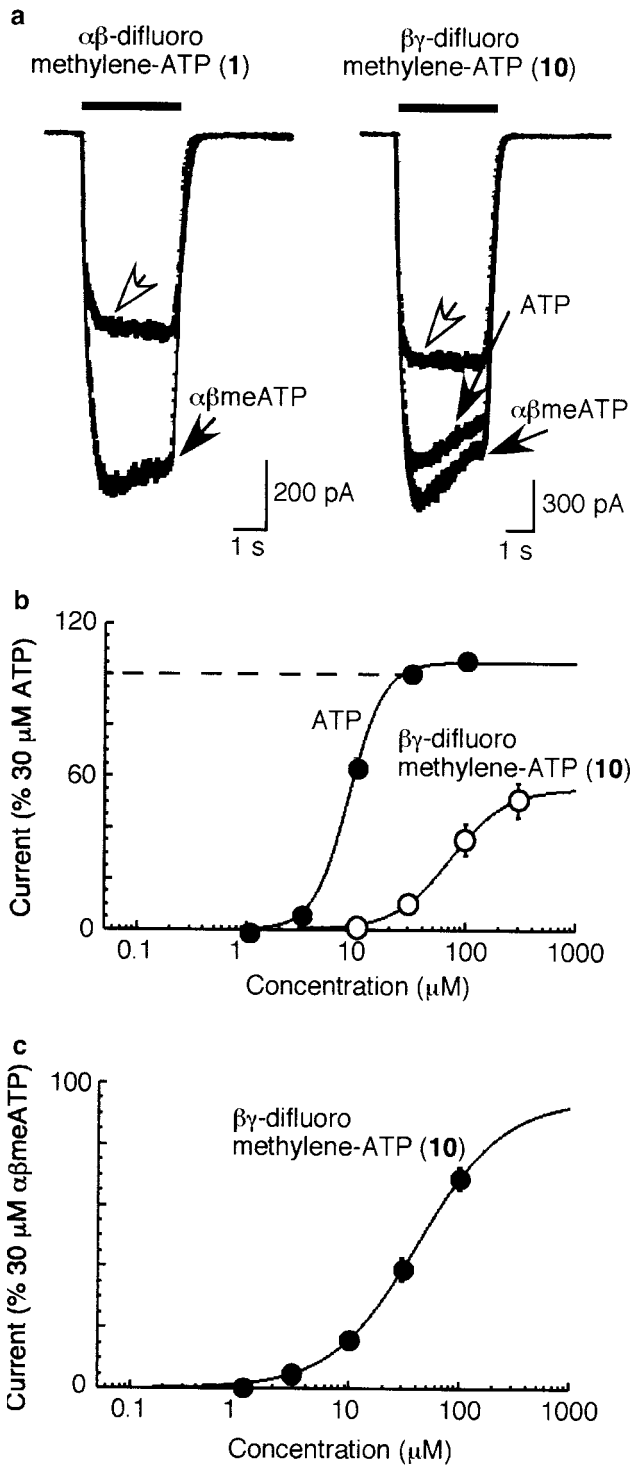

Figure 4 Difluoromethylene-substituted ATP analogues are agonists at both $P2X_2$ and $P2X_{2/3}$ receptors. (a) Cells transfected with $P2X_2$ and $P2X_3$ subunits. Currents evoked by $\alpha\beta$-difluoromethylene-ATP (100 μM)(left) and $\beta\gamma$-difluoromethyleneATP (100 μM)(right). The currents are indicated by open arrows. These are superimposed on currents evoked by $\alpha\beta$meATP and ATP in the same cells. Left, $\alpha\beta$meATP (5 μM). Right, $\alpha\beta$meATP (30 μM) and ATP (30 μM). (b) Cells transfected only with $P2X_2$ subunits. $\beta\gamma$-DifluoromethyleneATP is an agonist, though much less potent than ATP. (c) Cells transfected with $P2X_2$ and $P2X_3$ subunits. The solution contained 10 mM calcium to inhibit currents at homomeric $P2X_2$ receptors. $\beta\gamma$-difluoromethyleneATP showed agonist activity, which must therefore be at $P2X_{2/3}$ receptors.

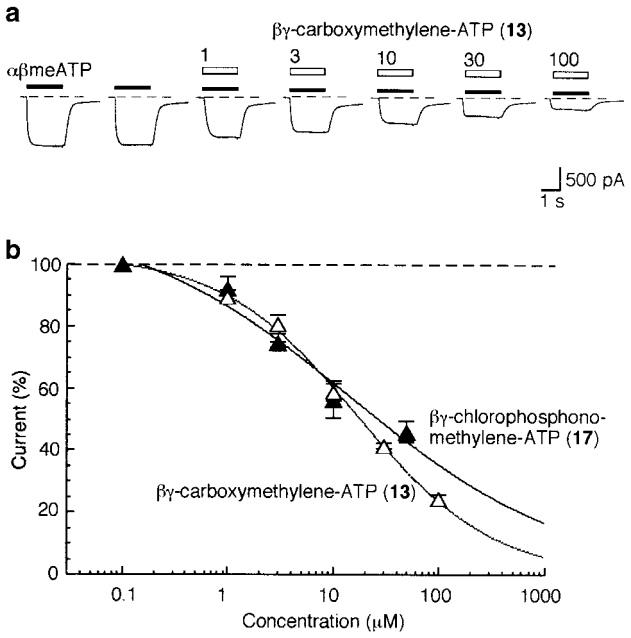

Figure 5 Antagonists at $P2X_{2/3}$ receptors. Inhibition of $\alpha\beta$meATP-evoked current by $\beta\gamma$-carboxymethylene-ATP and $\beta\gamma$-chlorophosphonomethylene-ATP. (a) Representative current traces; the currents shown were evoked by applying meATP in the presence of increasing concentrations of the antagonist. The first two traces show the control response in the absence of antagonist. (b) Antagonist concentration–inhibition curves. Currents are plotted as the per cent of that evoked by 30 μM meATP in the absence of antagonist.

(**20**); indeed, the corresponding AP_4 analogue is the only compound found that was more potent than $\alpha\beta$meATP itself (Figure 3). The oxygen atom in the $\gamma\delta$ position presumably contributes considerably to this, because $\beta\gamma,\gamma\delta$-bismethylene-AP_4 (**21**) was some ten-fold less effective than the $\alpha\beta,\beta\gamma$ substituted analogue. This could be because the partial charge on the oxygen atom of the $\gamma\delta$ bridge can substitute to a degree for that of the –OH group of the γ phosphate that normally contributes to the binding site. We do not understand why the introduction of chlorine atoms into the $\beta\gamma,\gamma\delta$-bis-substituted analogue reduces activity, whereas the same changes in the $\alpha\beta,\beta\gamma$-bis-substituted compounds increase activity.

The most useful compounds with respect to further elucidation of the function of sensory $P2X_{2/3}$ receptors will be selective antagonists. Our results indicate that it may be possible to find such compounds by relatively minor modification of the phosphate chain. Only two of the analogues tested had antagonist activity, and these inhibited the current evoked by $\alpha\beta$meATP with IC_{50} of about 10 μM. The antagonism was quickly reversible. The common feature of the two antagonists ($\beta\gamma$-carboxymethylene-ATP (**13**) and $\beta\gamma$-chlorophosphonomethylene-ATP (**17**); Table 1; Figure 4) is the additional negative charge on the $\beta\gamma$ methylene bridge. However, this 'supercharging' feature alone is not the full explanation, because the antagonist properties of $\beta\gamma$-chlorophosphonomethylene-ATP were not shared by $\beta\gamma$-fluorophosphonomethylene and $\beta\gamma$-phosphonomethylene (Table 1). Presumably, the chlorine atom contributes a steric effect that favourably positions the carboxylate, and that is not seen with fluorine or hydrogen. The effectiveness of the carboxylate attachment at the $\beta\gamma$-bridge suggests key positive charges at the binding site; the pattern of negative moieties is a common feature of other competitive $P2X_{2/3}$ receptor antagonists suramin, pyridoxal-5-phosphate-6-azo-2′4′-disulphonic acid and TNP-ATP (Spelta *et al*., 2002). The same features are observed in a recently described non-nucleotide receptor antagonist at $P2X_3$ and $P2X_{2/3}$ receptors (A-317491); this is a benzene ring bearing three carboxylates and one substituted carboxamide (Jarvis *et al*., 2002).

It would be desirable to be able to interpret the present results in the context of the structure of the ATP-binding site on the P2X receptor protein. Unfortunately, there is no relatedness in the amino-acid sequence of the P2X receptor and any other protein. Although some regions of the protein have been suggested to be involved in ATP binding (e.g. the conserved K–X–K–G beginning at Lys^{69} in the $P2X_2$ subunit; Jiang *et al*., 2000), none of the 'classical' nucleotide-binding motifs have been detected (Traut, 1994; Ramakrishnan *et al*., 2002). Among proteins that bind but do not hydrolyse ATP, there are several structures that show hydrogen bond formation between the bridging oxygen atoms and residues in the binding pocket (e.g. $\alpha\beta$ oxygen with NH_3^+ in ATP:co(I)rrinoid adenyltransferase; Bauer *et al*. (2001), and with oxygens of the C-terminus of the S1 subunit in pertussis toxin, Hazes *et al*., 1996). The present finding would certainly be consistent with a key role for such an interaction in the binding of ATP to P2X receptors, in which case the residue(s) involved might be expected to differ between the $P2X_1$ and $P2X_3$ subunits (where $\alpha\beta$meATP is effective) and the $P2X_2$ and other subunits (where it is not).

There have been only limited previous studies on the effects of phosphate chain-modified ATP analogues at P2 receptors. P2Y receptors, in general, are not activated or blocked by $\alpha\beta$meATP, $\beta\gamma$meATP and $\beta\gamma$imidoATP (Jacobson *et al*., 1999; Jacobson & Knutsen, 2001). We have found that replacement of the anhydride $\beta\gamma$ oxygen with a difluoromethylene unit retained activity at the $P2X_{2/3}$ receptor. This compound has previously been shown to be a partial agonist in the rabbit ear artery (Leff *et al*., 1993), where it presumably activates $P2X_1$ receptors. The $\beta\gamma$-difluoromethylene substitution is a feature of ARL67156 (6-*N*,*N*-diethyl-$\beta\gamma$-dibromomethylene-D-ATP), which is widely used as a blocker of ectoATPase. Futhermore, ARL67085 is a $\beta\gamma$-dichloromethylene derivative (2-propylthio-D-$\beta\gamma$-dichloromethylene-ATP), which acts as an antagonist with good selectivity for the platelet P2Y receptor ($P2Y_{12}$, formerly P2T) (Ingall *et al*., 1999).

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