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Kinetics of antagonist actions at rat $P2X_{2/3}$ heteromeric receptors

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1 Currents through heteromeric $P2X_{2/3}$ receptors were evoked by applying α,β-methylene-ATP to human embryonic kidney cells transfected with cDNAs encoding the $P2X_2$ and $P2X_3$ subunits. The concentration of α,β-methylene-ATP were $\leqslant 30\ \mu$M because higher concentrations can activate homomeric $P2X_2$ receptors.

2 The kinetics of action of three structurally unrelated antagonists were studied; these were 2′, 3′-O-(2,4,6,trinitrophenyl)-ATP (TNP-ATP), pyridoxal-5-phosphate-6-azophenyl-2′,4′-disulphonate (PPADS) and suramin.

3 The association and dissociation rate constants were determined by pre-applying the antagonist for various periods prior to the co-application of agonist and antagonist, or by changing the solution from one containing only the agonist to one containing both agonist and antagonist.

4 The high affinity of TNP-ATP for the $P2X_{2/3}$ receptor ($K_D \approx 2$ nM) results from fast binding ($k_{+1} \approx 100\ \mu M^{-1}\ s^{-1}$) rather than slow unbinding ($k_{-1} \approx 0.3\ s^{-1}$). For suramin ($K_D \approx 1\ \mu$M) the association rate constant ($\approx 1\ \mu M^{-1}\ s^{-1}$) was 100 times slower than that of TNP-ATP but the dissociation rate constant was similar ($k_{-1} \approx 1\ s^{-1}$). PPADS ($K_D \approx 0.1\ \mu$M) associated and dissociated some 100–10,000 times more slowly than the other antagonists.
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Abbreviations: $\alpha\beta$meATP, α,β-methylene-ATP; HEK, human embryonic kidney; PPADS, pyridoxal-5-phosphate-6-azophenyl-2′,4′-disulphonic acid; TNF-ATP, 2′,3′-O-(2,4,6-trinitrophenyl)-ATP

Introduction

Primary afferent neurons involved in the sensation of pain can be identified by the relatively small diameter of their cell bodies, and by their expression of binding sites for the isolectin B4, the vanilloid (capsaicin) receptor VR1, and the $P2X_3$ subunit (reviewed by Ding *et al*., 2000; Hamilton & McMahon, 2000; Bland-Ward & Humphrey, 2000; Burnstock, 2000). Gene knock-out experiments indicate that $P2X_3$ receptors are also involved in sensation of warmth and bladder distension (Souslova *et al*., 2000; Cockayne *et al*., 2000). Primary afferent neurons express other P2X receptor subunits, and the combination of $P2X_2$ and $P2X_3$ subunits is of particular interest (Vulchanova *et al*., 1998; Bradbury *et al*., 1998; Cook *et al*., 1997). In heterologous expression systems, this combination can form heteromeric P2X receptors (termed $P2X_{2/3}$) and these are marked by a unique phenotype with respect to the currents elicited by ATP and analogues (Lewis *et al*., 1995). Thus, low micromolar concentrations of ATP, though not α,β-methylene-ATP ($\alpha\beta$meATP), elicit currents in cells expressing only the $P2X_2$ subunits, and these show minimal desensitization over a period of 1–2 s. Both $\alpha\beta$meATP and ATP evoke currents in cells expressing only $P2X_3$ subunits, but when the concentration exceeds the EC_{50} these currents desensitize fully with applications continued for 1 s. A third phenotype is observed in cells expressing both $P2X_2$ and $P2X_3$ receptors; they respond to ATP and $\alpha\beta$meATP, but the current desensitizes very little (Lewis *et al*., 1995). This phenotype is also observed in nodose ganglion cells (Lewis *et al*., 1995; Thomas *et al*., 1998), in subsets of dorsal root ganglion cells (Grubb & Evans, 1999), in some trigeminal ganglion cells (Cook *et al*., 1997) and in some autonomic ganglion cells (Khakh *et al*., 1995; Zhong *et al*., 2000; 2001). It has recently been proposed that these properties are displayed by a set of capsaicin-insensitive primary afferents involved in sensing mechanical allodynia (Tsuda *et al*., 2000).

The substantiation of a role for ATP in primary afferent transmission depends very much on the availability of selective antagonists (see North & Surprenant, 2000). Those in most widespread use are 2′,3′-O-(2,4,6-trinitrophenyl)-ATP (TNP-ATP), suramin, and pyridoxal-5-phosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS). When studied in heterologous expression systems, TNP-ATP is particularly effective at blocking homomeric $P2X_1$, homomeric $P2X_3$ and heteromeric $P2X_{2/3}$ receptors (IC_{50} about 1 nM; Virginio *et al*., 1998). It is of intermediate effectiveness at heteromeric $P2X_{1/5}$ receptors (Surprenant *et al*., 2000) and relatively ineffective at homomeric $P2X_2$, $P2X_4$, and $P2X_7$ receptors (Virginio *et al*., 1998). Suramin shows less discrimination among the different receptors that have been studied, although the $P2X_4$ receptors are less sensitive than the others. PPADS is a submicrolar blocker of $P2X_1$ receptors (Jacobson *et al*., 1998), a micromolar blocker of $P2X_2$, $P2X_{2/3}$ and $P2X_5$ receptors, essentially ineffective at $P2X_4$ receptors and of intermediate effect at $P2X_7$ receptors (Buell *et al*., 1996; North & Surprenant, 2000).

Unfortunately, it is often difficult to compare the results of different experimenters with respect to the antagonists, because they have not been systematically compared in the

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same preparation. Various times of preincubation with antagonists have been used, and it has often not been determined whether these are sufficient to reach steady-state. There have been no comparative studies of the three antagonists on cells expressing $P2X_{2/3}$ receptors. Given the growing evidence that the $P2X_{2/3}$ heteromer may be a useful molecular target for drugs altering sensory processing, the aim of the present work was to determine the kinetics and mechanism of action of these three compounds at this particular P2X receptor subtype.

Methods

Cells and chemicals

HEK293 cells were used that had been stably transfected with rat $P2X_2$ cDNA (homomeric $P2X_2$) or stably transfected with rat $P2X_2$ and $P2X_3$ cDNAs in a bicistronic vector (Evans *et al*., 1995; Kawashima *et al*., 1998). The cells expressing heteromeric $P2X_{2/3}$ receptors also express homomeric $P2X_2$ receptors, but show no evidence of homomeric $P2X_3$ receptors (Lewis *et al*., 1995; Kawashima *et al*., 1998; Virginio *et al*., 1998). Culture media, sera and all cell culture reagents were obtained from Life Technologies (Paisley, U.K.), ATP and $\alpha\beta$meATP were from Sigma, TNP-ATP (2′-(or-3′)-O-(trinitrophenyl)adenosine 5′-triphosphate, trisodium salt) was from Molecular Probes (Eugene, OR, U.S.A.), suramin was from Bayer and PPADS (pyridoxal 5-phosphate-6-azophenyl-2′,4′-disulphonate, tetrasodium salt) was from Tocris (Cookson, U.K.).

Electrophysiology and drug applications

Standard whole-cell patch-clamp recordings were carried out at room temperature 24–72 h after passage of stable cell lines. Currents were recorded with an EPC9 amplifier (HEKA Elecktronik, Lambrecht, Germany). Unless otherwise stated, experiments were performed at a holding potential of −60 mV; series resistance, which ranged from 2–15 MΩ, was compensated by 60%. Patch pipettes (3–7 MΩ) were filled with (mM): NaF 145, EGTA 10, HEPES 10. The extracellular solution contained (mM): NaCl 147, KCl 2, $CaCl_2$ 2, $MgCl_2$ 1, HEPES 10 and glucose 13. Solutions were maintained at pH 7.3 and osmolarity 300–315 mOsmol l^{-1}. Current-voltage curves were obtained by applying 0.5 or 1 s ramp voltages from −130 to 50 mV. Agonists and antagonists were applied using the RSC 200 fast flow delivery system (Biologic Science Instruments, Grenoble, France). Agonists were normally applied at 2 min intervals. We measured the solution exchange time by observing the membrane current when switching from NMDG-Cl to NaCl in the continuous presence of ATP; the 10–90% time was 268±43 ms ($n=4$). For the experiments with antagonists, $\alpha\beta$meATP was applied at its EC_{50} concentration; this was determined each day from a sample of cells, and the values ranged from 4–9 μM. The antagonists were then applied at concentrations that gave an onset of inhibition which was clearly slower than the time of solution exchange. Concentrations of suramin lower than 0.3 μM were not used because they usually potentiated rather than inhibited the current evoked by $\alpha\beta$meATP.

Data analysis

Two methods were used to study the actions of the antagonists. First, current was evoked by an agonist application sustained for 20–25 s (always $\alpha\beta$meATP, at the EC_{50}). During this period, the solution was changed to one that contained both agonist and antagonist, for 5–10 s. Subtraction of the two currents gave the effect of the antagonist. Second, onset kinetics were measured by applying the antagonist for a variable period before (and also during) a test application of $\alpha\beta$meATP; the peak amplitude of the current evoked by $\alpha\beta$meATP was compared with and without the antagonist. Conversely, the recovery of the peak amplitude of the $\alpha\beta$meATP-evoked current was measured after the washout of the antagonist. The time courses of antagonist action were fitted by exponentials of time constants τ_{on} and τ_{off}. The dissociation rate constant (k_{-1}) was computed directly as $1/\tau_{off}$; the association rate constant (k_{+1}) was computed as $\{(1/\tau_{on}) - k_{-1}\}/[B]$ where [B] is the antagonist concentration. Results are presented as mean± s.e.mean, and the numbers refer to the number of individual cells tested. Differences among measured time constants were tested by Kruskal-Wallis's and Dunn's nonparametric tests.

Results

Isolation of responses mediated through heteromeric $P2X_{2/3}$ receptors

Cells transfected with both $P2X_2$ and $P2X_3$ cDNAs might be expected to express homomeric $P2X_2$, homomeric $P2X_3$ and one or more species of heteromeric $P2X_{2/3}$ receptors, and this has been shown to be the case previously (Thomas *et al*., 1998; Liu *et al*., 2001). We excluded involvement of homomeric $P2X_3$ receptors because (a) we did not observe any fast-desensitizing component to the currents and (b) responses at the homomeric $P2X_3$ receptor disappear when the agonist is applied repeatedly at intervals less than 4 min (Lewis *et al*., 1995). It is also possible that persistent release of ATP from HEK293 cells under these conditions contributes to desensitization of the receptors (Surprenant *et al*., 2000). To exclude a contribution from homomeric $P2X_2$ receptors, we determined the concentrations at which $\alpha\beta$meATP could be used, by carrying out control experiments on cells stably transfected only with the $P2X_2$ receptor. Concentrations of $\alpha\beta$meATP $\geqslant 100$ μM evoked inward currents in cells expressing only homomeric $P2X_2$ receptors (Figure 1). These results mean that it is not possible to use $\alpha\beta$meATP (at $\geqslant 100$ μM) to activate selectively heteromeric $P2X_{2/3}$ receptors in cells co-expressing $P2X_2$ and $P2X_3$ subunits, because of the concomitant activation of homomeric $P2X_2$ receptors. They also mean that the method of Schild analysis could not be used reliably to test for competitive interaction, because useful dose-ratio's could only be obtained by increasing the $\alpha\beta$meATP concentration into this non-selective range.

The current amplitude in response to 30 μM $\alpha\beta$meATP, in cells co-expressing both subunits, ranged from 25 to 90% (54±6%, $n=8$) of the current evoked by 30 μM ATP. These agonist concentrations are near-maximal for activation of heteromeric $P2X_{2/3}$ and homomeric $P2X_2$ receptors respec-

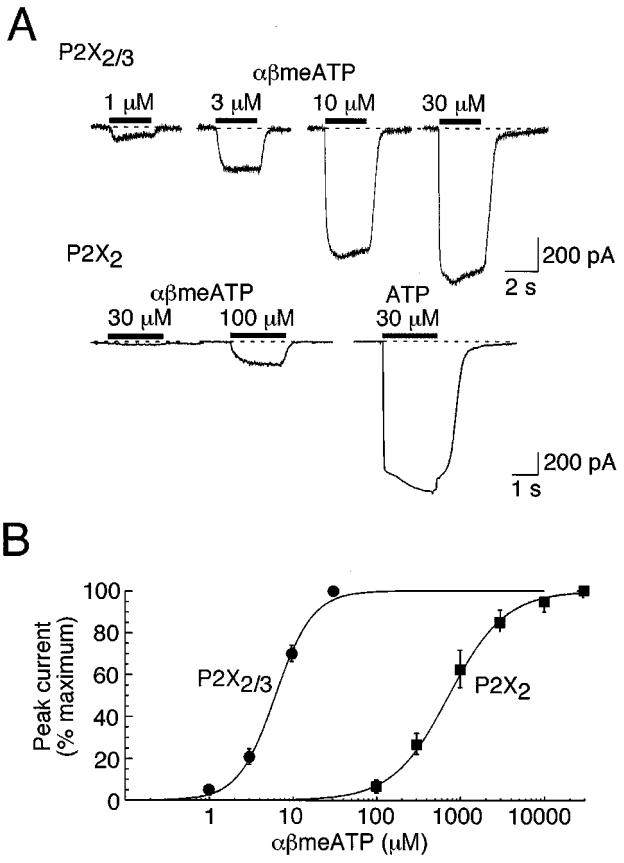

Figure 1 $\alpha\beta$meATP and ATP currents at heteromeric $P2X_{2/3}$ receptors and at homomeric $P2X_2$ receptors. (A) Representative current traces; period of agonist application indicated by bars above traces. (B) Graph compares currents activated by $\alpha\beta$meATP (filled circles) in HEK cell stably transfected with $P2X_2$ and $P2X_3$ with those in cells transfected only with the $P2X_2$ receptor cDNA. Currents are plotted as per cent of that evoked by 30 μM $\alpha\beta$meATP at the heteromeric $P2X_{2/3}$ receptor, or 30 μM ATP at the homomeric $P2X_2$ receptor (this is a maximal concentration).

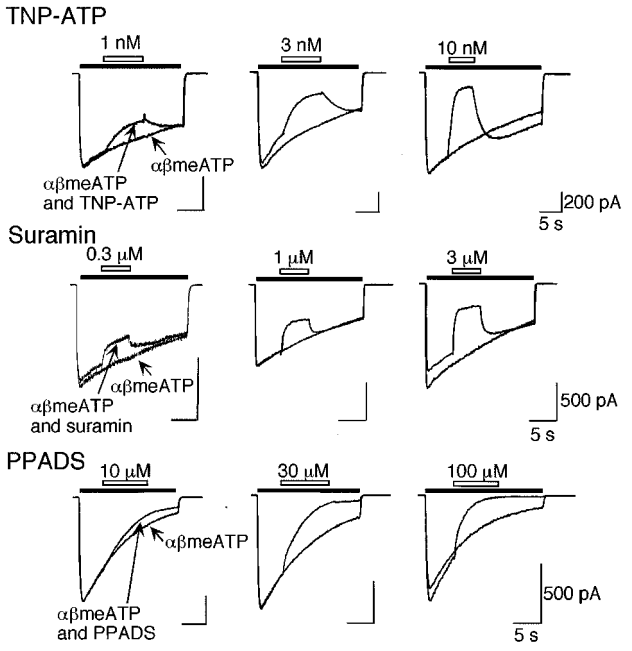

Figure 2 Inhibition of $\alpha\beta$meATP-evoked currents by TNP-ATP, suramin and PPADS at heteromeric $P2X_{2/3}$ receptors. In each panel, the two superimposed traces show the currents elicited by $\alpha\beta$meATP (at EC_{50}: filled bar); in one of the two traces, the antagonist was also applied during the $\alpha\beta$meATP application (period indicated by the open bar). Subtraction of traces such as these was used to estimate the onset and offset kinetics.

tively, so they provide a rough estimate of the proportion of heteromeric $P2X_{2/3}$ to homomeric $P2X_2$ receptors in a single cell. We estimate in this way that there is an approximately 45 : 55 ratio of heteromeric $P2X_{2/3}$ to homomeric $P2X_2$ receptors; this is similar to our previous study in which we estimated a 40 : 60 ratio using the same stably-transfected cell line (Thomas *et al*., 1998). Concentration-response curves for $\alpha\beta$meATP up to the concentration of 30 μM in these cells yielded an EC_{50} value of 6.5 ± 2.1 μM ($n = 18$; Figure 1). This EC_{50} value varied somewhat among cells, and in experiments on antagonist kinetics, the actual concentration of $\alpha\beta$meATP used was that determined to be the EC_{50} on cells tested on the same day. At this concentration, the $\alpha\beta$meATP-evoked current reached its peak amplitude typically in 0.98 ± 0.03 s ($n = 54$), and declined to $86 \pm 0.8\%$ ($n = 54$) during a 5 s application. With a 20 s application, the current declined to $39 \pm 2.3\%$ ($n = 38$) of control (see Figure 2).

Onset and offset of antagonist action

In the first series of experiments (antagonist co-application), $\alpha\beta$meATP was applied for periods of 20 or 25 s. This elicited an inward current that declined during the application. The decline, which we term slow desensitization (to distinguish it from the millisecond desensitization observed with homomeric $P2X_3$ receptors), was variable from cell to cell, but quite reproducible within the same cell (Figure 2). We then applied the antagonists TNP-ATP, suramin or PPADS during the maintained application of $\alpha\beta$meATP. The onset of the inhibition of the current was clearly dependent on the concentration of antagonist, and this experiment provided a range of values for τ_{on} (Figure 2). The offset of action was well fit by single exponential (τ_{off} for TNP-ATP was 4.7 ± 1.9 s (3), 3.8 ± 0.7 s (4), 1.9 ± 0.13 s (3) and 2.4 ± 0.13 s (4) for 1, 3, 10 and 30 nM: τ_{off} for suramin was 0.88 ± 0.13 s (3), 0.75 ± 0.06 s (5), 0.89 ± 0.17 s (4) and 0.84 ± 0.04 s (4) for 0.3, 1, 3 and 10 μM). There was no significant dependence on antagonists concentration ($P > 0.05$). We therefore pooled all the concentrations in our estimate of k_{-1}; we used this pooled estimate for the computation of k_{+1} and K_D values (Figure 3; Table 1).

Figure 4 illustrates the results of the second series of experiments (antagonist pre-application). Each of the traces shows the current elicited by $\alpha\beta$meATP applied for 5 s at the EC_{50} concentration; the application of $\alpha\beta$meATP was begun after a variable period of antagonist application (indicated above each trace in s). At the lowest concentration of TNP-ATP (1 nM), simultaneous application of TNF-ATP and $\alpha\beta$meATP (i.e. 0 s pre-application in Figure 4) caused minimal effect on the peak current, but it did inhibit the current measured at 5 s; at 10 nM, TNP-ATP profoundly reduced the current during the 5 s co-application. The altered time course of the agonist-induced current could be well accounted for the time course of onset of the antagonist, as

determined from the co-application experiments described above. Suramin (10 μM) appears to bind to the receptor almost as quickly as $\alpha\beta$meATP under these conditions, because even their simultaneous application there was a marked reduction in the initial peak response to agonist (Figure 4); however, at this concentration the rate of onset of action of suramin is unreliable because it is limited by the solution exchange time (see Methods).

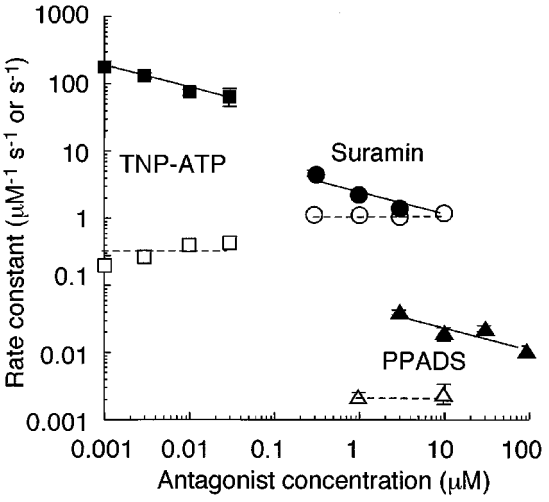

Figure 3 Summary data for the onset and offset of antagonist inhibition from experiments such as those shown in Figure 2. $n=3-5$ for each point. Forward rate constants (closed symbols) (k_{+1}) are in μM^{-1} s^{-1}. Backward rate constants (open symbols) (k_{-1}) are in s^{-1}. Backward rate constants for PPADS (open triangles) are from the experiments shown in Figure 5.

The onset of the inhibition by PPADS was measured in a similar manner, and the results are plotted in Figure 5A. The offset kinetics of PPADS was estimated by observing the recovery of the response to $\alpha\beta$meATP from its fully depressed state. The time course of recovery was the same at all concentrations tested (1, 3, 10 and 30 μM), and was well fit by an exponential of time constant 550 s (Figure 5B; Table 1). Note, however, that the recovery from inhibition by PPADS was never complete, reaching a steady level at which the current evoked by $\alpha\beta$meATP was about 50% of its initial value (Figure 5B). Figure 5 also shows that there was no change in the overall time course of the response to $\alpha\beta$meATP during the washout of PPADS, suggesting that antagonism by PPADS did not obviously change the slow desensitization of the receptor.

Lack of voltage dependence of antagonist action

Agonist-induced currents over the voltage range -130 to 50 mV were recorded during ramp commands in the absence and presence of increasing concentrations of TNP-ATP (1–30 nM) and suramin (0.3–10 μM). There was no voltage-dependence in the range -130 to -20 mV; the marked rectification of the current precluded accurate measurements at more positive potentials. Similar experiments were performed using PPADS (1 μM), with pre-application of 30–90 s; the percentage inhibition was the same at all voltages.

Discussion

The present experiments have determined the rates of association and dissociation of three antagonists at hetero-

Table 1 Association rate constants (k_{+1}), dissociation rate constants (k_{-1}) and dissociation equilibrium constants ($K_D = k_{-1}/k_{+1}$) for the antagonism of $\alpha\beta$meATP by TNP-ATP, suramin and PPADS at $P2X_{2/3}$ heteromeric receptors. For TNP-ATP and suramin, k_{-1} was measured as the mean value of $1/\tau_{off}$ for all concentrations tested

Antagonist	*Concentration* (μM)	k_{+1} ($\mu M^{-1} s^{-1}$)	k_{-1} (s^{-1})	K_D (μM)
TNP-ATP	0.001	85±13 (3)		0.002±0.0011 (3)
	0.003	142±29 (4)		0.002±0.0004 (4)
			0.31±0.05 (16)	
	0.01	79±10 (5)		0.004±0.0005 (5)
	0.03	67±2.3 (4)		0.005±0.0002 (4)
Suramin	0.3	4.3±0.66 (4)		0.33±0.03 (4)
	1	2.2±0.25 (5)	1.3±0.1 (13)	0.87±0.11 (4)
	3	1.3±0.09 (4)		1.7±0.19 (4)
PPADS	3	0.04±0.003 (3)		0.04±0.003 (3)
	10	0.02±0.006 (3)		0.10±0.002 (3)
			0.0018*±0.0001 (6)	
	30	0.02±0.003 (3)		0.07±0.006 (3)
	100	0.01±0.002 (3)		0.16±0.04 (3)

*Washout of PPADS was incomplete, with $\alpha\beta$meATP-induced currents recovering to about 50% of their initial value; k_{-1} here is the rate at which the currents reached this steady-state inhibited level.

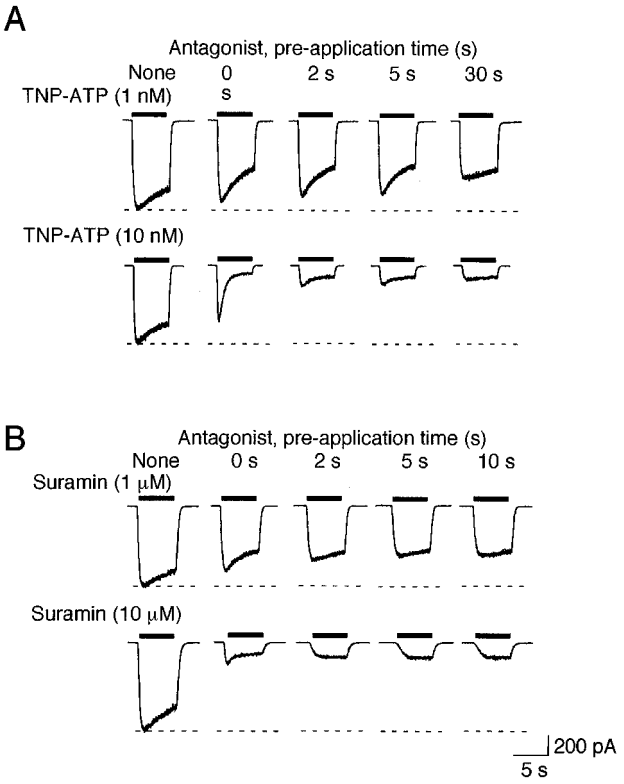

Figure 4 Onset kinetics for TNP-ATP (A) and suramin (B), determined by antagonist pre-application. The currents shown were evoked by co-applying $\alpha\beta$meATP and the antagonist, after a variable period of antagonist application (indicated in s above each trace). The first trace in each set shows the control response in the absence of antagonist.

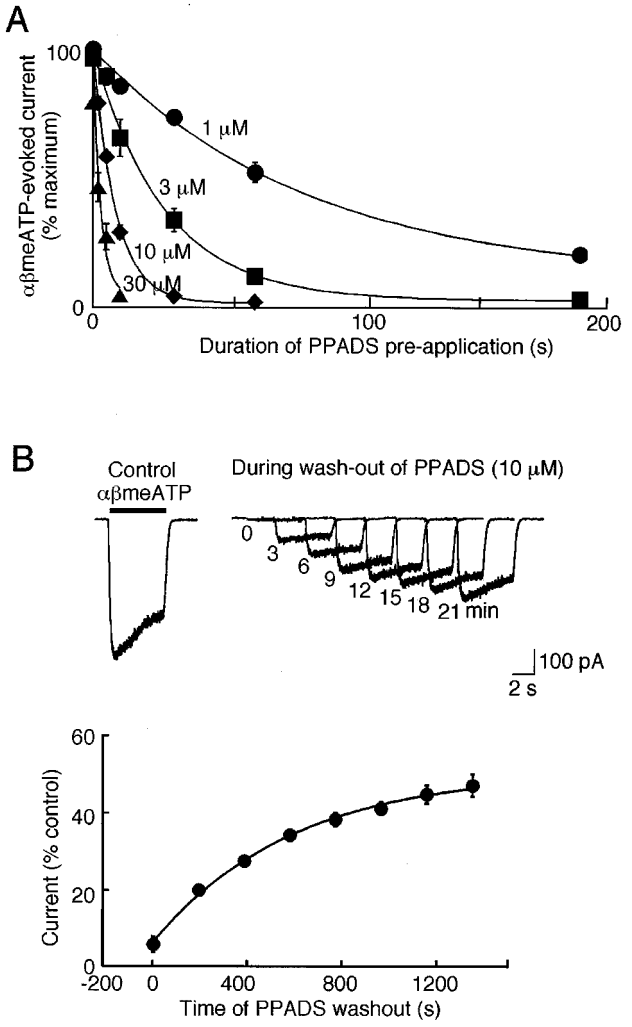

Figure 5 Onset and offset of PPADS inhibition. (A) The time course of inhibition by PPADS determined from experiments such as those shown in Figure 4. Time constants (τ_{on}) for onset of inhibition are 79 s (1 μM), 26 (3 μM), 8.3 (10 μM), 2.7 (30 μM), and 1.4 s (100 μM; not shown in figure), corresponding to k_{+1} of 0.013 $\mu M^{-1}\,s^{-1}$. (B) The recovery of the response to $\alpha\beta$meATP after washout of PPADS. The values in the graph are the means$\pm$s.e.mean for six experiments.

meric $P2X_{2/3}$ receptors. The first important step was the isolation of currents evoked at the heteromeric receptor, because cells transfected with $P2X_2$ and $P2X_3$ subunits clearly exhibit both homomeric $P2X_2$ receptors and heteromeric $P2X_{2/3}$ receptors (Thomas *et al*., 1998). Individual neurons also appear to express both such receptor classes (Thomas *et al*., 1998; Zhong *et al*., 2000). If the P2X receptor functions as a trimer (Nicke *et al*., 1998; Stoop *et al*., 1999), then two types of heteromeric receptor might be formed, which contain one or two copies of the $P2X_3$ subunit. We do not know whether one or both such receptor species are activated by $\alpha\beta$meATP in the present experiment; the Hill slope of the agonist dose-response curve (about 1.5; Figure 1) indicates that at least some of the heteromeric receptor(s) must contain more than one $\alpha\beta$meATP binding site. However, we are confident that homomeric $P2X_2$ receptors would not be activated by $\alpha\beta$meATP within the concentration range that we used (Figure 1). The limitation that the concentration of $\alpha\beta$meATP could not be increased by more than about 10 fold without leading to activation of homomeric $P2X_2$ receptors precluded the application of methods which depend on surmounting the antagonist action, such as Schild analysis. To be confident that antagonism is competitive, it is necessary to demonstrate that the block is surmountable for larger dose-ratio; a parallel shift in the dose-response curve for smaller dose-ratio is readily observed for non-competitive antagonism (Milner *et al*., 1982) and can be accounted for by the presence of spare receptors (see Foreman & Johansen, 1996).

When the agonist $\alpha\beta$meATP was applied alone at the EC_{50} concentration, the current peaked within 1 s; at the termination of the application, the currents declined exponentially with a time constant of about 250 ms (similar results were obtained by Burgard *et al*., 2000). On the other hand, the slow desensitization that was observed had a time constant of about 10–20 s. We used two methods to estimate the on- and off-rates for the antagonists. The first was co-application (Figure 2), where the antagonist was introduced when the channel was already opened by the continuous presence of the agonist. This method was useful for the onset of action of all the agonists, so long as their concentration was low enough (Figure 2); it was also useful for the offset of action of TNP-ATP and suramin. The second was pre-application (Figure 4), where the antagonist was applied alone before changing to co-application of agonist and antagonist. This method was useful for PPADS, but only for the lowest concentrations of TNP-ATP and suramin (Figure 4).

In the case of PPADS, there was reasonable agreement between the k_{+1} measured by co-application (10, 30, 100 μM; Figure 2; Table 1) and that measured by pre-application (Figure 5A; the k_{+1} values for these three concentrations are 0.013 μM^{-1} s^{-1}). At the lower concentration (3 μM) PPADS appeared to bind some three-times more rapidly to the open (co-application) than the closed (pre-application). The kinetics of the response to agonist was unaltered during the wash-out of PPADS (Figure 5) and this indicates that desensitization is unaffected by the presence of the antagonist.

The results of the present work are significant for two kinds of reasons. The first has to do with the practical aspect of using the antagonists experimentally to characterize P2X receptors. A recent paper by Li (2000) on bullfrog dorsal root ganglion cells reported that PPADS blocked the currents evoked by ATP with k_{+1} similar to that of the present study (0.06 μM^{-1} s^{-1} measured at 1 μM PPADS). He also reported that PPADS bound more rapidly to the channel when agonist was present than when agonist was absent. However, the rate of PPADS dissociation was somewhat faster than we observed, being described as complete after 8 min. The present work indicates that (Table 1) in the case of PPADS at the $P2X_{2/3}$ receptor, the dissociation equilibrium constant is about 0.1 μM, and at this concentration an application of 20–30 s would be sufficient for steady-state occupancy.

The second interest in the present results relates to the possible mechanism of inhibition at the $P2X_{2/3}$ receptor. Because of the need to isolate the currents through $P2X_{2/3}$ receptors, we have been constrained to use a single agonist concentration; we are therefore not able to say whether the antagonism is competitive. With a single agonist concentration, the simulations also do not discriminate between models in which agonist and antagonist can be bound to the same subunit at the same time (non-competitive), and those in which they can not (competitive). On the other hand, the inhibition by TNP-ATP that we have observed is consistent with competition for an overlapping binding site for ATP and TNP-ATP. However, the parts of the binding pocket that interact with the phosphate chain and the purine base must discriminate between ATP and TNP-ATP. The TNP-ATP analogues TNP-ADP, TNP-AMP and GTP-TNP are all antagonists in the nM concentration range, even though ADP, AMP and GTP do not activate or block the receptor (Virginio *et al*., 1998).

We had originally conjectured that the high affinity for TNP-ATP at the $P2X_{2/3}$ receptor (K_D about 3 nM) would result from a slow dissociation rate, and thought that the kinetics of the wash-out of antagonist action might provide information regarding the number of binding sites (Palma *et al*., 1996). In fact, the dissociation rate is only about 10 times slower than that of $\alpha\beta$meATP, and it is the 10 times faster association rate that accounts for its high affinity at the $P2X_{2/3}$ receptor. It is possible that TNP-ATP approaches the binding site more rapidly than $\alpha\beta$meATP because it has three additional negative charges; however, the lack of voltage-dependence to the action of TNP-ATP indicates that the site is probably outside the membrane electrical field. The finding that the association rate constant (k_{+1}) for TNP-ATP was faster at lower concentrations most likely reflects negative cooperativity in the binding steps, implying that more than one molecule of TNP-ATP must bind for effective blockade. There has recently been progress in identifying some of the residues that contribute to the ATP binding site for the $P2X_1$ (Ennion *et al*., 2000) and $P2X_2$ (Jiang *et al*., 2000) receptors. Given the negativity of the TNP moiety, it will be interesting to determine by mutagenesis whether removal of positive charges from the $P2X_3$ subunit can selectively reduce the affinity for TNP-ATP without changing the effectiveness of ATP.

Although the association rate of suramin is 30–100 times slower than that of TNP-ATP, it is about 100 times faster than that of PPADS. Suramin is a larger molecule than PPADS, so it is possible that this difference (as surmised for TNP-ATP) might result from electrostatic forces; there are six negative charges on the suramin molecule compared with three on PPADS (one phosphate, two sulphonates). The dissociation rate of PPADS is also about 10,000 times slower than that of suramin, and recovery remained incomplete after 21 min (about 50% recovery). This is very similar to the situation with the homomeric $P2X_2$ receptor, which also shows only a partial recovery from inhibition after 20 min washing (Evans *et al*., 1995; Buell *et al*., 1996). This has been interpreted as resulting from Schiff base formation with a lysine residue at position 246 of the $P2X_2$ receptor, because the washout of the antagonism occurs within 10 min (and is complete) for the mutant receptor K245E (Buell *et al*., 1996). The $P2X_3$ subunit has threonine rather than lysine at this position, and might therefore behave like $P2X_2$[K246E]. The present finding that recovery from PPADS inhibition occurred was only partial could result from the sum of two binding steps, one slowly reversible and the other irreversible.

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