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Potent desensitization of human $P2X_3$ receptors by diadenosine polyphosphates

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Abstract

In this study, the receptor desensitizing effects of diadenosine polyphosphates at recombinant human $P2X_3$ ($hP2X_3$) receptors were examined. Administration of Ap_3A, Ap_4A, Ap_5A or Ap_6A inhibited the $hP2X_3$ receptor-mediated response to a subsequent application of 3 μM αβ-methyleneATP (αβ-meATP), in a concentration-dependent manner, with IC_{50} values 2707, 42, 59 and 46 nM, respectively. These agonists did not desensitize αβ-meATP responses mediated by the slowly desensitizing heteromeric human $P2X_{2/3}$ receptor. $hP2X_3$ receptor desensitization was reversible and was not observed following the increase in intracellular Ca^{2+} levels produced by carbachol. A similar pattern of desensitization evoked by Ap_5A was also observed using electrophysiological recordings of *Xenopus* oocytes expressing $hP2X_3$ receptors. These data demonstrate that diadenosine polyphosphates, found endogenously in the central nervous system, can readily desensitize $hP2X_3$ receptors at nanomolar concentrations that are 10-fold lower than are required to produce agonist-induced receptor activation. Thus, $P2X_3$ receptor desensitization by diadenosine polyphosphates may provide an important modulatory mechanism of $P2X_3$ receptor activation in vivo.

Keywords: Diadenosine polyphosphate; Purinoceptor; ATP; $P2X_3$ receptor; Desensitization

1. Introduction

Diadenosine polyphosphates (Ap_nA) are molecules consisting of two adenosine moieties bridged via their 5′ position by a chain of two or more phosphates, where n represents the number of phosphates in the connecting chain ($n=2-6$) (for review, see, Miras-Portugal et al., 2001). These molecules are found endogenously and longer chain diadenosine polyphosphates [e.g. P^1,P^4-diadenosine tetraphosphate (Ap_4A) and P^1,P^5-diadenosine pentaphosphate (Ap_5A)] are present in high concentrations in the secretory granules of chromaffin cells (Rodriguez del Castillo et al., 1988) and in rat brain synaptic terminals (Pintor et al., 1992). Furthermore, upon administration of depolarizing agents, diadenosine polyphosphates are released from bovine adrenal glands and midbrain synaptosomes, in a Ca^{2+}-dependent manner (Castillo et al., 1992). Therefore, a potential role in neurotransmitter modulation has been proposed (Pintor et al., 1992, 2000; Miras-Portugal et al., 1998). In support of this, Castro et al. (1990) showed that Ap_4A and Ap_5A inhibited nicotine-evoked catecholamine secretion from isolated chromaffin cells and others have shown that diadenosine polyphosphates facilitate the release of acetylcholine from hippocampal synaptosomes (Pereira et al., 2000).

Various actions of diadenosine polyphosphates have been attributed to the activation of P2 receptors, a family of adenine nucleotide-sensitive receptors classified into P2X (ligand-gated ion channels) and P2Y (G-protein-coupled receptor) sub-families (Burnstock and King, 1996; Burnstock and Williams, 2000). P2X receptors have been implicated in essential cellular processes including smooth muscle contraction, fast excitatory neurotransmission and nociception (Williams and Jarvis, 2000; Burnstock et al., 2000; Burnstock and Williams, 2000). Pintor et al. (1996) demonstrated that Ap_4A evoked a full agonist response at recombinant rat

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$P2X_2$ receptors (derived from PC12 cells) expressed in *Xenopus* oocytes. Ap_4A, as well as some of the higher chain diadenosine phosphates [Ap_5A and P^1,P^6-diadenosine hexaphosphate (Ap_6A)], also have been shown to have agonist activity at rat and human $P2X_1$, $P2X_3$, $P2X_4$ receptors (Wildman et al., 1999; Bianchi et al., 1999).

$P2X_3$ receptors have a distinct physiological distribution with their high-density expression on sensory ganglia providing a potential role in pain signaling (Burnstock, 2000; Burnstock et al., 2000; Williams and Jarvis, 2000). Interestingly, $P2X_3$ (as well as $P2X_1$) receptors have very fast desensitizing properties (Cook and McCleskey, 1997; Cook et al., 1998) that can be modulated by a number of agents including extracellular Ca^{2+} and cibacron blue (Cook and McCleskey, 1997; Cook et al., 1998; Alexander et al., 1999).

Receptor desensitization plays an essential role in the modulation of neurotransmitter action. In effect, desensitization is an alternative method by which persistent receptor activation can be prevented. Specifically in the case of Ca^{2+}-permeable ion channels, desensitization could prevent excess intracellular Ca^{2+} influx that might result in cell death. However, the nature of the P2X receptor desensitization by diadenosine polyphosphates has not been characterized. Here, we have compared desensitization profiles of diadenosine polyphosphates, αβ-methyleneATP (αβ-meATP) and ATP at recombinant human $P2X_3$ receptors using Ca^{2+} influx measurement and electrophysiological recording. The desensitizing actions of diadenosine polyphosphates were examined by investigating their effects on the response to a maximally effective concentration of the P2X receptor-selective agonist αβ-meATP (3 μM).

2. Materials and methods

2.1. Cytosolic Ca^{2+} measurement

$P2X_3$ receptor function was determined on the basis of increases in cytosolic Ca^{2+} concentration as described in Bianchi et al. (1999) with minor modifications. The fluorescent Ca^{2+} chelating dye fluo-4 was used as an indicator of the relative levels of intracellular Ca^{2+} in a 96-well format using a Fluorescence Imaging Plate Reader (FLIPR, Molecular Devices, Sunnyvale, CA). Cells expressing recombinant $hP2X_3$ receptors (Bianchi et al., 1999) were grown to confluence and plated in 96-well black-walled tissue culture plates approximately 18 h before the experiment. One to 2 h before the assay, cells were loaded with fluo-4 AM (2.28 μM; Molecular Probes, Eugene, OR) in Dulbecco's phosphate-buffered saline (D-PBS) and maintained in a dark environment at room temperature. Immediately before the assay, each plate was washed twice with 250 μl D-PBS per well to remove extracellular fluo-4 AM. Two 50-μl additions of compounds (prepared in D-PBS) were made to the cells during each experiment. The first compound addition was made and incubation continued for the remainder of the experiment. The second compound addition of αβ-meATP (3 μM; Sigma, St. Louis, MO) occurred 3 min after the first, and measurement continued for 3 min after this final addition.

Re-sensitization experiments were performed by treating $hP2X_3$-expressing cells with 1 μM Ap_5A for 5 min, then washing the cells twice with 250 μl D-PBS per well. The plates were then incubated at 37 °C at varying times (2–60 min) and the cells were then re-challenged with various concentrations of αβ-meATP.

Fluorescence data were collected at 1- or 5-s intervals throughout the course of each experiment. Data shown are based on the peak increase in relative fluorescence units compared with basal fluorescence. Concentration–response data were analyzed using GraphPad Prism (San Diego, CA) and the consequent EC_{50} or IC_{50} values are derived from a single curve fit to the mean data of $n = 6$ wells.

2.2. Electrophysiology

Ovaries were removed from *Xenopus laevis* under tricaine (3-aminobenzoic acid ethyl ester methanesulfonate; Sigma) anesthesia (0.28% in deionized water) and oocytes were prepared according to procedures approved by Abbott Laboratories' Institutional Animal Care and Use Committee (Briggs and McKenna, 1998; Briggs et al., 1999). Oocytes were isolated and defolliculated following incubation for 1–2 h at room temperature in collagenase (Sigma type 1A, 2 mg/ml; Sigma) in low-Ca^{2+} Barth's solution (pH 7.55) containing 90 mM NaCl, 1.0 mM KCl, 0.66 mM $NaNO_3$, 0.82 mM $MgCl_2$, 2.4 mM $NaHCO_3$, 2.5 mM sodium pyruvate, 10 mM Na–HEPES buffer and 100 μg/ml gentamicin. Oocytes were maintained at 15 °C in normal Barth's solution (similar to the above except with 0.74 mM $CaCl_2$ added). Oocytes were injected with 10–12 nl $hP2X_3$ DNA (1 ug/ul) within 24 h of their preparation and were used within 3 days after injection.

Responses were measured using two-electrode voltage-clamp (−60 mV holding potential) in oocyte Ringer's (OR-2) solution (pH 7.4) containing 90 mM NaCl, 2.5 mM KCl, 2.5 mM $CaCl_2$, 1.0 mM $MgCl_2$ and 5 mM Na–HEPES buffer. Electrodes (0.1–1.2 MΩ) were filled with 3 M KCl and included a 0.2% agarose cushion in the tip. Activation and inhibition were measured using a combined protocol in which any agonist-like response was recorded during the first 5 s of test compound application and then incubation was continued for up to 200 s after which a challenge dose of 3 μM αβ-meATP was applied in the presence of test compound. Thus, both activation and inhibition were evaluated in the same oocytes for each compound at each concentration. Response stability was assessed by recording control responses to 3 μM αβ-meATP alone before and after the series of test compound applications. Responses were measured at peak amplitude and, in each oocyte, normalized to the control 3 μM αβ-meATP response.

3. Results

3.1. $hP2X_3$ versus $hP2X_{2/3}$ receptors

The desensitizing effects of Ap_4A on the agonist activity of 3 μM αβ-meATP in 1321 cells expressing $hP2X_3$ or $hP2X_{2/3}$ receptors were determined. When applied alone, Ap_4A had similar agonist potencies at $hP2X_3$ and $hP2X_{2/3}$ receptors (see Table 1). At a concentration that completely abolished the αβ-meATP agonist response at $hP2X_3$ receptors (300 nM, Fig. 1A), Ap_4A failed to affect the agonist activity of αβ-meATP at the slowly desensitizing $hP2X_{2/3}$ receptor (Fig. 1B). Moreover, at a concentration of 100 μM, Ap_4A had no effect on the response-mediated by $hP2X_{2/3}$ receptors even though this concentration of Ap_4A produced a near-maximal agonist response in both $hP2X_3$- and $hP2X_{2/3}$-expressing cells (Fig. 1A and B).

3.2. Desensitization concentration–response curves

Similar to the effects observed for Ap_4A, 3 min preincubation with αβ-meATP, ATP, or other diadenosine polyphosphates including Ap_3A, Ap_5A and Ap_6A, reduced the agonist effect of 3 μM αβ-meATP. As shown in Fig. 2, these inhibitory effects were concentration-dependent. αβ-meATP and ATP fully inhibited the subsequent αβ-meATP agonist response with IC_{50} values of 48 and 46 nM, respectively (Fig. 2; Table 1). Complete inhibition by Ap_4A, Ap_5A and Ap_6A of the subsequent αβ-meATP response was also observed, with the IC_{50} values 42, 59 and 46 nM, respectively (Table 1). Interestingly, although Ap_3A has been previously reported to be a partial agonist (Bianchi et al., 1999; Wildman et al., 1999), it was able to completely inhibit the αβ-meATP agonist effect with an IC_{50} value of 2.7 μM (Fig. 2; Table 1). Ap_2A was without effect.

3.3. Full and partial agonists versus antagonist profiles

The inhibitory effects evoked by diadenosine polyphosphates were distinct from the effects elicited by the P2X receptor antagonist 2′,3′-*O*-(2,4,6-trinitrophenyl) adenosine-5′-triphosphate (TNP-ATP). Fig. 3 shows intracellular Ca^{2+} traces illustrating the different profiles of the full agonists ATP and Ap_5A, the partial agonist Ap_3A, and the

Table 1
Potencies of αβ-meATP, ATP and diadenosine polyphosphates at recombinant $hP2X_{2/3}$ and $hP2X_3$ receptors

	$hP2X_{2/3}$ Agonist response pEC_{50}	$hP2X_3$ Agonist response pEC_{50}	$hP2X_3$ Desensitization pIC_{50}
αβ-meATP	5.88 ± 0.03	6.16 ± 0.07	7.32 ± 0.06
ATP	6.69 ± 0.08	6.51 ± 0.10	7.33 ± 0.07
Ap_2A	No effect	No effect	No effect
Ap_3A	4.27 ± 0.03	4.40 ± 0.09	5.57 ± 0.05
Ap_4A	6.40 ± 0.07	6.31 ± 0.09	7.37 ± 0.03
Ap_5A	5.85 ± 0.06	6.40 ± 0.07	7.23 ± 0.05
Ap_6A	5.96 ± 0.06	6.45 ± 0.09	7.33 ± 0.05

Desensitization was determined by the inhibition of the αβ-meATP (3 μM)-evoked agonist response 3 min following administration of the test compound. Potencies are shown as mean pEC_{50} or pIC_{50} ± S.E.M.

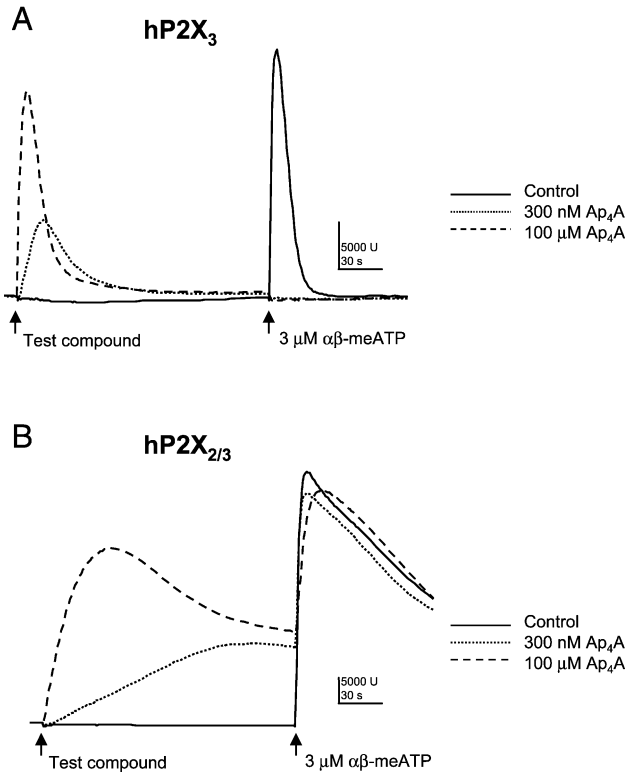

Fig. 1. Kinetics and magnitude of response of Ca^{2+} influx into cells expressing (A) $hP2X_3$ or (B) $hP2X_{2/3}$ receptors measured using fluo-4 Ca^{2+} influx assay. Effect of 300 nM and 100 μM Ap_4A on baseline and αβ-meATP (3 μM)-stimulated Ca^{2+} influx. Test compounds were applied at the first arrow, incubated for 3 min and αβ-meATP was applied at the second arrow. Vertical scale bars represent fluorescence units, the horizontal bars represent time.

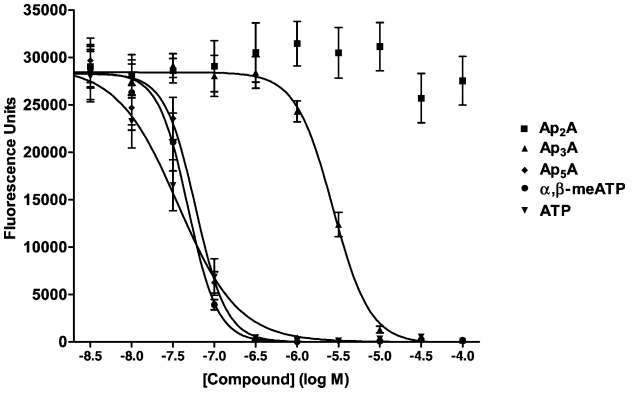

Fig. 2. Effect of increasing concentrations of Ap_2A (squares), Ap_3A (triangles), Ap_5A (diamonds), αβ-meATP (circles) or ATP (inverted triangles) on the agonist response evoked by αβ-meATP (3 μM) via $hP2X_3$ receptors measured using fluo-4 Ca^{2+} influx assay. Effects of Ap_4A and Ap_6A are not shown for reasons of clarity. Cells were treated with test compound 3 min before the addition of 3 μM αβ-meATP. Data shown are the mean fluorescence units ± S.E.M. ($n = 6$).

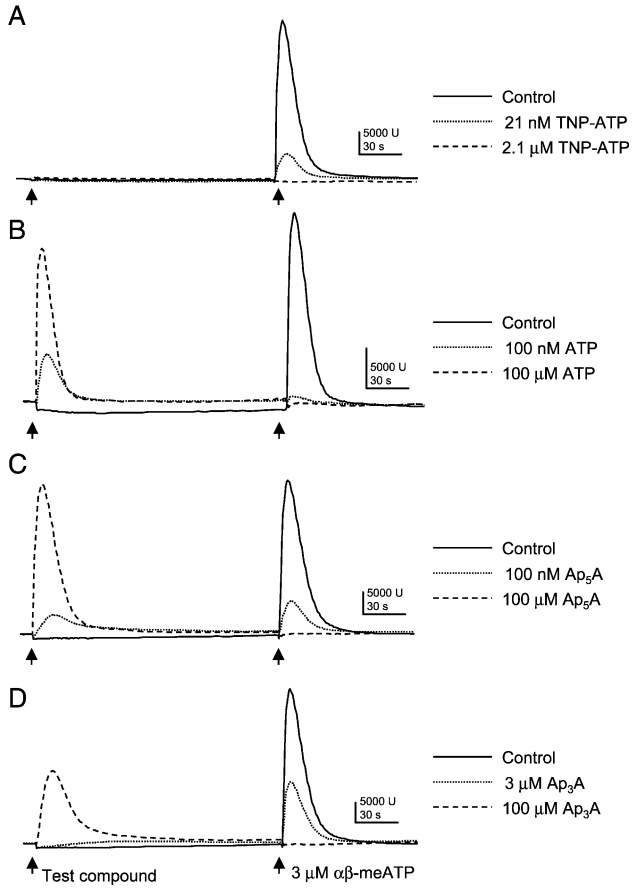

Fig. 3. Kinetics and magnitude of response of Ca^{2+} influx into cells expressing $hP2X_3$ receptors measured using fluo-4 Ca^{2+} influx assay. Effect of (A) TNP-ATP, (B) ATP, (C) Ap_5A and (D) Ap_3A on baseline and αβ-meATP (3 μM)-stimulated Ca^{2+} influx. Test compounds were applied at the first arrow, incubated for 3 min and αβ-meATP was applied at the second arrow. Vertical scale bars represent fluorescence units, the horizontal bars represent time.

antagonist TNP-ATP in evoking agonist and inhibitory actions at $hP2X_3$ receptors. TNP-ATP evoked a concentration-dependent antagonism of the αβ-meATP agonist effect and did not elicit a detectable agonist response, even at concentrations 100-fold greater than that required to markedly reduce the αβ-meATP agonist response (Fig. 3A). In contrast, ATP and Ap_5A both produced significant agonist effects alone and both of these full agonists inhibited the subsequent αβ-meATP response (Fig. 3B and C). At a concentration of 100 nM, ATP or Ap_5A evoked only a sub-maximal agonist response but almost completely inhibited the agonist response to 3 μM αβ-meATP (Fig. 3B and C). At a higher concentration of 100 μM, ATP or Ap_5A produced maximal agonist effects and completely eliminated the subsequent αβ-meATP (3 μM) response. The partial agonist Ap_3A elicited a limited agonist effect, reaching only 51% of the ATP response at the maximal concentration tested (Fig. 3D). Although Ap_3A evoked only partial agonist actions, the compound was able to fully inhibit the agonist response of 3 μM αβ-meATP (Fig. 3D).

3.4. Agonist concentration–response curves

Consistent with previous pharmacological characterizations of mammalian $P2X_3$ receptors (Bianchi et al., 1999; Wildman et al., 1999), ATP and αβ-meATP evoked concentration-dependent increases of intracellular Ca^{2+} in $hP2X_3$ receptor-expressing 1321 cells, with EC_{50} values of 311 and 655 nM, respectively (Fig. 4A; Table 1). P^1,P^2-diadenosine pyrophosphate (Ap_2A) did not produce an agonist effect and Ap_3A was a partial agonist, producing an agonist response that was only 51% that of the maximal response to ATP, with an EC_{50} value of 40 μM (Fig. 4A; Table 1). AP_4A, Ap_5A and Ap_6A evoked maximal responses of 70%, 83% and 88% of the ATP response, with EC_{50} values of 487, 400 and 357 nM, respectively (Fig. 4A; Table 1). No agonist effects of Ap_5A, Ap_6A, αβ-meATP or ATP were observed in null 1321 cells (data not shown). The agonist responses to Ap_5A and αβ-meATP (both 3 μM) were reduced, in a concentration-dependent manner by 2′-(or-3′)-*O*-(trinitrophenyl) adenosine-5′-triphosphate (TNP-ATP) (Fig. 4B). The IC_{50} values for TNP-ATP versus 3 μM Ap_5A and αβ-meATP were 6 and 8 nM, respectively (Fig. 4B).

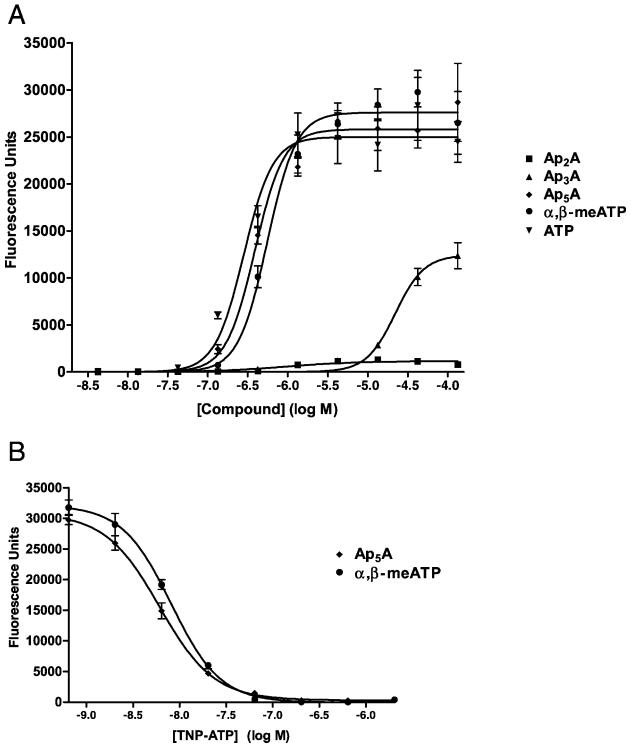

Fig. 4. (A) Agonist characterization of diadenosine polyphosphates, αβ-meATP and ATP on $hP2X_3$ receptor function was measured using fluo-4 Ca^{2+} influx assay. Cells were treated with increasing concentrations of Ap_2A (squares), Ap_3A (triangles), Ap_5A (diamonds), αβ-meATP (circles) or ATP (inverted triangles). Data shown are the mean fluorescence units ± S.E.M. ($n=6$). (B) Effect of increasing concentrations of TNP-ATP on the agonist responses evoked by Ap_5A (diamonds) and αβ-meATP (circles) via $hP2X_3$ receptors measured using fluo-4 Ca^{2+} influx assay. Cells were treated with TNP-ATP 3 min before the addition of Ap_5A or αβ-meATP. Data shown are the mean fluorescence units ± S.E.M. ($n=6$).

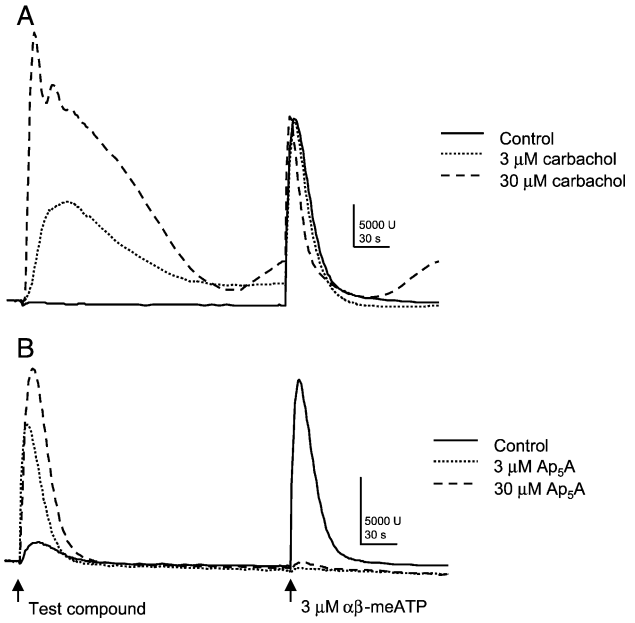

Fig. 5. Kinetics and magnitude of response of Ca^{2+} influx into cells expressing $hP2X_3$ receptors measured using fluo-4 Ca^{2+} influx assay. Effect of (A) carbachol and (B) Ap_5A (both at 3 or 30 μM) on baseline and αβ-meATP (3 μM)-stimulated Ca^{2+} influx. Test compounds were applied at the first arrow, incubated for 3 min and αβ-meATP was applied at the second arrow. Vertical scale bars represent fluorescence units, the horizontal bars represent time.

3.5. Lack of effect of carbachol

The non-selective agonist at muscarinic and nicotinic cholinergic receptors, carbachol, evoked a concentration-dependent increase in intracellular Ca^{2+} in $hP2X_3$-expressing 1321N1 cells. As shown in Fig. 5A, carbachol was without effect on the subsequent agonist response evoked by 3 μM αβ-meATP. Even at a concentration of carbachol that elicited a full agonist response (30 μM), the magnitude of the αβ-meATP was unchanged. In contrast, the same concentration of Ap_5A completely abolished the subsequent αβ-meATP response (Fig. 5B).

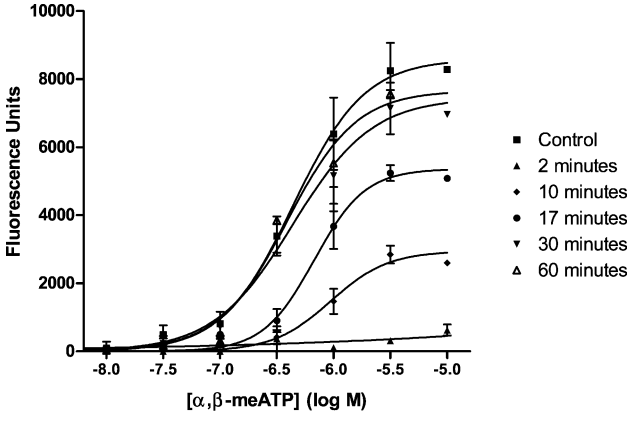

Fig. 6. Time-dependent reversal of Ap_5A-evoked desensitization of $hP2X_3$ receptors. $hP2X_3$-expressing cells in a 96-well plate were treated with 1 μM Ap_5A or D-PBS for 5 min and then washed twice with 250 μl D-PBS per well to remove extracellular Ap_5A. The plates were then incubated at 37 °C for varying time periods and the cells were then re-challenged with αβ-meATP. The resulting concentration response curves to αβ-meATP, after incubation times of 2, 10, 17, 30 or 60 min, are shown. The control curve shows the αβ-meATP agonist response mediated via mock desensitized cells (pretreated with D-PBS).

3.6. Re-sensitization profile

To determine if the inhibitory effects of diadenosine polyphosphates on the αβ-meATP Ca^{2+}-influx response were reversible, a re-sensitization experiment was performed by treating $hP2X_3$-expressing cells in a 96-well plate with 1 μM Ap_5A or D-PBS for 5 min, then washing the cells twice with 250 μl D-PBS per well to remove extracellular Ap_5A. The plates were incubated at 37 °C for varying time periods (2–60 min) and the cells were then re-challenged with αβ-meATP. The resulting concentration–response curves to αβ-meATP, after varying incubation times, are shown in Fig. 6. This shows a time-dependent reversal of $hP2X_3$ receptor

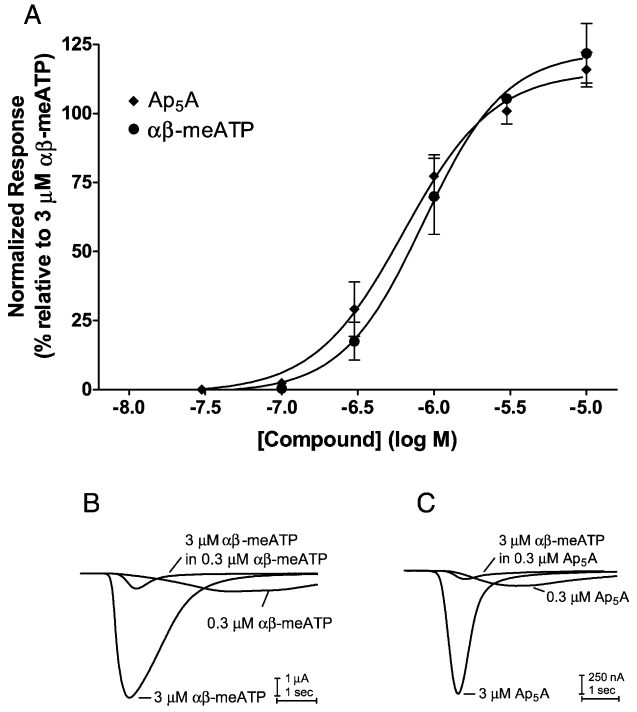

Fig. 7. Electrophysiological characterization of agonist responses and desensitization by αβ-meATP and Ap_5A. (A) Voltage-clamp responses to increasing concentrations of αβ-meATP and of Ap_5A were measured in oocytes expressing $hP2X_3$ receptors ($n=3$ and 5, respectively). The response was measured at peak amplitude and normalized to reference 3 μM αβ-meATP responses measured in each of the same oocytes. Data are shown as mean ± S.E.M. (B) Typical recordings from an oocyte expressing $hP2X_3$ receptors show the large, rapidly desensitizing response to 3 μM αβ-meATP, the smaller and slower response to 0.3 μM αβ-meATP, and inhibition of the response to 3 μM αβ-meATP following exposure to 0.3 μM αβ-meATP for 60–200 s. Similar results were observed in three cells. (C) Likewise, 0.3 μM AP_5A elicited a small, slow response compared to 3 μM AP_5A, but markedly inhibited the response to 3 μM αβ-meATP. Similar results were observed in five cells.

desensitization. The control curve represents the response from cells that were preincubated with D-PBS. The additional curves represent data from cells pretreated with Ap_5A, washed and incubated for 2–60 min. After 10 min incubation, there was a partial reversal of the desensitization. Following 30–60 min incubation, the αβ-meATP response was not different from that mediated by the non-desensitized $hP2X_3$ receptors.

3.7. Electrophysiology

Fig. 7A shows the agonist activation curves resulting from application of αβ-meATP or Ap_5A to oocytes expressing $hP2X_3$ receptors. In agreement with the Ca^{2+} influx data presented above, EC_{50} values for αβ-meATP and Ap_5A were 854 and 632 nM, respectively (Fig. 7). Furthermore, αβ-meATP and Ap_5A also inhibited the response to subsequent application of 3 μM αβ-meATP with IC_{50} values of 209 and 148 nM. Also shown in Fig. 7 are electrophysiological records illustrating the limited agonist responses of 300 nM αβ-meATP (B) or Ap_5A (C) compared with their almost complete inhibition (at the same concentration) of the subsequent response to 3 μM αβ-meATP. At 300 nM, αβ-meATP evoked an agonist response only 18% of the maximal, but produced 75% inhibition of the subsequent αβ-meATP response. Similarly, 300 nM Ap_5A elicited 29% of the maximal agonist response but reduced the αβ-meATP response by 89%.

4. Discussion

In the present study, the receptor desensitization properties of diadenosine polyphosphates, αβ-meATP, and ATP were examined at $hP2X_3$ receptors. Experiments were performed with stably transfected 1321 cells and *Xenopus* oocytes expressing recombinant $hP2X_3$ receptors, using intracellular Ca^{2+} measurement and electrophysiological recording, respectively.

Desensitization of P2X receptors by diadenosine polyphospates has been previously studied in guinea pig and rat vas deferens (Stone and Paton, 1989) and in isolated rat sensory neurons (Krishtal et al., 1988). Agonist-induced desensitization in these preparations is likely mediated via $P2X_1$ and/or $P2X_3$ receptors. Consistent with the data observed in native tissues, the present data demonstrate that specific diadenosine polyphosphates produced desensitization of $hP2X_3$ receptors. The inhibitory effects of diadenosine polyphosphates, ATP, and αβ-meATP were distinct from receptor antagonism produced by TNP-ATP. Unlike the agonists, TNP-ATP failed to produce an agonist response at $hP2X_3$ receptors even at concentrations 100-fold greater than were required to completely block the αβ-meATP agonist response. This observation is consistent with other demonstrations that TNP-ATP functions as a full competitive antagonist of $P2X_3$ receptors (Burgard et al., 2000; Jarvis et al., 2001). Furthermore, αβ-meATP agonist responses mediated by the slowly desensitizing heteromeric $hP2X_{2/3}$ receptor were not reduced by prior administration of diadenosine polyphosphates, indicating that the inhibitory effects at $hP2X_3$ were specific to the fast-desensitizing homomeric subtype. The $P2X_3$ receptor desensitizing effects of Ap_5A were reversible, implying that the diadenosine polyphosphate-evoked reductions in αβ-meATP responses were not mediated via cytotoxicity. The cholinergic receptor agonist carbachol increased intracellular Ca^{2+} levels in $hP2X_3$-expressing 1321N1 cells but failed to affect a subsequent agonist response to αβ-meATP. This suggests that the desensitizing effects of prior P2X agonist exposure were specific to the $hP2X_3$ receptor expressed in 1321N1 cells.

The agonist potencies of ATP, αβ-meATP and diadenosine polyphosphates presented here are consistent with our previous data (Bianchi et al., 1999). Similarly, Wildman et al. (1999) showed that Ap_4A, Ap_5A and Ap_6A had EC_{50} values of 0.8, 1.3 and 1.6 μM at recombinant rat $P2X_3$ receptors expressed in *Xenopus* oocytes. Furthermore, this group also showed that Ap_2A was inactive and Ap_3A was a partial agonist at rat $P2X_3$ receptors (Wildman et al., 1999). The agonist potencies of diadenosine polyphosphates reported here and in the Wildman et al. (1999) study are approximately 3- to 10-fold higher than previously described in other preparations including dorsal root ganglion neurons, chromaffin cells, and brain synaptosomes (Rae et al., 1998; Castro et al., 1990; Gomez-Villafuertes et al., 2000; Diaz-Hernandez et al., 2000). However, the reported potencies in these latter studies do not necessarily reflect exclusive activation of $P2X_3$ receptors because diadenosine polyphosphates have agonist activity at other suramin- and PPADS-sensitive P2X receptors (Westfall et al., 1997; Bianchi et al., 1999; Wildman et al., 1999; Lewis et al., 2000). Furthermore, re-uptake of diadenosine polyphosphates, additional receptor interactions or distinct receptor distribution profiles may also account for apparent agonist potencies. The agonist effects of diadenosine polyphosphates shown here are unlikely to be mediated via hydrolysis of the compounds to ATP and/or adenosine as it has been shown that these agents are relatively resistant to degradation (Pintor et al., 1996). In isolated rat brain synaptic terminals, Ap_4A proved to be resistant to ecto-enzymatic hydrolysis showing only 2% hydrolysis compared with 75% hydrolysis of ATP under similar conditions (Gomez-Villafuertes et al., 2000).

Interestingly, all compounds with agonist activity at $hP2X_3$ receptors were able to evoke receptor desensitization. Even Ap_3A, a partial agonist eliciting a response of only 51% of the maximal ATP response, was able to fully desensitize the receptor. Consistent with these observations, partial nicotinic receptor agonists have been shown to readily inhibit fast desensitizing α7 nicotinic acetylcholine receptors (Briggs and McKenna, 1998). Also, in common with the nicotinic receptor data, the agonist concentrations required for the activation of the $hP2X_3$ receptor were greater than those required to produce desensitization.

These data demonstrate that diadenosine polyphosphates, αβ-meATP, and ATP are agonists at $hP2X_3$ receptors and can readily desensitize $hP2X_3$ receptors at nanomolar concentrations that are approximately 10-fold lower than are required to observe functional receptor activation. The existence of diadenosine polyphosphate-sensitive purinergic receptors that are not inhibited by classical P2 receptor antagonists has been proposed (Pintor and Miras-Portugal, 1995; Miras-Portugal et al., 1998, 1999). However, these 'P_4' or 'dinucleotide' receptors are yet to be cloned and thus their pharmacological properties have not been fully characterized. The present data demonstrate that, in addition to their other putative sites of action, diadenosine polyphosphates can potently interact with $P2X_3$ receptors at low nanomolar concentrations.

The fact that $P2X_3$ receptors can undergo agonist-induced receptor desensitization at agonist concentrations that produce only marginal receptor activation provides an additional level of complexity in the interpretation of $P2X_3$ functional pharmacology. For example, intraplantar administration of α,β-meATP has been shown to induce nociception when administered alone (Bland-Ward and Humphrey, 1997; Hamilton et al., 1999; Jarvis et al., 2001), and to significantly attenuate the nociceptive effects of a subsequent administration of α,β-meATP (Bland-Ward and Humphrey, 1997; Tsuda et al., 1999a,b). Thus, $P2X_3$ receptor desensitization appears to be a functionally relevant mechanism for the regulation of nociceptive signaling.

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