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Selective Agonism of Group I P2X Receptors by Dinucleotides Dependent on a Single Adenine Moiety

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ABSTRACT

We have investigated the activity of naturally occurring high-performance liquid chromatography-purified diadenosine polyphosphates (Ap_nA, $n = 5–6$), adenosine polyphospho guanosines (Ap_nG, $n = 5–6$), and diguanosine polyphosphates (Gp_nG, $n = 5–6$) under voltage-clamp conditions at recombinant rat $P2X_{1-4}$ purinoceptor subtypes expressed in *Xenopus laevis* oocytes. At $rP2X_1$ and $rP2X_3$ receptors, Ap_nAs and Ap_nGs evoked concentration-dependent inward currents. Gp_nGs were not active at these receptors. At $rP2X_2$ and $rP2X_4$ receptors, dinucleotides did not show significant activity. For the $rP2X_1$ receptor, Ap_nAs and Ap_nGs were partial agonists; for the $P2X_3$ receptor, only Ap_5G was full agonist, whereas the other tested substances were partial agonists. The rank order of potency at $rP2X_1$ was ATP $\geq Ap_6A \geq Ap_5A \geq Ap_6G \geq Ap_5G$, and rank order of efficacy was ATP $\geq Ap_5A \geq Ap_6A > Ap_5G > Ap_6G$, whereas at $rP2X_3$ the rank order of potency was ATP $> Ap_5G \geq Ap_5A \geq Ap_6A \geq Ap_6G$ and the rank order of efficacy was ATP $\approx Ap_5G \geq Ap_5A \approx Ap_6A \geq Ap_6G$. For $rP2X_1$ and $rP2X_3$ it is evident that receptor agonism depended on the presence of at least one adenine moiety in the dinucleotide, while the presence of a guanine moiety had a significant impact and decreased agonist efficacy. The data suggest that naturally occurring Ap_nAs and Ap_nGs may play an important physiological role in different human tissues and systems by activating group I P2X receptors.

Diadenosine polyphosphates (Ap_nAs) and adenosine polyphospho guanosines (Ap_nGs) have been detected in human tissues (Schlüter et al., 1994, 1998; Jankowski et al., 1999) where they have been shown in many cases to mediate vasoconstrictor and vasodilator actions via P2X and P2Y receptors, respectively (Schlüter et al., 1994; Ralevic et al., 1995; van der Giet et al., 1997, 1999). Some dinucleotides, for example, diadenosine hexaphosphate (Ap_6A), may also exert negative chronotropic and inotropic effects on the mammalian heart (Vahlensieck et al., 1996). Diadenosine polyphosphates (Ap_nA, $n = 3–6$) are known to regulate the growth of rat mesangial cells grown in culture, an effect that may have a bearing on renal glomerular function in vivo and, subsequently, on renal control of blood pressure (Heidenreich et al., 1995; Schulze-Lohoff et al., 1995). Additionally, Ap_nAs can exert direct vasoconstrictor effects on renal blood vessels (van der Giet et al., 1997, 1999), as well as Ap_nGs showing vasoconstrictor actions in other blood vessels (e.g., mesenteric artery) where they can elicit fast inward currents in isolated vascular smooth muscle cells (Lewis et al., 2000).

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P2X receptors are nucleotide-gated ion channels permeable to monovalent (Na^+, K^+) and divalent (Ca^{2+}) ions and, in vascular tissues, cause both depolarization and Ca^{2+} influx sufficient to initiate smooth muscle contraction (Benham and Tsien, 1987). Of the P2X subunits cloned from mammalian tissues (Humphrey et al., 1998), transcripts for $P2X_1$, $P2X_2$, and $P2X_4$ have been found in rat cardiovascular tissues (Bogdanov et al., 1998; Nori et al., 1998), while $P2X_3$ transcripts are present mainly in mammalian sensory nerves but also are found in human cardiac tissue (Chen et al., 1995; Garcia-Guzman et al., 1997; Bogdanov et al., 1998). $P2X_1$ and $P2X_3$ receptors belong to group I P2X receptors and are characterized by agonism by ATP and α, β-meATP, with suramin blockade of agonist-evoked fast-desensitizing inward currents (Humphrey et al., 1998). $P2X_2$ (group II) and $P2X_4$ (group III) receptors are sensitive to ATP but not α, β-meATP, the former evoking slowly desensitizing currents that are blocked by either suramin or pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid at $P2X_2$ receptors but not $P2X_4$ receptors (Humphrey et al., 1998).

Dinucleotide activation of P2X receptors in blood vessels has been shown to depend on the length of the polyphosphate

ABBREVIATIONS: Ap_nA, diadenosine polyphosphate; Ap_nG, polyphospho guanosine; Ap_6A, diadenosine hexaphosphate; α, β-meATP, α, β-methylene-adenosine 5'-triphosphate; Gp_nG, diguanosine polyphosphate; Ap_5A, diadenosine pentaphosphate; Ap_5G, adenosine pentaphospho guanosine; Ap_6G, adenosine hexaphospho guanosine; Gp_5G, diguanosine pentaphosphate; Gp_6G, diguanosine hexaphosphate.

chain of these naturally occurring substances, with compounds possessing four or more phosphates causing potent vasoconstriction (Ralevic et al., 1995; van der Giet et al., 1999; Lewis et al., 2000). A recent study has indicated that blood pressure-regulating effects of dinucleotides might involve the activation of more than one type of P2X receptor in renal blood vessels (van der Giet et al., 1999). Thus, a P2X receptor mediating a transient fast constriction in the perfused kidney was tentatively identified as the $P2X_1$ receptor, while another unidentified yet pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid-sensitive P2X receptor was thought to mediate a sustained vasoconstriction (van der Giet et al., 1999).

The present study focused on the agonist properties at recombinant $P2X_{1-4}$ receptors of those Ap_nA and Ap_nG compounds that evoke vasoconstriction in blood vessels. The agonist properties of a recently isolated third group of naturally occurring dinucleotides, diguanosine polyphosphates (Gp_nGs), were also studied at these recombinant P2X receptors, to establish the pharmacological activity of these three families of dinucleotides at molecularly defined P2X receptors and, indirectly, to shed further light on their vasoconstrictor actions in vivo.

Materials and Methods

Oocyte Preparation. *Xenopus laevis* frogs were anesthetized in tricaine (0,4% w/v) and killed by decapitation. The ovarian lobes were removed surgically and stored at 4°C in Barth's solution [pH 7.45; 110 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 7.5 mM Tris-HCl, 0.33 mM $Ca(NO_3)$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 50 μg/l gentamycine sulfate]. Mature oocytes were taken from ovarian sacs and were defolliculated by a two-step process involving collagenase treatment (type IA, 2 mg/ml in a Ca^{2+}-free Ringer's solution, for 2–3 h), after which the follicular cell layer was stripped away using fine forceps. Defolliculated oocytes were stored in Barth's solution (4°C, pH 7.5). Defolliculation was necessary to remove the native purinoceptors found on the follicle cell monolayer enveloping oocytes (King et al., 1996). Cells were injected cytosolically with cRNA (capped RNA) for $rP2X_1$ (Valera et al., 1994), $rP2X_2$ (Brake et al., 1994), $rP2X_3$ (Chen et al., 1995), or $rP2X_4$ (Bo et al., 1995) (40 nl, 1 μg/μl), and then incubated at 18°C in Barth's solution for 48 h to allow full receptor expression and stored at 4°C in Barth's solution for up to 12 days.

Electrophysiology. Membrane currents were measured from cRNA-injected oocytes using a twin-electrode voltage-clamp amplifier (Axoclamp 2A; Axon Instruments, Foster City, CA). The holding potential (V_h) was −50 mV, unless stated otherwise. The voltage-recording and current-recording microelectrodes (1–5 Ω-tip resistance) were filled with 3 M KCl. Oocytes were placed in an electrophysiological chamber (0.5-ml volume) and superfused (at 5 ml/min) with Ringer's solution containing 110 mM NaCl, 2.5 mM KCl, 5 mM HEPES, 1.8 mM $BaCl_2$. The pH of the bathing Ringer's solution was adjusted to 7.5 by the addition of either 1.0 N HCl or 1.0 N NaOH. Electrophysiological data were stored on magnetic tape using a DAT recorder (Sony 1000ES; Tokyo, Japan) and displayed using a pen recorder (Gould 2200S; Cleveland, OH).

Solutions. All nucleotides were prepared in Ringer's solution and the pH of stock solutions readjusted to the desired level. Agonists were superfused by gravity flow from independent reservoirs placed above the preparation. All drugs were added for 120 s or until the evoked current reached a peak and then washed off with Ringer's solution for a period of 20 min. Data were normalized to the maximum current (I_{max}) evoked by the drug. The agonist concentration that evoked 50% of the maximum response (EC_{50}) was taken from Hill plots of the transform $\log(I/I_{max} - I)$, where I is the current evoked by each concentration of agonist. The Hill coefficient (n_H) was also taken from the slope of Hill plots.

Statistics and Graphs. Data are presented as mean ± S.E.M. of four to six (except for ATP, $n = 16$) sets of data from different oocyte batches. Concentration-response curves were fitted by nonlinear regression analysis using Prism, version 2.0 (GraphPad, San Diego, CA). Significant differences of potencies and efficacies were analyzed by using Kruskal-Wallis test (Instat, version 2.05A; GraphPad).

Drugs. ATP was purchased from Roche Molecular Biochemicals (Mannheim, Germany) and GTP disodium salt was purchased from Sigma Chemical Co. (Poole, Dorset, UK). Diadenosine pentaphosphate (Ap_5A), Ap_6A, adenosine pentaphospho guanosine (Ap_5G), adenosine hexaphospho guanosine (Ap_6G), diguanosine pentaphosphate (Gp_5G), and diguanosine hexaphosphate (Gp_6G) were synthesized and purified as previously described (Heidenreich et al., 1995; Schlüter et al., 1998).

Results

Effects at Rat $P2X_1$ Receptor. Like ATP, Ap_5A, Ap_5G, Ap_6A, and Ap_6G evoked fast inward membrane currents that rapidly inactivated in defolliculated *X. laevis* oocytes expressing recombinant rat $P2X_1$ receptors (Fig. 1, A and B, original tracing for Ap_6A not shown). Gp_5G, Gp_6G, and GTP failed to

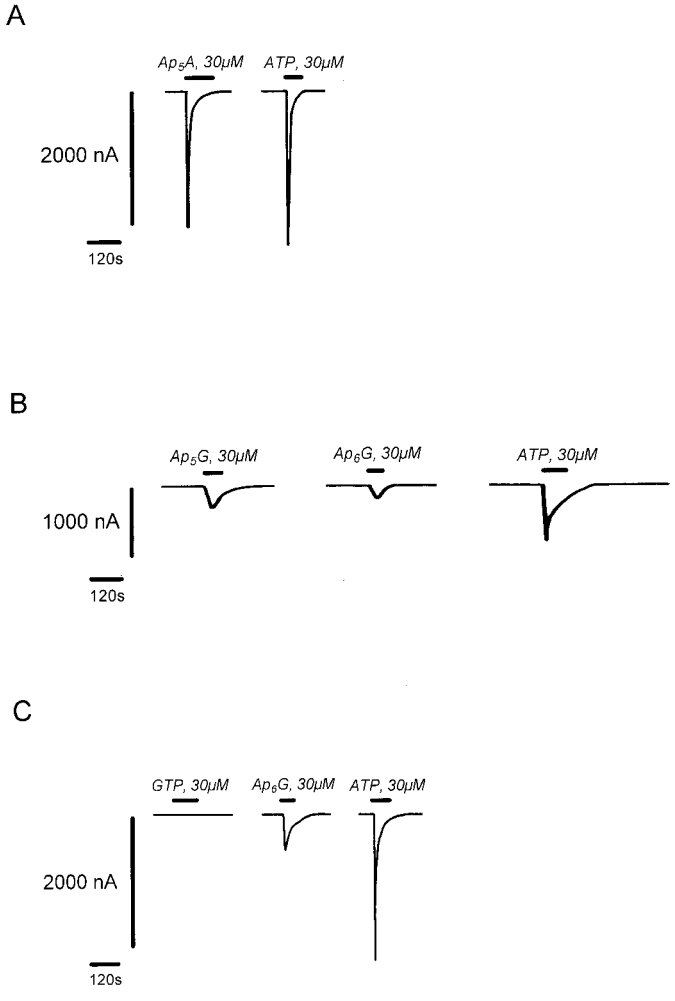

Fig. 1. Dinucleotide agonism at $rP2X_1$ receptors. Representative traces of Ap_5A-activated inward currents compared with activation by ATP (A), Ap_5G and Ap_6G were only partial agonists at $rP2X_1$ (B), GTP failed to evoke a measurable inward current at $rP2X_1$ (C). All agents were applied at 30 μM for 120 s. $V_h = -50$ mV.

evoke significant membrane currents (Fig. 1C, original traces for Gp_5G and Gp_6G not shown).

The EC_{50} value for Ap_5A (Table 1) was 2- to 3-fold less potent than ATP. At supermaximal concentrations (30 μM), Ap_5A evoked 84.0 ± 1.0% of maximum ion flux evoked by ATP (30 μM) (Fig. 3A). Ap_5G was a partial agonist, evoking 50.8 ± 6.3% of the maximal ATP effect. Ap_5G (Table 1) was 4-fold less potent than ATP.

Ap_6A (Table 1) was slightly less potent than ATP but a full agonist (Figs. 2C and 3A), whereas Ap_6G was a partial agonist (27.7 ± 3.2% of maximal ATP response, Fig. 3A). ATP was 3- to 4-fold more potent than Ap_6G (Table 1).

EC_{50} and 95% confidence interval values are summarized in Table 1. The rank order of potency at the $rP2X_1$ receptor was ATP ≥ Ap_6A ≥ Ap_5A ≥ Ap_6G ≥ Ap_5G ≥ GTP = Gp_5G = Gp_6G (inactive), while the rank order of efficacy was ATP ≥ Ap_5A ≥ Ap_6A > Ap_5G > Ap_6G ≥ GTP = Gp_5G = Gp_6G (inactive).

Effects at Rat $P2X_3$ Receptor. Like ATP, Ap_5A, Ap_5G, Ap_6A, and Ap_6G evoked fast inward currents that rapidly inactivated in defolliculated *X. laevis* oocytes expressing recombinant rat $P2X_3$ receptors (Fig. 4, A and B, original tracing for Ap_6A not shown). Gp_5G, Gp_6G, and GTP failed to evoke significant membrane currents (Fig. 4C, original tracing for Gp_5G and Gp_6G not shown).

The EC_{50} value for Ap_5A (Table 2) was approximately 2- to 3-fold less potent than ATP. At supermaximal concentrations (30 μM), Ap_5A evoked 86.1 ± 1.4% of maximum ion flux caused by ATP (30 μM) (Fig. 3B). Ap_5G caused 92.4 ± 4.0% of the maximal ATP effect at $rP2X_3$ (Fig. 3B), and it was 2-fold less potent than ATP (Table 2).

Ap_6A was 3- to 4-fold less potent (Table 2) than ATP but caused 83.6 ± 1.0% of the maximal ATP effect (Fig. 3B). Ap_6G caused 72.6% of the maximal ATP effect (Fig. 3B) and was 4- to 5-fold less potent than ATP (Table 2).

EC_{50} and 95% confidence interval values are summarized in Table 2. The rank order of potency at the $rP2X_3$ receptor was ATP > Ap_5G ≥ Ap_5A ≥ Ap_6A ≥ Ap_6G ≥ GTP = Gp_5G = Gp_6G (inactive). The rank order of efficacy at the $rP2X_3$ receptor was ATP ≈ Ap_5G ≥ Ap_5A ≈ Ap_6A ≥ Ap_6G ≥ GTP = Gp_5G = Gp_6G (inactive) (Fig. 5).

Effects at Rat $P2X_2$ Receptor. Ap_5A, Ap_5G, Ap_6A, Ap_6G, and GTP (all 30 μM) failed to activate membrane currents in defolliculated *X. laevis* oocytes expressing recombinant rat $P2X_2$ receptors (data not shown). ATP was a full agonist (EC_{50} = 18.27 μM; confidence interval, 15.29–21.84 μM).

Effects at Rat $P2X_4$ Receptor. Ap_5A, Ap_5G, Ap_6A, Ap_6G, and GTP (all 30 μM) failed to evoke inward membrane currents in defolliculated *X. laevis* oocytes expressing recombinant rat $P2X_4$ receptors. ATP was a full agonist (EC_{50} = 3.33 μM; confidence interval, 2.17–5.10 μM).

TABLE 1

Agonist potency of ATP and dinucleotides at $rP2X_1$

EC_{50} values and Hill coefficients are presented as mean ± S.E.M.

	EC_{50}	95% Confidence Interval EC_{50}	Hill Coefficient
	μM		
ATP	0.60	0.47–0.77	0.8 ± 0.1
Ap_5A	1.50	1.15–1.95	1.1 ± 0.1
Ap_5G	2.26	1.80–2.83	1.4 ± 0.2
Ap_6A	0.97	0.70–1.36	0.8 ± 0.1
Ap_6G	2.16	1.52–3.08	1.0 ± 0.2

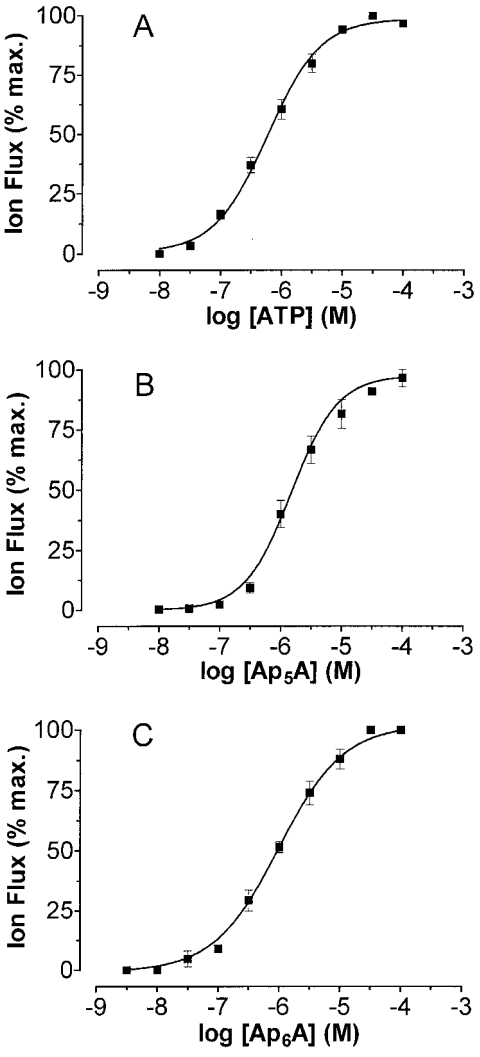

Fig. 2. Nucleotide potency at rat $P2X_1$ receptors. Concentration-response curves for ATP (A), Ap_5A (B), and Ap_6A (C). Agonist activity was normalized to its own maximal response in each experiment (n = 6). EC_{50} values and Hill coefficients are given in Table 1. Curves were fitted using the Hill equation, as defined by Prism, version 2.0 (GraphPad); where error bars are not observed, they fall within the symbol size.

Discussion

The present results confirmed that highly purified Ap_5A and Ap_6A are agonists at $P2X_1$ and $P2X_3$ receptors, being relatively selective for these two group I P2X receptors and failing to activate either homomeric $P2X_2$ or $P2X_4$ receptors. Like ATP, these two diadenosine polyphosphates evoked fast inward currents that rapidly inactivated in their continued presence. Like ATP, they also required long washout periods of up to 20 min before reproducible inward currents could be evoked by a second challenge. It proved difficult to discriminate between the actions of Ap_nA compounds and ATP at $P2X_1$ and $P2X_3$ receptors, since they all yielded broadly similar EC_{50} values in the low micromolar range. Their maximal responses were also broadly similar and each nucleotide could be considered as a partial agonist. Furthermore, there seemed little opportunity to exploit Ap_nA compounds as a means of distinguishing $P2X_1$ receptors from $P2X_3$ receptors, since any differences in their potency and intrinsic efficacy were very minor. It remains the case that the dinucleotidic

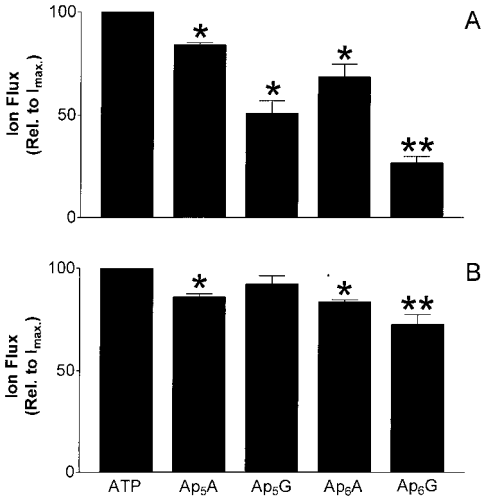

Fig. 3. Nucleotide efficacy at group I P2X receptors. Maximum responses evoked by dinucleotides at supramaximal concentrations (30 μM) as a percentage of the maximal response evoked by ATP (30 μM) at $rP2X_1$ (A) and $rP2X_3$ (B) receptors. Agonist activity was normalized to ATP response, comparing each dinucleotide with ATP in the same cell ($n = 4$). $^*P < 0.05$, $^{**}P < 0.01$, significant differences ATP versus dinucleotide.

antagonist diinosine pentaphosphate represents the most effective means to differentiate $P2X_1$ and $P2X_3$ receptors (King et al., 1999).

The bis-nucleotides Ap_5G and Ap_6G were also found to be selective agonists of $P2X_1$ and $P2X_3$ receptors, since they, too, were quite ineffective at homomeric $P2X_2$ and $P2X_4$ receptors. These naturally occurring substances evoked fast inward currents that rapidly inactivated and were thus similar to ATP. Our results showed that the mononucleotide GTP was inactive at $P2X_1$ and $P2X_3$ receptors, a finding in keeping with earlier studies of these two P2X receptors (Valera et al., 1994; Chen et al., 1995). Therefore, the observed agonist activity of Ap_nG compounds appeared to depend on the solitary adenine moiety. The EC_{50} values of Ap_nGs were similar to corresponding Ap_nA compounds, the removal of one adenine moiety having no significant bearing on agonist potency. However, the maximal response (or intrinsic efficacy) evoked by Ap_nGs was significantly reduced at $P2X_1$ receptors. This observation suggests that the guanine moiety can interfere in some way with the actions of the adenine moiety. This point was borne out where Gp_nGs were tested and found to be quite inactive at $P2X_1$ and $P2X_3$ receptors (also $P2X_2$ and $P2X_4$ receptors). Thus, group I P2X receptors require the presence of at least one adenine moiety in dinucleotidic compounds to bind to the ligand-docking site and cause the necessary conformational change to open (or keep open for a significant period of time) the intrinsic ion channel.

Our pharmacological data on Ap_nA compounds were comparable with the results of an earlier study of recombinant $P2X_{1-4}$ receptors (Wildman et al., 1999), while Ap_nG and Gp_nG have not previously been tested on these ionotropic ATP receptors. The present study had the advantage of using high-performance liquid chromatography-purified dinucleotides, whereas earlier surveys of dinucleotide activity have relied on commercially available compounds. This difference aside, the EC_{50} values for Ap_5A and Ap_6A fell in the low micromolar range and were indistinguishable statistically from the reported values of Wildman et al. (1999). We did notice that the maximal response to Ap_5A at $P2X_1$ receptors

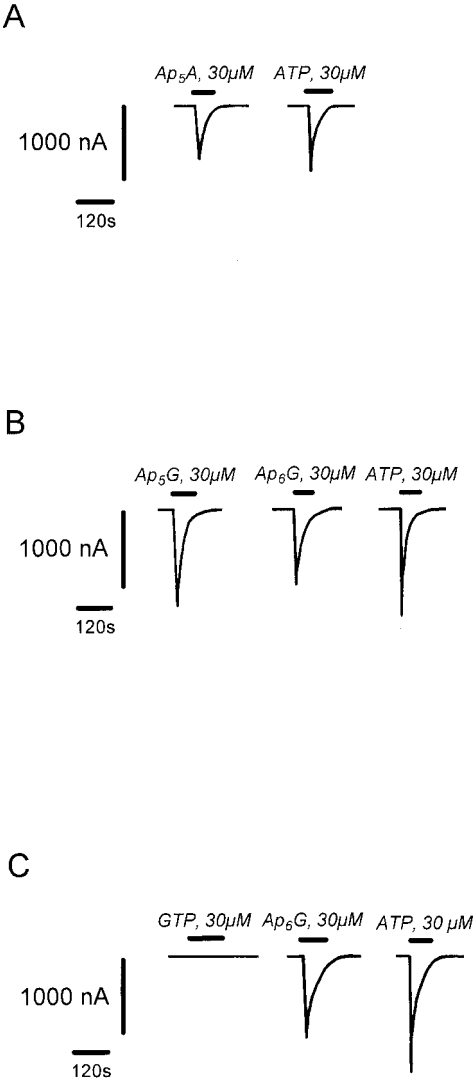

Fig. 4. Dinucleotide agonism at $rP2X_3$ receptors. Representative traces of Ap_5A-activated inward currents compared with activation by ATP (A), Ap_5G and Ap_6G were partial agonists at $rP2X_3$ (B), GTP failed evoke a measurable inward current at $rP2X_3$ (C). All agents were applied at 30 μM for 120 s. $V_h = -50$ mV.

TABLE 2
Agonist potency of ATP and dinucleotides at $rP2X_3$
EC_{50} values and Hill coefficients are presented as mean ± S.E.M.

	EC_{50}	95% Confidence Interval EC_{50}	Hill Coefficient
	μM		
ATP	1.30	1.06–1.67	0.8 ± 0.1
Ap_5A	2.89	2.40–3.47	1.0 ± 0.1
Ap_5G	2.76	2.09–3.66	1.0 ± 0.1
Ap_6A	4.32	3.57–5.23	1.0 ± 0.0
Ap_6G	6.24	4.70–8.27	1.2 ± 0.1

was significantly greater in our hands than reported earlier for human and rat orthologs of $P2X_1$ (Evans et al., 1995; Wildman et al., 1999). It should be borne in mind that $P2X_1$ receptors are rapidly desensitized by both mononucleotides and dinucleotides, and minor differences in rates of recovery from desensitization could account for the observed discrepancies in the intrinsic efficacy of these compounds. Our latest data suggest that Ap_5A and Ap_6A are partial agonists at $P2X_1$ and $P2X_3$ receptors.

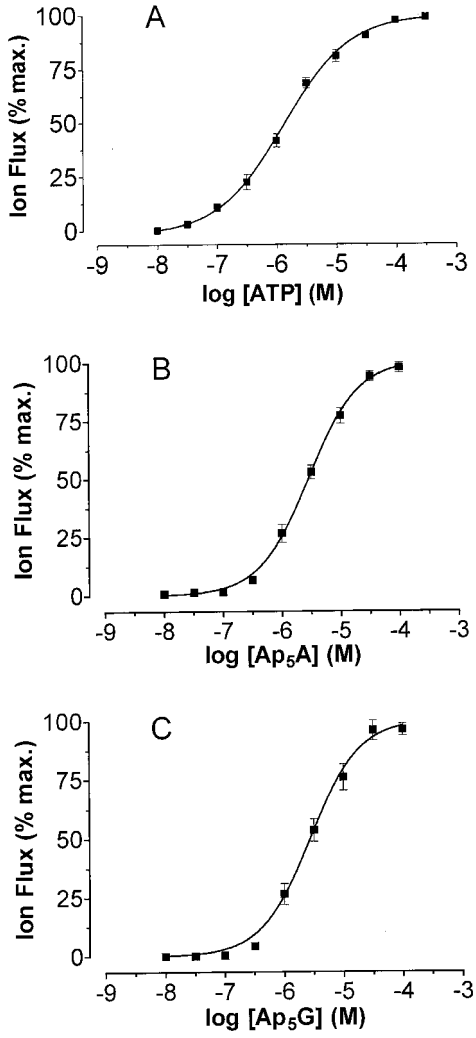

Fig. 5. Nucleotide potency at rat $P2X_3$ receptors. Concentration-response curves for ATP (A), Ap_5A (B), and Ap_5G (C). Agonist activity was normalized to its own maximal response in each experiment ($n = 6$). For EC_{50} values and Hill coefficients are given in Table 2. Curves were fitted using the Hill equation, as defined by Prism, version 2.0 (GraphPad).

None of the active dinucleotides evoked sustained inward currents at $P2X_{1-4}$ receptors and, accordingly, it remains a difficult prospect to explain how these compounds evoke a sustained vasoconstriction in perfused rat kidney (van der Giet et al., 1999). Of the $P2X_1$, $P2X_2$, and $P2X_4$ messenger RNAs found in arterial tissues (Nori et al., 1998), only $P2X_2$ and $P2X_4$ transcripts could result in nondesensitizing homomeric P2X receptors. However, our present results confirmed that the pentaphosphate and hexaphosphate of each dinucleotide were inactive at $P2X_2$ and $P2X_4$ receptors. It is possible that heteromeric P2X receptors with nondesensitizing properties could occur in renal vascular tissues, but the stoichiometry of such P2X subunit assemblies remains unknown. It is improbable that $P2X_1$ and $P2X_2$ subunits were involved, since an earlier study indicated that their coexpression failed to produce a nondesensitizing heteromer (Lewis et al., 1995). Similarly, biochemical evidence indicates that heteromeric $P2X_{1/4}$ and $P2X_{2/4}$ receptor assemblies are highly unlikely (Torres et al., 1998). The recently discovered heteromeric $P2X_{1/5}$ receptor represents a more interesting candidate, since this receptor produces biphasic α, β-meATP responses involving transient and sustained inward currents (Haines et al., 1999; Le et al., 1999; Surprenant et al., 2000). Furthermore, a recent study has likened the heteromeric $P2X_{1/5}$ receptor to native P2X receptors in guinea pig submucosal arterioles, on the basis of common pharmacological and operational profiles (Surprenant et al., 2000). However, $P2X_5$-like immunoreactivity has not been reported in renal vasculature (Chan et al., 1998), nor has it been observed in the vascular supply of the adjacent adrenal gland (Afework and Burnstock, 1999). $P2X_5$ transcripts have been found in rat mesenteric artery (Phillips and Hill, 1999), but Ap_nA and Ap_nG compounds do not elicit nondesensitizing responses in this arterial preparation (Lewis et al., 2000). Thus, it is unlikely that $P2X_{1/5}$ receptors could account for the sustained agonism by these dinucleotides in renal vasculature.

Both Ap_5G and Ap_6G, as well as Ap_5A and Ap_6A, have been found in the secretory vesicles of human blood platelets (Schlüter et al., 1994, 1998). Dinucleotide concentrations in the range of 0.5 to 3 μM have been found in the supernatant following platelet aggregation (Schlüter et al., 1998), although the local concentration at the site of release might be 10-fold higher (Beigi et al., 1999). Such concentration levels would saturate the P2X receptor population in the renal vasculature, where agonist-evoked transient and sustained vasoconstrictor responses are maximal in the low micromolar range (van der Giet et al., 1999). Our present results indicate that the fast vasoconstrictor effects of Ap_nGs and Ap_nAs was consistent with the activation $P2X_1$ receptors since, of the two group I P2X receptors that respond to these compounds, only $P2X_1$ receptors are present in smooth muscle. The mediator of slow response cannot yet be accounted for, but the occurrence of a nondesensitizing P2X receptor in kidney has important implications in the development and maintenance of essential hypertension by naturally occurring dinucleotides that potentially can be released in physiologically relevant concentrations.

Acknowledgments

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