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Sensitization by Extracellular Ca^{2+} of Rat $P2X_5$ Receptor and Its Pharmacological Properties Compared with Rat $P2X_1$.

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ABSTRACT

The recombinant rat $P2X_5$ ($rP2X_5$) receptor, a poorly understood ATP-gated ion channel, was studied under voltage-clamp conditions and compared with the better understood homomeric $rP2X_1$ receptor with which it may coexist in vivo. Expressed in defolliculated *Xenopus laevis* oocytes, $rP2X_5$ responded to ATP with slowly desensitizing inward currents that, for successive responses, ran down in the presence of extracellular Ca^{2+} (1.8 mM). Replacement of Ca^{2+} with either Ba^{2+} or Mg^{2+} prevented rundown, although agonist responses were very small, whereas reintroduction of Ca^{2+} for short periods of time (<300 s) before and during agonist application yielded consistently larger responses. Using this Ca^{2+}-pulse conditioning, $rP2X_5$ responded to ATP and other nucleotides (ATP, 2-methylthio-ATP, adenosine-5′-*O*-(thiotriphosphate), 2′-&-3′-*O*-(4-benzoylbenzoyl)-ATP, α,β-methylene-ATP, P^1-$P^{(4)}$-diadenosine-5′-phosphate, and more) with pEC_{50} values within 1 log unit of respective determinations for $rP2X_1$. Only GTP was selective for $rP2X_5$, although 60-fold less potent than ATP. At $rP2X_5$, lowering extracellular pH reduced the potency and efficacy of ATP, whereas extracellular Zn^{2+} ions (0.1–1000 μM) potentiated then inhibited ATP responses in a concentration-dependent manner. However, these modulators affected $rP2X_1$ receptors in subtly different ways–with increasing H^+ and Zn^{2+} ion concentrations reducing agonist potency. For P2 receptor antagonists, the potency order at $rP2X_5$ was pyridoxal-5-phosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS) > 2′,3′-*O*-(2,4,6-trinitrophenyl)ATP (TNP-ATP) > suramin > reactive blue 2 (RB-2) > diinosine pentaphosphate (Ip_5I). In contrast, the potency order at $rP2X_1$ was TNP-ATP = Ip_5I > PPADS > suramin = RB-2. Thus, the Ca^{2+}-sensitized homomeric $rP2X_5$ receptor is similar in agonist profile to homomeric $rP2X_1$—although it can be distinguished from the latter by GTP agonism, antagonist profile, and the modulatory effects of H^+ and Zn^{2+} ions.

Extracellular ATP acts as a signaling molecule within the central, peripheral, and enteric nervous systems; elsewhere at neuroeffector junctions of the autonomic nervous system; and locally as a paracrine or autocrine humoral agent (Ralevic and Burnstock, 1998). Irrespective of its sites of action, ATP exerts its effects through two main classes of P2 receptors—the P2X and P2Y families. For the P2X receptor class, seven subunits ($P2X_{1-7}$) have been identified thus far (Burnstock and King, 1996). P2X subunit proteins have intracellular N and C termini with two membrane-spanning domains connected by a large cysteine-rich extracellular loop. Three, possibly four, P2X subunits assemble to form homomeric complexes that function as nonselective cation channels gated by extracellular ATP (Kim et al., 1997; Nicke et al., 1998). Different P2X receptor subunits can also coassemble to form heteromeric complexes with pharmacological and operational profiles distinct from the homomeric P2X receptors made by the constituent subunits. So far, five functional P2X heteromers ($P2X_{1/2}$, $P2X_{1/5}$, $P2X_{2/3}$, $P2X_{2/6}$, and $P2X_{4/6}$) have been characterized (Brown et al., 2002), of a series of 11 heteromers predicted by immunoprecipitation studies

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Part of this work has been presented at the joint meeting of the German, Scandinavian, and British Physiology Societies at Tübingen Germany, 2002 March 17–19 (Wildman et al., 2002, in References). Data on $rP2X_1$ also appears in a PhD thesis titled "Pharmacological agents that distinguish between P2X receptor subtypes" by Sean Gerard Brown (University College London, 2001).

ABBREVIATIONS: 2MeSATP, 2-methylthioATP; meATP, methyleneATP; $rP2X_1$, rat $P2X_1$ receptor; C/R, concentration/response; PBST. phosphate-buffered saline with Triton X-100; Ip_5I, diinosine pentaphosphate; TNP-ATP, 2′,3′-*O*-(2,4,6-trinitrophenyl)ATP; BAPTA-AM, 1,2-bis(2-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid (acetoxymethyl ester); ATPγS, adenosine-5′-*O*-(thiotriphosphate); BzATP, 2′-&-3′-*O*-(4-benzoylbenzoyl)-ATP; PPADS, pyridoxal-5-phosphate-6-azophenyl-2′,4′-disulfonic acid; RB-2, reactive blue 2; Ap_xA, P^1-$P^{(x)}$-diadenosine-5′-phosphate (x = 2–6); HEK, human embryonic kidney; PKC, protein kinase C; PKD, polycystic kidney disease.

(Torres et al., 1999). The formation of heteromeric P2X receptors has helped explain in part, but not totally, some pharmacological aspects of purinergic signaling at endogenous P2X receptors in whole tissues.

The $P2X_5$ receptor was first cloned from rat celiac ganglia (Collo et al., 1996). Homomeric $P2X_5$ was viewed as a slowly desensitizing receptor sensitive to ATP and 2MeSATP, but insensitive to α,β-meATP and many other nucleotides. Agonist responses were noted to be exceedingly small (by a factor of 100) compared with the responses of other recombinant P2X receptors tested under similar conditions, which raised questions over the functional role of homomeric $P2X_5$ receptors in vivo (Collo et al., 1996). Also, $P2X_5$ transcripts and protein have a restricted distribution, with expression mainly in the trigeminal mesencephalic nucleus of the brainstem; a lesser presence in sensory neurons, cervical spinal cord, and some blood vessels; and a limited presence in kidney, heart, skeletal muscle, adrenal gland, retina, and assorted epithelial cell types (Collo et al., 1996; Garcia-Guzman et al., 1996; Brändle et al., 1998; Phillips et al., 1998; Gröschel-Stewart et al., 1999; Phillips and Hill, 1999; Taylor et al., 1999; Gitterman and Evans, 2000; Ryten et al., 2001). However, many of these tissues and cell types contain mRNA for other P2X isoforms and, on occasion, this includes transcripts for the $P2X_1$ subunit. $P2X_1$ receptors are abundant in smooth muscle yet also occur in heart, sensory neurons, spinal cord, duodenal submucosa, blood platelets, and megakaryocytes. Functionally, homomeric $P2X_1$ receptors are viewed as rapidly desensitizing, α,β-meATP-sensitive P2X receptors that are blocked by suramin (Khakh et al., 2001). Interestingly, a novel $P2X_{1/5}$ receptor can form when $P2X_1$ and $P2X_5$ subunits are coexpressed in the same cell (Torres et al., 1998; Haines et al., 1999; Lê et al., 1999; Surprenant et al., 2000). This observation, together with proposed assemblies of $P2X_{2-4}$ subunits with the $P2X_5$ subunit (Torres et al., 1999), has lent weight to the suggestion that the main physiological role of the $P2X_5$ subunit probably rests with the construction of heteromeric P2X receptors.

Earlier histochemical investigations have revealed $P2X_5$-like immunoreactivity ($P2X_5$-ir), without the colocalization of other P2X subunit proteins, in the apical border of epithelial cells lining the collecting duct of the rat nephron (Chan et al., 1998; Bailey et al., 2000; Schwiebert and Kishore, 2001). This site is also the locus of a pernicious disease, called polycystic kidney disease (PKD), in which a prominent up-regulation of $P2X_5$-ir has been observed in human and rat PKD cell cultures (Schwiebert, 2001; Schwiebert and Kishore, 2001). In a broader context, dense $P2X_5$-ir has also been associated with the proliferating and differentiating cell layers of rat squamous epithelial tissues (Gröschel-Stewart et al., 1999) and with developing (E15–18) rat embryo skeletal myotubes (Ryten et al., 2001). Thus, we have taken a fresh look at recombinant $P2X_5$ receptors to see under what circumstances homomeric $P2X_5$ assemblies might be able to function. The positive outcome of such experiments then led us to compare homomeric $P2X_5$ receptors with homomeric $P2X_1$, because the possibility exists that multiple P2X subtypes of close pharmacological profile, yet dissimilar operational properties, are present elsewhere in rat kidney—specifically the intrarenal vasculature (van der Giet et al., 1999). Part of this study has been presented elsewhere (Wildman et al., 2002).

Materials and Methods

Oocyte Preparation. *Xenopus laevis* frogs were anesthetized in tricaine (0.4% w/v), killed by decapitation, and their ovarian lobes were removed surgically. Oocytes (stages V and VI) were defolliculated by a 2-step process involving collagenase treatment (Type IA, 2 mg/ml in a Ca^{2+}-free Ringer solution, for 2 h) followed by a mechanical stripping away of the follicular layer with fine forceps (in double-strength Ca^{2+}-free Ringer solution). Defolliculated oocytes were stored mainly in a (Ca^{2+}-free) Ba^{2+}-containing Ringer solution, pH 7.5 at 4°C, containing 110 mM NaCl, 2.5 mM KCl, 5 mM HEPES, and 1.8 mM $BaCl_2$. In a few experiments, $BaCl_2$ was replaced with equimolar $CaCl_2$. Gentamicin sulfate was not present in storage solutions. Defolliculated oocytes were injected cytosolically with either rat $P2X_5$ or rat $P2X_1$ cRNA (40 nl, 1 μg/μl), incubated for 24 to 48 h at 18°C in a Ba^{2+}-containing Ringer solution then kept at 4°C for up to 7 days until used in electrophysiological experiments. Only those $P2X_5$-injected oocytes with maximal ATP-evoked whole-cell currents of 20 nA and greater were used in this investigation.

Electrophysiological Recordings. Agonist-activated membrane currents ($V_h = -95$ mV) were recorded from cRNA-injected oocytes using a twin-electrode voltage-clamp amplifier (Axoclamp 2A; Axon Instruments, Union City, CA). The voltage-recording and current-recording microelectrodes (1–5 MΩ tip resistance) were filled with 3.0 M KCl. Oocytes were superfused with a Ba^{2+}-Ringer solution (10 ml/min, pH 7.5, at 18°C). In some cases, $MgCl_2$ or $CaCl_2$ (1.8 mM) was added to the superfusate, either in place of or in addition to $BaCl_2$, to investigate the effects of different divalent cations in the superfusate. Where stated, the pH of the bathing solution was adjusted using either 1.0 N HCl or NaOH to achieve the desired level. Electrophysiological data were stored on a computer using a MP100 WSW interface (Biopac Systems, Goleta, CA) and analyzed using the software package Acqknowledge III (Biopac Systems).

Data Analysis. Agonists were prepared in a Ca^{2+}-containing Ringer solution (1.8 mM Ca^{2+}) and superfused (12 ml/min) by a gravity-fed continuous flow system that allowed rapid addition and washout. In some cases, agonists were prepared in a Ba^{2+}-containing Ringer solution (1.8 mM Ba^{2+}) to investigate $P2X_5$ receptor rundown. Agonists were added for 90 s or until the current reached a peak, then washed for a period of 15 and 20 min for $rP2X_5$ and $rP2X_1$ receptors, respectively. For concentration-response (C/R) curves, data were normalized to the maximum current (I_{max}) evoked by ATP (100 μM). 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 taken from the slope of the Hill plots. The activity of P2 antagonists was tested by adding each antagonist in cumulative concentrations (as mentioned in the text), each concentration applied 15 min or 20 min ($rP2X_5$ and $rP2X_1$, respectively) before the addition of a submaximal concentration of ATP ($\sim EC_{70}$ concentration, 1 μM ATP). The antagonist concentration that reduced ATP-responses by 50% (IC_{50}) was taken from inhibition curves. The reversibility of receptor blockade was tested after prolonged washout of antagonists.

The effects of extracellular Zn^{2+} were also investigated on agonist activity. C/R curves for the modulatory activity of Zn^{2+} ions were constructed using a submaximal concentration of ATP and data were normalized to the amplitude of control ATP responses. These experiments were carried out for different preincubation periods for Zn^{2+} (ie, 0 or 15 min and 0 or 20 min for $rP2X_5$ and $rP2X_1$ receptors, respectively) before the addition of ATP. Data are presented as mean ± S.E.M. of three or more sets of data using different oocyte batches. Significant differences were determined by Student's t test (Instat v2.05a; GraphPad Software, San Diego, CA). Concentration-response curves and inhibition curves were fitted by nonlinear regression analysis using commercial software (Prism v2.0, GraphPad).

Immunohistochemistry. Defolliculated oocytes were injected with either cRNA for rP2X$_5$ or sterile H_2O (control), stored in either a Ba^{2+}- or Ca^{2+}-containing Ringer solution (18°C, for 48 h) and then prepared for immunohistochemistry. All reactions were performed on free-floating whole oocytes. Injected and control oocytes were fixed for 2 min in paraformaldehyde [4% (w/v) in a 0.1 M phosphate buffer] and washed in phosphate-buffered saline containing 0.1% Triton X-100 (PBST). Oocytes were preincubated for 30 min at room temperature with 10% normal rabbit serum in phosphate-buffered saline (with 1% Triton X-100 added), followed by overnight incubation at 4°C with a goat polyclonal antibody for P2X$_5$ (Santa Cruz, CA) diluted 1:100 in PBST and containing 1% normal rabbit serum. Oocytes were washed in PBST and incubated for 1 h at room temperature with biotinylated rabbit anti-goat IgG (Vector Laboratories, Peterborough, UK) diluted 1:200 in PBST containing 1% normal rabbit serum and 1% bovine serum albumin. After washing with PBST, oocytes were incubated for 1 h at room temperature with a preformed avidin-biotinylated alkaline phosphatase complex (Vector Labs) diluted according to kit instructions with PBST. A *Vector Red* alkaline phosphatase substrate kit was used to visualize immunoreactivity. Stained oocytes were washed in distilled water, placed onto slides and covered in *Histotec*, a permanent aqueous mountant (Serotec Ltd., Oxford UK). Fluorescent immunostaining was viewed with a Leica DMR microscope fitted with a Rhodamine filter (515–560 nm). Digital pictures were taken with a Nikon CoolPix 990 digital camera; fluorescence images were converted from color to grayscale, and then inverted (white-on-black to black-on-white) using Photoshop v5.5 (Adobe Systems Inc., San José, CA).

Drugs. ATP, related nucleotides, and other drugs, including tricaine, were obtained from Sigma-Aldrich (Poole, Dorset, UK). Suramin was a gift from Bayer plc (Newbury, Berkshire, UK), Ip$_5$I was a gift from Dr. J. Pintor (Universidad Complutense, Madrid, Spain) and TNP-ATP was obtained from Molecular Probes (Eugene, OR). All reagents were AnalaR grade from Sigma-Aldrich. Contaminating ATP was removed from ADP stocks by adding hexokinase (50 U/ml) and glucose (25 mM) for 2 h before assay as described in Bogdanov et al. (1998).

DNA Constructs. The rP2X$_5$ receptor cDNA was a kind gift from Dr. Xuenong Bo and Professor Alan North (University of Sheffield, South Yorkshire, UK) and the rP2X$_1$ receptor cDNA from Dr. Gary Buell (Ares Sereno, Geneva, Switzerland). The rP2X$_5$ DNA was resequenced by MWG Biotech Ltd (Milton Keynes, Buckinghamshire, UK). Using BLAST analysis of a comprehensive database

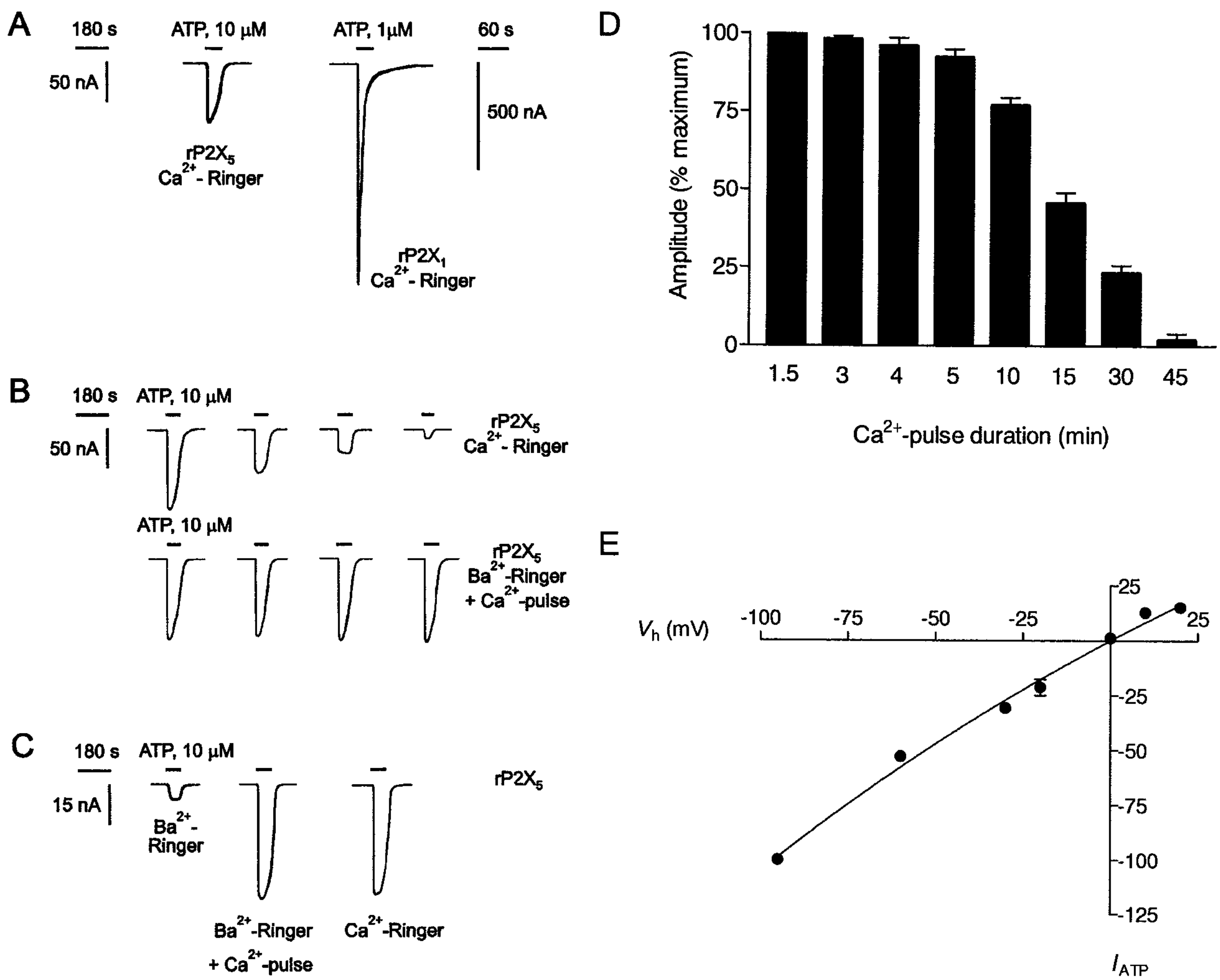

Fig. 1. Activation of homomeric P2X receptors. A, whole cell inward currents activated by near saturating concentrations of ATP at homomeric rP2X$_5$ and rP2X$_1$ receptors [holding potential (V_h), −95 mV]. B, rundown of successive ATP responses at the rP2X$_5$ receptor bathed in a Ringer solution containing Ca^{2+} ions (1.8 mM) (upper row), and consistent-amplitude ATP responses where a 90-s conditioning Ca^{2+}-pulse (1.8 mM) was added to a superfusate containing Ba^{2+} ions (1.8 mM) (lower row). C, maximum ATP responses at the rP2X$_5$ receptor in either Ba^{2+}-Ringer, or Ba^{2+}-Ringer and 90-s conditioning Ca^{2+}-pulse, or Ca^{2+}-Ringer. D, mean amplitude of maximum ATP-evoked responses (n = 4) at the rP2X$_5$ receptor after conditioning Ca^{2+}-pulses of various durations before agonist application. E, current-voltage relationship ATP evoked inward currents at the rP2X$_5$ receptor after a 90-s conditioning Ca^{2+}-pulse (n = 3).

(957,033 sequences), the sequence exactly matched the rat $P2X_5$ purinoceptor (GenBank accession number X92069).

Results

Whole-Cell Inward Currents. ATP (10 and 1 μM) elicited maximal inward membrane currents in defolliculated oocytes expressing $rP2X_5$ and $rP2X_1$ receptors, respectively. At the $rP2X_5$ receptor, the evoked inward current was slowly desensitizing with a maximum amplitude of the order of 75nA (at −95 mV), whereas, at the $P2X_1$ receptor, the evoked inward current was rapidly desensitizing with a maximum amplitude of approximately 1 μA at the same holding potential (Fig. 1A). At the $rP2X_5$ receptor, successive ATP-activated inward currents (I_{ATP}) showed nearly complete and irreversible rundown (92 ± 8% of the initial ATP response) over a period of 45 to 60 min when oocytes were exposed to a Ca^{2+}-containing Ringer solution (Ca^{2+}, 1.8 mM) (Fig. 1B). However, rundown could be avoided if a Ba^{2+}-containing Ringer solution (Ba^{2+}, 1.8 mM) was used during washout periods and Ca^{2+} ions (1.8 mM) returned to the superfusate (with Ba^{2+} present in the background; 1.8 mM) during the period of agonist application (Fig. 1B). If Ca^{2+} was omitted during agonist application and only Ba^{2+} was present, subsequent agonist responses were significantly decreased in amplitude (by 10-fold) although there was no further rundown of ATP responses (Fig. 1C). There was no significant difference between the amplitude of agonist responses evoked in the presence of either Ba^{2+} and Ca^{2+} together or Ca^{2+} alone (Fig. 1C). Similar results were obtained when Ba^{2+} was substituted for Mg^{2+} ions (data not shown). At the $rP2X_1$ receptor, substitution of Ca^{2+} with equimolar Ba^{2+} in the Ringer solution caused no significant difference in the amplitude of agonist responses or rundown of ATP responses. Additionally, using a Ba^{2+}-Ringer superfusate during inter-agonist periods and Ba^{2+}/Ca^{2+}-Ringer solution during agonist application (90-s conditioning Ca^{2+}-pulse), no significant difference was observed in the amplitude of agonist responses at $P2X_1$ (data not shown).

Analysis of the inhibitory effect of conditioning Ca^{2+}-pulse at $rP2X_5$ receptors showed that perfusion periods greater than 5 min were necessary to initiate rundown, 15 min to cause half-maximal inhibition, and 45 min to cause maximal inhibition (Fig. 1D). Thus, all further agonist responses at $rP2X_5$ were evoked after a 90-s conditioning Ca^{2+}-pulse. Current/voltage analysis of I_{ATP} (1 μM ATP, approximately EC_{70} at pH 7.5) after a 90-s conditioning Ca^{2+}-pulse revealed a reversal potential (E_{rev}) of 2.0 ± 3.0mV (n = 3) at the $rP2X_5$ receptor (Fig. 1E). Furthermore, preincubation with BAPTA-AM (30 μM for 30 min, in either Ca^{2+} or Ba^{2+}-Ringer) failed to alter the amplitude, time constant or degree of rundown of I_{ATP} evoked (10 μM, preceded by a 90-s Ca^{2+}-Ringer pulse) by $P2X_5$ receptors (data not shown).

Immunohistochemistry. Relative amounts of membrane bound $P2X_5$-like material were assessed in $rP2X_5$ cRNA-injected oocytes stored for 48 h in either a Ba^{2+}- or Ca^{2+}-containing Ringer solution (Fig. 2). A dense and even layer of $P2X_5$-like immunoreactive material was observed in the plasmalemma of whole cRNA-injected oocytes that had been kept in a Ba^{2+}-containing Ringer solution (Fig. 2A). In contrast, immunostaining was much less dense and markedly punctate for cRNA-injected oocytes stored in a Ca^{2+}-containing Ringer solution (Fig. 2C). Little, if any, immunostaining was observed in control (H_2O-injected) oocytes (Fig. 2E), and where the anti-$P2X_5$ antibody was omitted, very little alkaline phosphatase surface activity was seen in either cRNA-injected or control oocytes (Fig. 2, B, D, and F).

Comparison of I_{ATP} at Homomeric P2X Receptors. All agonist responses at $rP2X_5$ receptors were evoked after a 90-s conditioning Ca^{2+}-pulse. The maximum ATP-evoked inward current was 74 ± 22 nA at the $rP2X_5$ homomer (n = 15), compared with 950 ± 260 nA at the $rP2X_1$ receptor (n = 15) (Fig. 3A). Increasing the concentration of injected $rP2X_5$ cRNA 5-fold (from 1 to 5 μg/μl) did not result in larger whole-cell currents for maximum ATP responses (data not shown). The area-under-the-curve ($I \times t = Q$, or charge transfer) for the $rP2X_5$ receptor was 583 ± 212 × 10^{-9} Q,

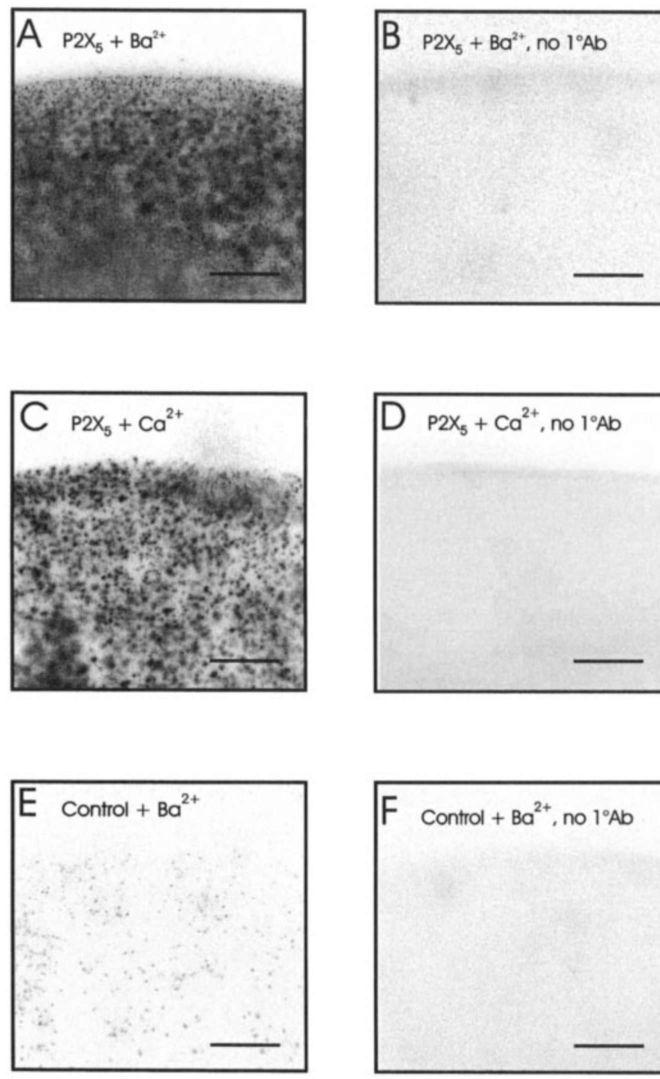

Fig. 2. Immunostaining in $P2X_5$ cRNA-injected oocytes. A, $P2X_5$-like immunoreactivity in a cRNA-injected oocyte stored for 48 h in a Ba^{2+}-containing bathing solution before immunohistochemical processing. B, the primary antibody (anti-$P2X_5$) was omitted. Experiments were repeated for cRNA-injected oocytes stored for 48 h in Ca^{2+}-containing bathing solution (C, D) and for H_2O-injected control oocytes for 48 h in a Ba^{2+}-containing bathing solution (E, F). The plasmalemmal accumulation of $rP2X_5$-like material in cRNA-injected oocytes was affected by prolonged exposure to extracellular Ca^{2+} ions, resulting in reduced immunohistochemical staining and formation of punctate islands of $P2X_5$-like protein. Scale bar, 50 μm.

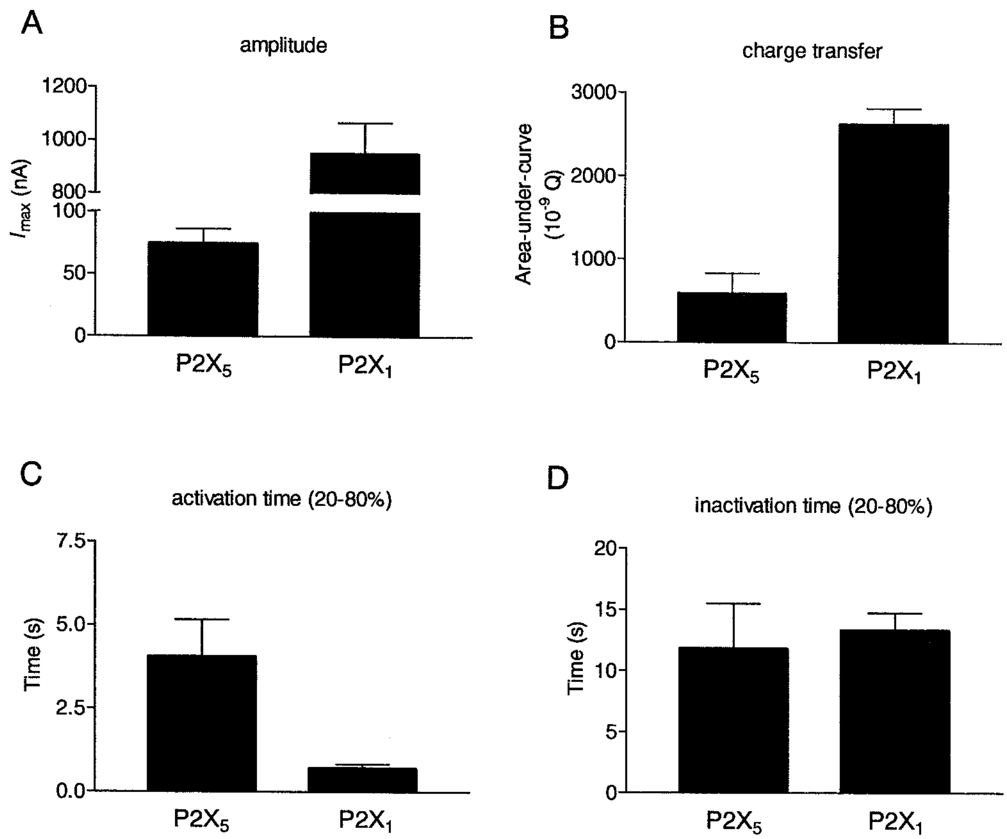

Fig. 3. ATP responses at homomeric P2X receptors. The maximum amplitude (A), area-under-the-curve ($I \times t = Q$, charge transfer) (B), activation-time (20–80%) (C), and inactivation-time (20–80%) (D) of ATP responses (10 or 1 μM, respectively) at homomeric rP2X$_5$ and rP2X$_1$ receptors. Data are expressed as mean ± S.E.M., n = 15.

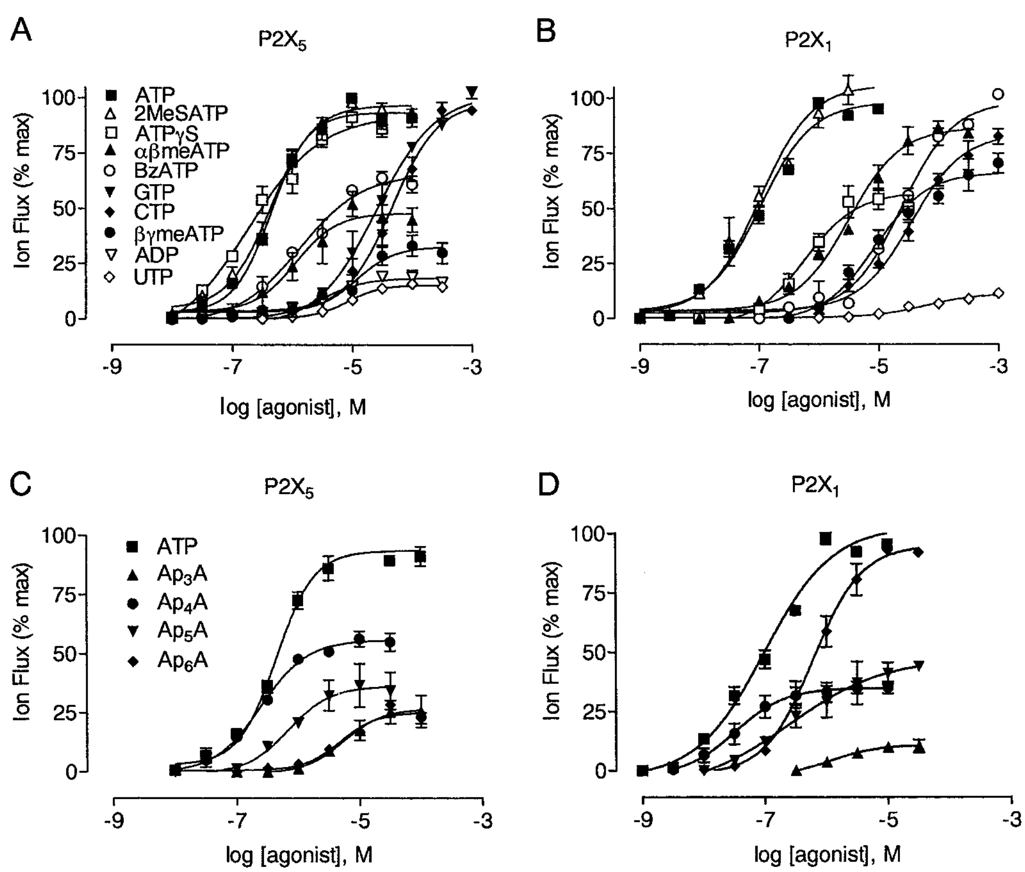

Fig. 4. Agonism of homomeric P2X receptors. C/R curves for nucleotide agonists of homomeric rP2X$_5$ receptors (A and C) and rP2X$_1$ receptors (B and D) at pH 7.5. Data were normalized to the amplitude of maximum ATP responses (10 and 1 μM for rP2X$_5$ and rP2X$_1$, respectively). EC$_{50}$ and n_H values are given in Table 1. Data are expressed as mean ± S.E.M. (n = 4 or more).

TABLE 1
Agonist potency at homomeric P2X receptors.
EC_{50} and n_H values at pH 7.5.

	rP2X$_5$		rP2X$_1$	
	EC_{50}	n_H	EC_{50}	n_H
	μM		μM	
ATP	0.44 ± 0.05	1.4 ± 0.2	0.10 ± 0.07	0.8 ± 0.3
2MeSATP	0.44 ± 0.15	1.2 ± 0.1	0.10 ± 0.03	0.7 ± 0.2
ATPγS	0.29 ± 0.17	0.7 ± 0.2	0.59 ± 0.23[a]	1.0 ± 0.2
α,β-meATP	1.1 ± 0.2[a]	1.3 ± 0.6	3.2 ± 0.2[a]	0.8 ± 0.4
BzATP	1.3 ± 0.9[a]	0.8 ± 0.2	24.2 ± 0.2	0.8 ± 0.1
GTP	25.1 ± 0.1	0.9 ± 0.3	Inactive	
CTP	46.2 ± 0.9	1.1 ± 0.2	35.1 ± 0.2[a]	0.6 ± 0.3
β,γ-meATP	11.8 ± 0.2[a]	1.4 ± 0.6	8.7 ± 0.2[a]	0.6 ± 0.4
ADP	3.6 ± 0.3[a]	1.3 ± 1.0	Inactive	
UTP	8.2 ± 0.1[a]	1.5 ± 0.7	61.2 ± 0.3[a]	0.4 ± 0.4
ITP	Inactive		Inactive	
UDP	Inactive		Inactive	
Ap$_2$A	Inactive		Inactive	
Ap$_3$A	5.4 ± 0.2[a]	1.4 ± 0.6	1.2 ± 0.5[a]	1.1 ± 0.3
Ap$_4$A	0.25 ± 0.1[a]	1.1 ± 0.2	0.04 ± 0.01[a]	1.0 ± 0.3
Ap$_5$A	0.69 ± 0.2[a]	1.3 ± 0.6	0.90 ± 0.10[a]	0.9 + 0.1
Ap$_6$A	4.8 ± 0.2[a]	1.5 ± 0.6	0.60 ± 0.07	1.0 ± 0.2
PPADS	0.20 ± 0.02	0.6 ± 0.1	0.12 ± 0.07	1.5 ± 0.3
TNP-ATP	0.45 ± 0.18	0.6 ± 0.2	0.001 ± 0.0007	0.8 ± 0.1
Suramin	1.5 ± 0.20	0.7 ± 0.2	1.7 ± 0.72	0.9 ± 0.1
RB-2	18.3 ± 0.81	0.9 ± 0.1	2.3 ± 1.00	1.1 ± 0.3
Ip$_5$I	>30		0.003 ± 0.0008	0.9 ± 0.2

[a] Partial agonist.

compared with $2667 \pm 209 \times 10^{-9}$ Q for the rP2X$_1$ receptor (Fig. 3B). At the rP2X$_5$ receptor, the time for I_{ATP} to activate and deactivate (20–80%) was 4.1 ± 0.9 s and 11.8 ± 3.9 s, respectively (n = 15). At the rP2X$_1$, the activation time (20–80%) was 0.7 ± 0.2 s, and inactivation time (20–80%) was 13.3 ± 1.6 s (n = 15) (Fig. 3C, 3D). Maximal amplitude, area-under-the-curve, and activation times were statistically different ($p < 0.05$) for inward currents evoked by the two P2X receptor subtypes.

Agonist Potency at Homomeric P2X Receptors. A broad range of nucleotides was tested for agonist activity at homomeric rP2X$_5$ and rP2X$_1$ receptors. C/R curves for both P2X subtypes were determined under conditions of a Ba^{2+}-containing superfusate during interagonist periods and Ba^{2+}/Ca^{2+}-containing solution just before (90-s conditioning Ca^{2+}-pulse) and during agonist application. For ATP, the EC_{50} (and n_H) values were 441 ± 49 nM (1.4 ± 0.2) and 104 ± 65 nM (0.8 ± 0.3) at rP2X$_5$ and rP2X$_1$ receptors, respectively (n = 12). For other agonists, the potency order at the rP2X$_5$ was (by comparison of EC_{50} values): ATP = 2MeSATP = ATPγS > α,β-meATP = BzATP > ADP > UTP > β,γ-meATP > GTP > CTP. Here, α,β-meATP, BzATP, β,γ-meATP, ADP, and UTP were partial agonists and ITP and UDP were inactive (Fig. 4A, Table 1). At the rP2X$_1$ receptor, the agonist potency order was: ATP = 2MeSATP > ATPγS > α,β-meATP > β,γ-meATP > BzATP > CTP > UTP. Here, ATPγS, α,β-meATP, CTP, β,γ-meATP, and UTP were partial agonists and GTP, ADP, ITP, and UDP were inactive (Fig. 4B, Table 1). EC_{50} values for most agonists were within 1 log unit of concentration for the two P2X subtypes, except for GTP (which activated only rP2X$_5$) and BzATP and UTP (which were significantly more potent at rP2X$_5$). Of these three agents, only GTP was a full agonist at homomeric rP2X$_5$ receptors.

A range of adenine dinucleotides was tested for agonist activity at homomeric rP2X$_5$ and rP2X$_1$ receptors. At the rP2X$_5$ receptor, Ap$_4$A was a partial agonist and slightly more potent than ATP (Fig. 4C, Table 1). Ap$_3$A, Ap$_5$A and Ap$_6$A were partial agonists but none were more potent than ATP. Ap$_2$A (0.1–100 μM) was inactive as an agonist and, furthermore, neither antagonized nor potentiated ATP responses. At the rP2X$_1$ receptor, only Ap$_6$A was a full agonist but 6-fold less potent than ATP (Fig. 4D, Table 1). Ap$_3$A, Ap$_4$A, and Ap$_5$A were partial agonists and only Ap$_4$A was more potent than ATP. Ap$_2$A (0.1–30 μM) was inactive as an agonist and, furthermore, failed to modulate ATP-responses.

Antagonism of Homomeric P2X Receptors. A series of known P2 receptor antagonists was tested against I_{ATP} evoked by homomeric rP2X$_5$ and rP2X$_1$ receptors. At the rP2X$_5$ receptor, the potency order for five antagonists was: PPADS > TNP-ATP > suramin > RB-2 ≫ Ip$_5$I (Fig. 5A, Table 1). Notably, PPADS was active at submicromolar concentrations (mean IC_{50}, 200 nM), whereas TNP-ATP, suramin, and RB-2 were 3-, 8-, and 90-fold less potent. Ip$_5$I was a weak antagonist (15 ± 7% inhibition at 30 μM) and, because of limited availability, was not tested at higher concentrations. At the rP2X$_1$ receptor, the potency order was: TNP-ATP = Ip$_5$I > PPADS > suramin = RB-2 (Fig. 5B, Table 1). Both TNP-ATP and Ip$_5$I were active in the nanomolar concentration range and virtually equipotent. Relative to these two compounds, PPADS was about 10-fold less potent, whereas suramin and RB-2 were about 1000-fold less potent. Where data for homomeric rP2X$_5$ and rP2X$_1$ receptors were compared, TNP-ATP and Ip$_5$I are relatively selective for rP2X$_1$ receptors. PPADS and suramin were equally effective at the two P2X subtypes. RB-2 was about 9-fold more potent at rP2X$_1$ than rP2X$_5$ receptors. The blocking actions of TNP-ATP, Ip$_5$I, and suramin at both P2X receptors subtypes were reversible with washout periods of about 1 h, whereas blockade by either RB-2 or PPADS was only partially reversible after 1-h washout.

Cationic Modulation of Homomeric P2X Receptors. The potency and efficacy of ATP were re-examined with different divalent cations present in the bathing solution. Four extracellular solutions, containing either Ba^{2+}, Ca^{2+}, or Mg^{2+} ions (1.8 mM) or using a conditioning Ca^{2+}-pulse (1.8 mM, for 90 s) with background presence of Ba^{2+} ions (1.8 mM) were examined at homomeric rP2X$_5$ and rP2X$_1$ receptors.

As established previously, the rP2X$_5$ receptor was most sensitive to ATP when conditioned by a brief Ca^{2+}-pulse (Fig. 6A, Table 2). The use of bathing solutions containing either Mg^{2+} or Ba^{2+} alone resulted in a significant reduction ($p < 0.05$) of agonist efficacy but did not significantly alter ATP potency (Table 2). Use of a solution containing Ca^{2+} alone resulted in a reduction of efficacy and a 10-fold reduction of ATP potency (Table 2). Here, Ca^{2+}-dependent rundown was avoided by limiting each P2X$_5$-expressing oocyte cell to two ATP challenges (15 min apart) and each concentration assessed on different oocytes (n = 4). When each of the four extracellular solutions was retested at rP2X$_1$ receptors, there was no significant effect on ATP potency and only a slight decrease (16 ± 5%, n = 4) in ATP efficacy in the presence of Mg^{2+} ions (Fig. 6B, Table 2).

Proton Modulation of Homomeric P2X Receptors. The potency and efficacy of ATP was reassessed at homomeric rP2X$_5$ and rP2X$_1$ receptors at four different levels of extracellular pH (pH 8.0, 7.5, 6.5 and 5.5). At rP2X$_5$, alkaline conditions (pH 8.0) had no effect on ATP activity, whereas acidic conditions (pH 6.5 and 5.5) caused a progressive reduction of ATP efficacy (Fig. 6C, Table 2). A 3- to 4-fold reduction of ATP potency was observed at pH 5.5 (Table 2). At rP2X$_1$, alkaline conditions also had no effect on ATP activity, whereas acidic conditions caused a progressive reduction of ATP potency without affecting efficacy (Fig. 6D, Table 2).

Zinc Modulation of Homomeric P2X Receptors. The modulatory effect of extracellular Zn^{2+} ions (0.01–1000 μM) on ATP responses was examined at homomeric rP2X$_5$ and rP2X$_1$ receptors. At rP2X$_5$, Zn^{2+} ions (1–100 μM; EC$_{50}$, 42.6 ± 23.9 μM) potentiated membrane currents to ATP, by approximately 2-fold, when coapplied with the agonist (Fig. 6E). Higher concentrations (300–1000 μM) inhibited ATP responses without full blockade at the higher concentration tested. When applied 15 min before the agonist, Zn^{2+} caused the same concentration-dependent effects. The modulatory effects of Zn^{2+} were reversed after washout. At rP2X$_1$, Zn^{2+} ions (0.01–300 μM) inhibited ATP responses in a concentration-dependent and reversible manner (Fig. 6F). The inhibitory effect of Zn^{2+} was also dependent on preincubation time: IC$_{50}$, 9.3 ± 0.6 μM (0 min preincubation) and 0.8 ± 0.1 μM (20 min preincubation).

Discussion

In the present study, it was shown that homomeric rP2X$_5$ receptors could produce small-amplitude slowly desensitizing inward currents when activated by ATP. In the past, investigators have found it difficult to generate functional P2X$_5$ receptors—for example, where this subtype was studied originally by Collo et al. (1996), and in the control experiments of investigators studying heteromeric P2X$_{1/5}$ receptors (Torres et al., 1998; Haines et al., 1999; Lê et al., 1999; Surprenant et al., 2000). If there has been any prior success with homomeric P2X$_5$, the resultant agonist-evoked responses have been consistently small [<100 pA in HEK293 cells at −70mV (Collo et al., 1996; Garcia-Guzman et al., 1996), and <100 nA in *X. laevis* oocytes at −100mV (Lê et al., 1999)]. We tried to increase receptor levels and agonist responses by injecting greater amounts of rP2X$_5$ transcripts into oocytes. This procedure failed here, although it has been successful for us in the past when studying other P2X subtypes (Liu et al., 2001).

Difficulties in expressing rP2X$_5$ in oocytes were overcome by removing Ca^{2+} ions from the extracellular solution, yet Ca^{2+} was later found to be important when activating this homomeric receptor. So, extracellular Ca^{2+} had a dual role—or sensitizing and desensitizing actions—at rP2X$_5$ receptors. Extracellular Ca^{2+} ions had this effect, Ba^{2+} and Mg^{2+} did not, whereas Zn^{2+} acted somewhat like Ca^{2+} (see below). When the exposure time to extracellular Ca^{2+} was brief (5 min or less, before agonist addition), ATP routinely

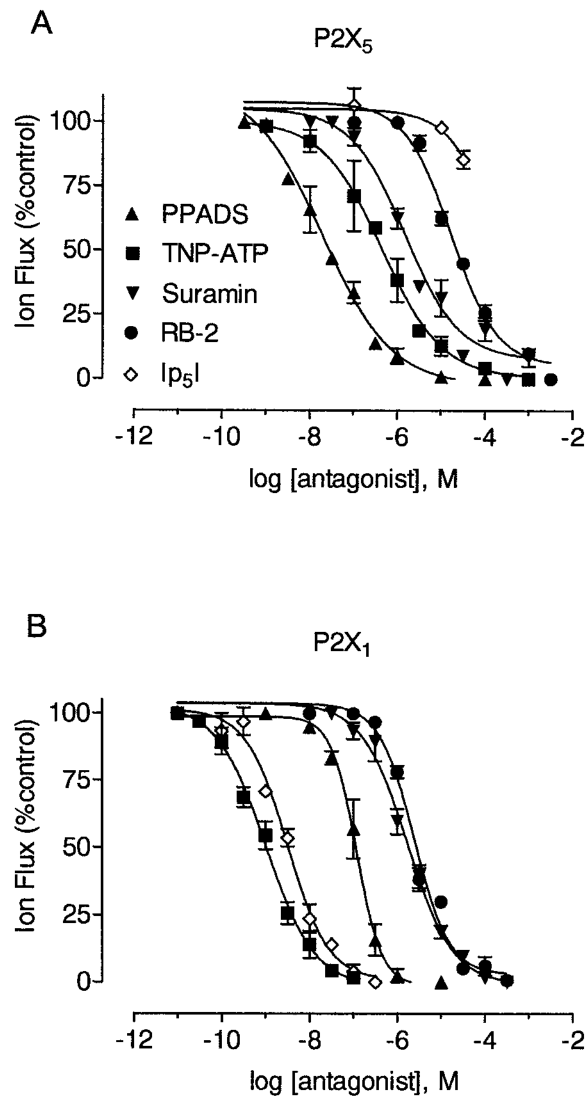

Fig. 5. Antagonism of homomeric P2X receptors. Inhibition curves for antagonists of homomeric rP2X$_5$ receptors (A) and rP2X$_1$ receptors (B). Data were normalized to the amplitude of control ATP responses (at 1 μM, approximately EC$_{70}$ at pH 7.5). IC$_{50}$ and n_H values are given in Table 1. Data are expressed as mean ± S.E.M. (n = 4).

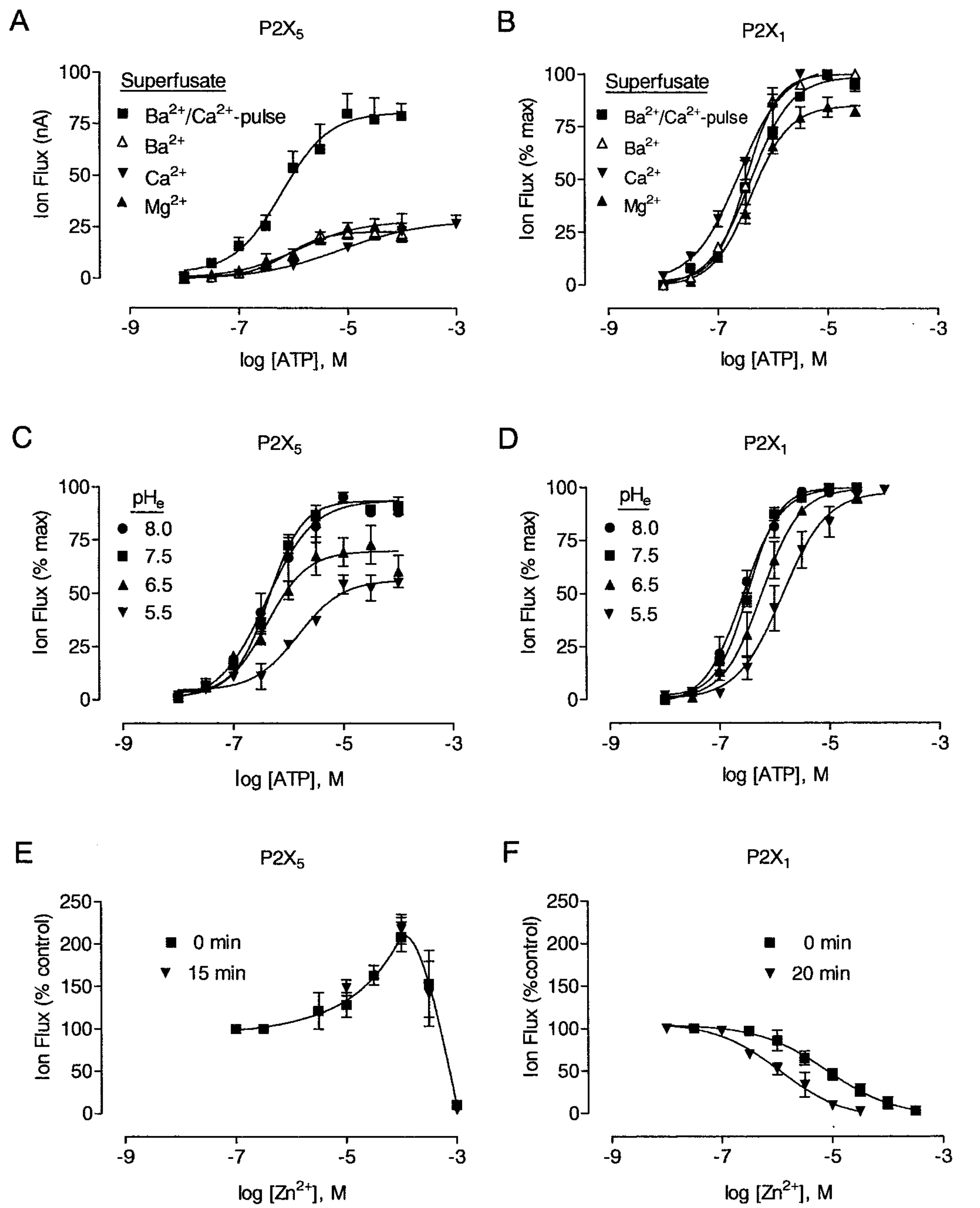

Fig. 6. Modulators of homomeric P2X receptors. A and B, C/R curves for ATP (at pH 7.5), in the presence of either extracellular Ba^{2+}, Ca^{2+}, or Mg^{2+} ions (each 1.8 mM) or in the presence of Ba^{2+} ions and with a conditioning Ca^{2+}-pulse (Ba^{2+}/Ca^{2+}-pulse, each 1.8 mM) at homomeric $rP2X_5$ and $rP2X_1$ receptors. In C and D, C/R curves for ATP at four levels of extracellular pH (pH_e) (8.0, 7.5, 6.5, 5.5) at homomeric $rP2X_5$ and $rP2X_1$ receptors. In E and F, C/R curves for modulation of agonist responses by extracellular Zn^{2+} ions (0.01–1000 μM) at homomeric $rP2X_5$ and $rP2X_1$ receptors activated by 300 nM ATP, pH 7.5. Zn^{2+} ions were added to the superfusate and applied either simultaneously with ATP (0 min preincubation), or before and during application of ATP (15 or 20 min preincubation). Data in A are amplitudes of measured currents. Data in B, C, and D are normalized to the maximum ATP response at pH 7.5. Data in E and F are normalized to the control ATP response at pH 7.5. EC_{50} and n_H values are given in Table 2. Data expressed as mean ± S.E.M. (n = 4).

TABLE 2
Modulators of homomeric P2X receptors

	ATP Agonism			
	$rP2X_5$		$rP2X_1$	
	EC_{50}	n_H	EC_{50}	n_H
	μM		μM	
Cation modulation				
Ba^{2+}/Ca^{2+}-pulse	0.63 ± 0.23	0.9 ± 0.2	0.38 ± 0.21	1.1 ± 0.3
Ca^{2+} alone	7.2 ± 0.4	0.6 ± 0.3	0.27 ± 0.11	1.1 ± 0.3
Ba^{2+} alone	0.78 ± 0.28	1.2 ± 0.5	0.10 ± 0.07	0.8 ± 0.3
Mg^{2+} alone	1.3 ± 0.3	0.8 ± 0.3	0.40 ± 0.18	1.2 ± 0.2
H^+ modulation				
pH 8.0	0.39 ± 0.09	1.0 ± 0.2	0.27 ± 0.16	1.2 ± 0.2
pH 7.5	0.45 ± 0.05	1.4 ± 0.2	0.38 ± 0.21	1.1 ± 0.3
pH 6.5	0.40 ± 0.15	1.1 ± 0.3	0.59 ± 0.07	1.2 ± 0.2
pH 5.5	1.5 ± 0.4	1.1 ± 0.3	1.4 ± 1.0	1.0 ± 0.2

evoked consistent-amplitude responses. Exposure times to Ca^{2+} greater than 5 min caused a progressive rundown of agonist-evoked inward currents. Even when storing oocytes, the long-term presence of extracellular Ca^{2+} had a deleterious effect and later diminished the mean amplitude of ATP-activated currents at the rP2X$_5$ receptor. Immunostaining revealed that prolonged exposure to extracellular Ca^{2+} affected the density of P2X$_5$-like material in the membrane of injected oocytes and also led to the formation of punctate islands of P2X$_5$-like protein. The occurrence of puncta was reminiscent of ATP-induced P2X$_2$ receptor translocation and internalization in rP2X$_2$-GFP transfected rat embryonic hippocampal neurons (Khakh et al., 2001b). Movement of P2X$_2$ receptors and formation of puncta in hippocampal neurons was prevented by the T18A mutation of rP2X$_2$ subunits, thereby deleting an N terminus binding motif for Ca^{2+}-dependent PKC (Khakh et al., 2001b). Our immunostaining results, showing lowered P2X$_5$-ir density and clumping in the presence of extracellular Ca^{2+}, suggested a similar movement of P2X$_5$ receptors in oocytes—a possibility that we are continuing to investigate.

The phenomenon of sensitization and desensitization by extracellular Ca^{2+} at rP2X$_5$ was also reminiscent of earlier findings at P2X$_3$ receptors. Short pulses of extracellular Ca^{2+} (10 mM, for 30–90 s) rapidly resensitized P2X$_3$-like receptors in sensory neurons and recombinant rP2X$_3$ receptors in HEK293 cells (Cook and McCleskey, 1997; Cook et al., 1998), although the mechanism for this phenomenon remains unexplained. On the other hand, influx of extracellular Ca^{2+} plays a role in the inactivation of rP2X$_3$ receptor (King et al., 1997). This Ca^{2+}-dependent mechanism again may be due to the intracellular PKC site (T*X*[K/R]) conserved in the N terminus of all P2X$_{1-7}$ subunits and shown to be important in the inactivation of wild-type and mutant P2X receptors (Boué-Grabot et al., 2000; Khakh et al., 2001b). Our attempts to alter intracellular Ca^{2+} levels by exposing oocytes to BAPTA-AM (30 μM for 30 min) were largely inconclusive, although our paradigm exactly followed another oocyte study in which Ca^{2+}/PKC-dependent potentiation of *N*-methyl-D-aspartate receptors was shown to be BAPTA-AM–sensitive (Skeberdis et al., 2001). More convincingly, the rate of desensitization was significantly reduced by lowering intracellular Ca^{2+} in HEK293 cells expressing chick P2X$_5$ receptors (Ruppelt et al., 2001). Thus, we believe that difficulties in recording inward currents from homomeric P2X$_5$ receptors may be due to the complex actions of Ca^{2+} ions at extracellular and intracellular loci on the P2X$_5$ subunit.

For homomeric rP2X$_1$ receptors, it was comparatively easier to evoke large-amplitude ATP responses, and the substitution of Ca^{2+} with either Ba^{2+} or Mg^{2+}, or use of Ca^{2+}-pulses in the presence of extracellular Ba^{2+}, did not alter the position of the ATP C/R curve. Thus, a crucial difference between homomeric rP2X$_5$ and rP2X$_1$ receptors is the sensitization of the former by extracellular Ca^{2+} ions. We have previously investigated the modulatory effects of extracellular H^+ and Zn^{2+} ions at the rP2X$_1$ receptor (Wildman et al., 1999) and repeated these experiments in the present study for both P2X receptor subtypes. For rP2X$_1$, reduction of extracellular pH (pH$_e$) reduced the potency but not efficacy of ATP, whereas, at the rP2X$_5$ receptor, lowering pH reduced both potency and efficacy. At the rP2X$_1$ receptor, agonist activity was inhibited in a concentration- and time-dependent manner by extracellular Zn^{2+} because of a reduction in agonist potency without a concomitant change in agonist efficacy (Wildman et al., 1999). At the rP2X$_5$ receptor, extracellular Zn^{2+} (0.1–100 μM) caused a potentiation of ATP responses and, at higher concentrations (100–1000 μM), a potent inhibition of agonist responses. The potentiating and inhibitory effects of Zn^{2+} at rP2X$_5$ superficially resembled the sensitizing and desensitizing effects of extracellular Ca^{2+}. However, the effects of the former were time-independent, whereas the latter were time-dependent.

A greater range of nucleotides than previously realized could activate homomeric rP2X$_5$ receptors. In our hands, ATP, 2MeSATP, and ATPγS were full and potent agonists. CTP and GTP also were full agonists although some 60-fold less potent than ATP. Of the remainder, α,β-meATP, β,γ-meATP, BzATP, ADP, and UDP are partial agonists and of intermediate potency. α,β-meATP activation of the rP2X$_5$ receptor was not seen in earlier studies (Collo et al., 1996). However, rather elaborate ionic conditions have allowed us to gain insight into the effects of this agonist, whereas earlier investigations may have been hampered by the Ca^{2+}-dependent desensitization of P2X$_5$ receptors. Of the dinucleotides with three or more phosphates tested at rP2X$_5$, all were partial agonists and only Ap$_4$A was as potent as ATP.

The agonist properties of rP2X$_1$ receptors were reassessed in the hope of identifying selective agonists for rP2X$_5$ and rP2X$_1$ subtypes. When comparing EC$_{50}$ values, few compounds were wholly selective for homomeric rP2X$_5$. This led us to the conclusion that these two P2X receptor subtypes might be viewed as pharmacologically similar, although, structurally, their respective subunits are only 36% identical (Khakh et al., 2001a). Closer inspection of the agonist profiles revealed that GTP will activate rP2X$_5$ but not rP2X$_1$. However, there are no selective agonists that will activate rP2X$_1$ and not rP2X$_5$. Our attention turned to antagonists to identify similarities or differences in their activities at these two P2X subtypes. Differences were most obvious for TNP-ATP and Ip$_5$I, which blocked rP2X$_1$ receptors in the nanomolar range, whereas TNP-ATP was ~500 fold less potent at rP2X$_5$ and Ip$_5$I was relatively ineffective. In contrast, the potency of PPADS, suramin, and RB-2 was not vastly different at rP2X$_5$ and rP2X$_1$ receptors. Thus, if it was difficult to distinguish P2X$_1$ from P2X$_5$ receptors by agonist activity, it was easier to do so using TNP-ATP and Ip$_5$I as antagonists.

Setting aside the pharmacology of these two P2X receptor subtypes, it is important to consider whether or not homomeric P2X$_5$ receptors can exist naturally. From the Ca^{2+}-sensitizing and desensitizing phenomena described here, it is difficult to see how homomeric P2X$_5$ receptors could be functional in the long term. P2X$_5$-ir occurs on the apical border of epithelial cells of the collecting duct in the rat kidney (Chan et al., 1998; Bailey et al., 2000; Schwiebert and Kishore, 2001); therefore, there may be circumstances in which the tubular fluid of the nephron may allow the resensitization of P2X$_5$ receptors, as shown here. We are in the process of determining the composition of tubular fluid in the collecting ducts of normal rats and in PKD models, in the hope of testing such extracellular fluids against the P2X$_5$ receptor. Others have shown that P2X$_1$ and other P2X subtypes are internalized after agonist activation and, thereafter, slowly externalized (Dutton et al., 2000; Li et al., 2000; Ennion and Evans, 2001; Khakh et al., 2001b; Bobanovic et al., 2002).

Under such circumstances and with extracellular Ca^{2+} present, it may still be possible to activate freshly externalized $P2X_5$ receptors in the short term with ATP. This might be important where $P2X_5$ expression is up-regulated in developing and proliferating tissues. However, where $P2X_5$ subunits have been found in blood vessels, it seems likely that these subunits will be involved in heteromeric $P2X_{1/5}$ receptors. This heteromeric assembly, like homomeric $P2X_1$ receptors, is not believed to be sensitive to extracellular Ca^{2+} and differs from $P2X_1$ only by supporting a steady-state current in the continued presence of ATP. A last possibility is a post-translational modification of $P2X_5$ subunits to strip away Ca-desensitization, but there is no credible evidence yet for such a modification in mammalian kidney cells. Thus, the physiological role of the homomeric $P2X_5$ receptor remains a curious enigma, probably requiring $P2X_5$-knockout to unravel, although the present pharmacological data puts us in a better position to identify such assemblies wherever they might occur.

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