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PHARMACOLOGY OF CLONED P2X RECEPTORS

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■ **Abstract** There are seven P2X receptor cDNAs currently known. Six homomeric ($P2X_1$, $P2X_2$, $P2X_3$, $P2X_4$, $P2X_5$, $P2X_7$) and three heteromeric ($P2X_2/P2X_3$, $P2X_4/P2X_6$, $P2X_1/P2X_5$) P2X receptor channels have been characterized in heterologous expression systems. Homomeric $P2X_1$ and $P2X_3$ receptors are readily distinguishable by their rapid desensitization, the agonist action of αβmethyleneATP, and the block by 2′,3′-O-(2,4,6-trinitrophenyl)-ATP. $P2X_2$ receptors are unique among homomeric forms in their potentiation by low pH. Homomeric $P2X_4$ receptors are much less sensitive to antagonism by suramin and pyridoxal 5-phosphate-6-azo-2′,4′-disulfonic acid. Homomeric $P2X_7$ receptors are the only form in which 2′,3′-O-(4-benzoylbenzoyl)-ATP is more potent than ATP. The heteromeric $P2X_2/P2X_3$ receptor resembles $P2X_2$ in slow desensitization kinetics and potentiation by low pH and is similar to $P2X_3$ with respect to agonism by αβmethyleneATP and block by 2′,3′-O-(2,4,6-trinitrophenyl)-ATP. Other agonists, antagonists, and ions that can be used to differentiate among the receptors are discussed.

INTRODUCTION

P2X receptors are membrane ion channels activated by the binding of extracellular adenosine 5′-triphosphate (ATP). This action of ATP, the direct gating of a cation selective channel, was first demonstrated some 15 years ago (1–3); since then there have been many additional reports of such actions of exogenous ATP [reviewed elsewhere (4–7)]. Extracellular ATP can also activate P2Y receptors. Because few of the available agonists or antagonists are very selective between P2X and P2Y receptors, the main criteria used to define the involvement of P2X receptors have been the time course of the response and/or the observation of unitary currents in outside-out patches. Thus, the opening of a cation-conducting pathway within a few milliseconds of applying the ATP indicates involvement of a P2X receptor. Such effects have now been described for a wide range of mammalian cells, including neurons, striated, smooth and cardiac muscles, epithelia, bone, and many different leukocytes. The properties of the unitary currents flowing through single ion channels have been described in several cases, and the

range in values suggests considerable receptor heterogeneity [PC12 cells (8), smooth muscle (9, 10), and hippocampal (11) and autonomic (12–14) neurons]. Where it is not possible to obtain such direct kinetic demonstration of the involvement of a ligand-gated ion channel, pharmacological tests become important. Much reliance has been placed on the use of available agonists and antagonists to identify actions mediated by P2X receptors.

The availability of selective antagonists becomes even more critical when addressing the functional role for endogenous ATP at P2X receptors. The initial evidence for a transmitter role for ATP was provided at the autonomic neuroeffector junction, with direct recording of the excitatory junction potential and block by the desensitizing agonist αβmethyleneATP (αβmeATP) or by the antagonist suramin (15–17); similar approaches have been used to imply that ATP mediates synaptic transmission at neuro-neuronal junctions (18, 19).

Seven P2X receptor subunit cDNAs have been cloned; Figure 1 illustrates the relatedness of the deduced amino acid sequences. Several splice variants have also been described, but these are not discussed here because many have not been functionally expressed and most have not been tested with a range of agonists and antagonists. The cDNAs have been expressed in oocytes (DNA or RNA injection), HEK293 cells (transfection or Semliki forest virus infection), or insect cells (baculovirus infection); there seem to be no obvious consistent differences among the expression systems. When expressed singly, $P2X_1$ through $P2X_4$ subunits assemble into ion channels, which provide robust currents when activated with ATP. $P2X_5$ receptors also express, but the currents are much smaller. Expression of homomeric $P2X_6$ receptors has been reported only in a small fraction of transfections (20) and is not considered further.

There are important kinetic differences among the currents evoked by ATP in cells expressing P2X receptors, and these mimic the variability also observed in native cells [reviewed elsewhere (5–7)]. When ATP is applied briefly (1–2 s) to cells expressing $P2X_3$ receptors, lower concentrations (<1 μM) elicit inward currents, which are maintained throughout the application. However, currents decline almost to zero during the application of higher concentrations. Subsequent applications within the next few minutes produce much smaller responses (sometimes called run-down). For the $P2X_1$ receptor, the time constant of desensitization itself is about 300 ms at maximal concentrations; recovery from desensitization occurs over 10–30 min. For the $P2X_3$ receptor, there is also little or no desensitization with low ATP concentrations (100–300 nM), but higher concentrations evoke currents that decline even more quickly than that observed for $P2X_1$ receptors (time constant $\approx$100 ms), and recovery from desensitization requires up to 15 min. In contrast, $P2X_2$ and $P2X_4$ receptors show little or no desensitization on this timescale (1–2 s), even with maximal concentrations. However, when the ATP application is continued for several seconds, the currents decline, and this occurs more rapidly for the $P2X_4$ receptor [reviewed elsewhere (6, 20)].

A further complication is that the permeability of the ionic channel can change during ATP applications that are continued for several seconds [$P2X_2$ and $P2X_4$

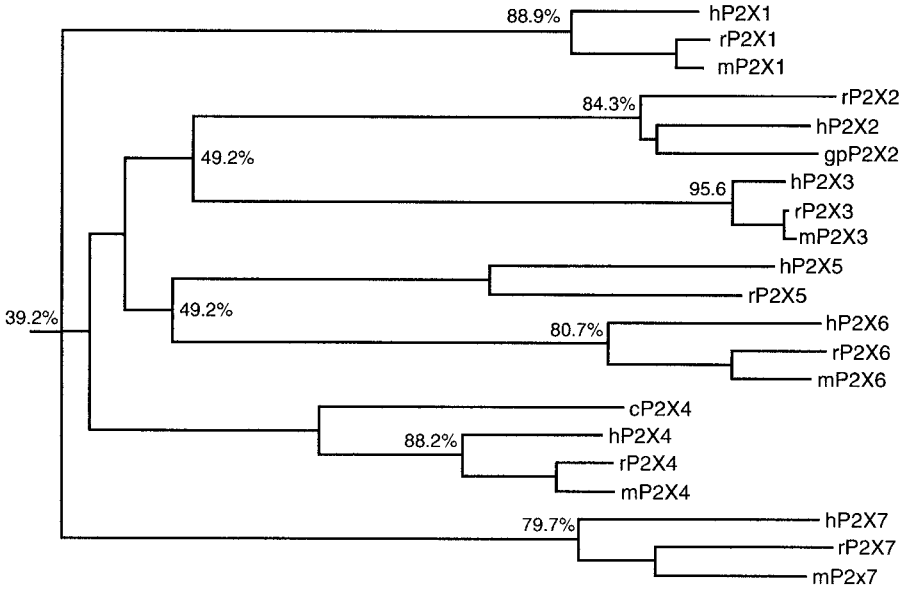

Figure 1 Relatedness of P2X receptor subunits. Amino acid sequences including both transmembrane domains and the extracellular domain were aligned by ClustalW (default parameters) and displayed by Treeview. Percentage of identical amino acids is shown between human and rat receptors for $P2X_1$, $P2X_2$, $P2X_3$, $P2X_4$, $P2X_6$, and $P2X_7$, and between $rP2X_2$ and $rP2X_3$, $rP2X_5$ and $rP2X_6$, and $rP2X_1$ and $rP2X_7$. National Center for Biotechnology Information accession numbers of these sequences are as follows: $hP2X_1$, P51575 (79); $rP2X_1$, P47824 (80); $mP2X_1$, P51576 (79); $hP2X_2$, AAD42947; $gpP2X_2$, O70397 (81); $rP2X_2$, P49653 (82); $hP2X_3$, P56373 (83); $rP2X_3$, P49654 (25, 84); $mP2X_3$ (85); $hP2X_4$, NP002551 (57); $rP2X_4$, S62359 (60); $mP2X_4$, AAC95601 (86); $cP2X_4$, AAD01645; $hP2X_5$, Q93086 (87); $rP2X_5$, CAA63052 (20); $hP2X_6$, O15547 (88); $rP2X_6$, P51579 (20); $mP2X_6$, O54803; $hP2X_7$, Q99572 (64); $rP2X_7$, Q64663 (23); $mP2X_7$, CAA08853 (89). Abbreviations: h, human; r, rat; m, mouse; gp, guinea pig; c, chicken.

receptors (21, 22); $P2X_7$ receptors (23, 24)]. The permeability increases so as to allow the passage of fluorescent cations such as quinolinium,4-[(3-methyl-2-(3H)-benzoxazolylidene)methyl]-1-[3-(triethylammonio)propyl]di-iodide (YOPRO-1) and has proven particularly useful for studies of the $P2X_7$ receptor. Except where stated, the pharmacological properties described in this chapter refer to measurements of membrane currents when agonists are applied briefly (for 1–2 s).

There is both functional and biochemical evidence for P2X receptor formation as heteromultimers; this includes $P2X_2/P2X_3$ (25, 26), $P2X_1/P2X_5$ (27, 28), and $P2X_4/P2X_6$ (22, 29). Agonists, antagonists, or modulators that are selective among these many possible subtypes of P2X receptor are needed for at least three reasons. First, they might be used to identify further physiological roles for endogenous ATP. It is particularly important in this respect to recognize that some of

the currently available antagonists block other ion channel receptors at concentrations similar to those that block P2X receptors (30). Second, it may be possible to use them to determine the subunit composition of the native multimeric receptors (31). Third, P2X antagonists have several potential therapeutic applications. In this chapter we review what is currently known of such molecules, with respect to their actions at heterologously expressed P2X receptors.

HOMOMERIC RECEPTORS

$P2X_1$ Receptors

Agonists The defining features of homomeric $P2X_1$ receptors are high sensitivity to αβmeATP [50% effective concentration (EC_{50}) ~1 μM] (Table 1) and the rapid desensitization of the current during agonist applications lasting 1–2 s. αβmeATP is about equally active at $P2X_3$ receptors, whereas βγmeATP shows about 30-fold selectivity for $P2X_1$ compared with $P2X_3$ (25, 32) (Table 1). Diadenosine polyphosphates (Ap_nA) allow further distinctions to be made among rat homomeric receptors $P2X_1$ through $P2X_4$ (33, 34). In the case of the rat $P2X_1$ receptor, activity increases with an increasing number of phosphate moieties: Ap_6A is a full agonist, whereas Ap_5A and Ap_4A are partial agonists (EC_{50} is close to that of ATP); Ap_3A has a very weak effect, and Ap_2A has no effect at 30 μM. A similar result was reported for Ap_5A in human $P2X_1$ receptors (32).

Antagonists Antagonists selective for $P2X_1$ receptors have been reported. MRS2220 (cyclic pyridoxine-α4,5-monophosphate-6-azo-phenyl-2′,5′disulfonate) blocks at a concentration of ~10 μM, whereas similar concentrations of the parent cyclic pyridoxal phosphate analog (cyclic pyridoxine-α4,5-monophosphate) potentiate responses at $P2X_1$ receptors (35). These compounds have no effect on currents evoked at $P2X_2$ or $P2X_4$ receptors (or $hP2Y_2$, $hP2Y_4$, or $rP2Y_6$) (35).

2′,3′-O-(2′,4′,6′)-trinitrophenyl-ATP (TNP-ATP) is 1000-fold more effective when blocking ATP-induced currents at $P2X_1$ receptors [50% inhibitory concentration (IC_{50}) ~1 nM] than at $P2X_2$, $P2X_4$, and $P2X_7$ (36) (Table 2). This action of TNP-ATP is shared by TNP-ADP and TNP-AMP, though not by TNP-adenosine. The nanomolar affinity at $P2X_1$ (and $P2X_3$) receptors has led to its use in characterizing receptors on native tissues (31, 37). In nodose ganglion, TNP-ATP inhibits ATP-evoked currents with a biphasic inhibition curve, implying at least two receptors on a single cell (31). TNP-ATP also antagonizes the action of αβmeATP to induce currents in dissociated mesenteric artery smooth muscle, with an IC_{50} of 2 nM; this is consistent with a $P2X_1$ receptor. On the other hand, it is much less effective to inhibit nerve-evoked contractions of the muscle, indicating either that the synaptic receptors are not $P2X_1$ receptors or perhaps that TNP-ATP is rapidly degraded in intact tissue preparations (37).

TABLE 1 Agonist sensitivities of cloned P2X receptors[a]

Receptor	ATP	ADP	αβmeATP	βγmeATP	2meSATP	BzATP	References
$P2X_1$	1	30	1-3	10	1	3	79, 80
		80%	100%	40%	100%	60%	
$P2X_2$	10	≈300	>100	>300	3	30	82
		100%	<5%	<10%	100%	60%	
$P2X_3$[b]	1	≈50	1	>300	0.3	—	25, 83, 84
		>80%	100%	—	100%	—	
$P2X_4$[c]	10	>>100	>>100	—	10–100	—	53–57, 60
		—	<10%	—	30–80%	—	
$P2X_5$[d]	10	≈300	>>100	—	10	>500	20, 83, 87
		>80%	—	—	—		
$P2X_7$[e]	100	>>300	>>300	>100	10	3	23, 64, 89
		—	—	—	80%	300%	
$P2X_2/P2X_3$[f]	1	—	1	—	—	—	25
$P2X_1/P2X_5$[f]	1	10	5	—	—	—	28, 71
$P2X_4/P2X_6$[f,g]	10	—	30	—	—	—	29, 58

[a]The upper of the two values in each cell is the concentration eliciting 50% of maximal response to that agonist (micromolar) [50% effective concentration (EC_{50})]; the lower value is the maximal response evoked by that agonist as a fraction of the maximal response evoked by ATP. There are differences among EC_{50} reported for agonists that range up to 10-fold. These differences occur between laboratories and also at various times from the same laboratory; the reasons for the differences are not known but may include seasonal and other differences in host cells, and the purity and stability of agonists, variable rates of desensitization, and differences in the divalent ion concentrations used (values reported are in presence of 1–2 mM calcium and magnesium) (see Table 3). The values presented here are approximate averages of the published value; they refer to rat receptors because those data are most complete. However, there are often species differences and some of these have been highlighted in the notes.

[b]EC_{50} for CTP of 18 μM at $hP2X_3$ (63) but >100 μM at $rP2X_3$ (84).

[c]EC_{50} for 2MeSATP at $rP2X_4$ varies [≈10 μM (60); 20 μM but only 30% maximum (55); ≈100 μM (56)].

[d]$hP2X_5$ receptors so far described are missing either exon X ($P2X_{5A}$) or both exons III and X ($P2X_{5B}$); these do not form functional channels. A human/rat chimeric receptor has been expressed that would have all the extracellular regions of $hP2X_5$ (87).

[e]Human $hP2X_7$ receptors are 10-fold less sensitive to BzATP and ATP than are rat receptors (64, 68), and mouse receptors are approximately twofold less sensitive than are human receptors (89) when ionic currents are measured.

[f]When a mixture of $P2X_2$ and $P2X_3$ subunits is expressed, the cell might be expected to make homomeric $P2X_2$ and $P2X_3$ channels in addition to hetermeric $P2X_2/P2X_3$ channels. ATP would activate all three species of channel, and EC_{50} values are therefore difficult to interpret without further information (e.g. kinetics). Because αβmeATP does not activate $P2X_2$ receptors, and because currents at $P2X_3$ receptors desensitize fully within a second. Any current measured at 2 s after applying αβmeATP is assumed to result from $P2X_2/P2X_3$ heteromers. Similar considerations apply for the other heteromers.

[g]In oocytes expressing both $P2X_4$ and $P2X_6$ subunits, αβmeATP evokes a maximum current that is 13% that evoked by ATP; for $P2X_4$ homomers this is 7% (29).

The suramin analog 8,8′-(carbonylbis(imino-3,1-phenylene carbonylimino)bis(1,3,5-naphthalenetrisulfonic acid) (NF023) also shows selectivity for $P2X_1$ receptors (38). Both $rP2X_1$ and $hP2X_1$ have an IC_{50} of ~200 nM, which is about 20-fold more sensitive than $P2X_3$ and over 50-fold more sensitive than $P2X_2$ and $P2X_4$ receptors. In summary, NF023 and TNP-ATP are useful tools for iden-

TABLE 2 Antagonist sensitivities of cloned P2X receptors

	Suramin	NF023	PPADS	TNP-ATP	References
$P2X_1$	1 μM	200 nM	1 μM	6 nM	32, 38, 79, 80
$P2X_2$	10 μM	~100 nM	1 μM	1 μM	38, 80
$P2X_3$	3 μM	1 μM	1 μM	1 nM	25, 84
$P2X_4$	>300 μM	>100 μM	>300 μM	15 μM	36, 38, 53–57, 60
$P2X_5$	4 μM	—	3 μM	—	23
$P2X_7$[c]	~500 μM	—	50 μM	>30 μM	23, 39, 68, 89
$P2X_2/P2X_3$[c]	—	1 μM	~5 μM	7 nM	25, 38
$P2X_1/P2X_5$[a]	—	—	—	~200 nM	28, 71
$P2X_4/P2X_6$[b]	—	—	—	—	29

Values are expressed as concentration causing 50% inhibition of current evoked by ATP (IC_{50}). Concentrations of ATP vary, but submaximal concentration has been chosen where possible.
[a]Values reported (28) report 200 nM for $P2X_1$ and 64 nM for $P2X_1/P2X_5$ but without any preincubation of antagonist; these values are much higher than those found by Virginio et al. for $P2X_1$ (36).
[b]There is no selective way to activate $P2X_4/P2X_6$ heteromers separately from $P2X_4$ homomers.
[c]αβmeATP used as agonist to avoid homomeric $P2X_2$ receptors, and currents measured after desensitzation of homomeric $P2X_3$ receptors.

tifying the participation of $P2X_1$ receptors, although in each case care must be taken with the concentrations used, and $P2X_3$ components should be eliminated by further tests.

Ions The effects of ions have not been systematically studied on expressed $P2X_1$ receptor subunits (Table 3). Calcium has little or no inhibitory effect up to 100 mM, which is in pronounced contrast to the $P2X_2$ receptor (39). The current evoked by ATP at homomeric $P2X_1$ receptors is about 50% inhibited by a 10-fold increase in proton concentration (pH change from 7.3 to 6.3) (40). Gadolinium and lanthanum also inhibit currents at $P2X_1$ receptors (41).

$P2X_2$ Receptors

Agonists and Antagonists There are no agonists or antagonists that selectively recognize homomeric $P2X_2$ receptors. The EC_{50} for ATP is typically about 10-fold higher than for $P2X_1$ receptors, although there is considerable variability among published values. They are not activated by αβmeATP, at least at concentrations up to 300 μM (Table 1). They are sensitive to suramin and pyridoxalphosphate-6-azophenyl-2′,4′-disulfonate (PPADS), but not TNP-ATP (Table 2).

Ions $P2X_2$ receptors have a unique phenotype with respect to ions (Table 3). Thus, they are the only P2X receptor at which the response to ATP is increased by acidification of the extracellular solution (40, 42–44). Low pH does not affect the amplitude of unitary $P2X_2$ receptor currents, but it introduces more brief

TABLE 3 Ion sensitivities of cloned P2X receptors[a]

Receptor	Calcium	Magnesium	Zinc	Copper	Hydrogen	References
$P2X_1$	No effect	—	—	—	Decrease	39, 40
	>100 mM				$pK_a \approx 6.3$	
$P2X_2$	Decrease	—	Increase	Increase	Increase	40, 45, 82
	5 mM		20 μM	16 μM	$pK_a \approx 7.3$	
$P2X_3$	Decrease	—	—	—	Decrease	40, 46
	90 mM				$pK_a \approx 6.0$	
$P2X_4$	—	—	Increase	No effect	Decrease	45, 55, 56, 62
			2 μM	to 50 μM	$pK_a \approx 7.0$	
$P2X_7$	Decrease	Decrease	Decrease	Decrease	Decrease	
	3 mM	500 μM	10 μM	0.5 μM	$pK_a \approx 6.1$	
$P2X_2/P2X_3$	Decrease	—	—	—	Increase	46
	15 mM				$pK_a \approx 7.3$	

[a]The effects shown are those of increasing the ion concentration, and the values are the concentrations that decrease by 50% (pKa in the case of hydrogen) or cause 50% of the maximal increase in response to ATP. There are no studies of $P2X_5$ or $P2X_1/P2X_5$ receptors. Studies on $P2X_4/P2X_6$ are difficult to interpret because it is not possible to separate components of current through homomeric $P2X_4$ receptors.

closings into the channel openings (44). In this way, the potentiation by protons was similar to the effect of increasing the ATP concentration, which suggests that protons increase the affinity of the channel for ATP.

ATP-induced currents are potentiated by both zinc and copper at low micromolar concentrations; this allows the receptors to be distinguished from $P2X_4$ receptors, which are less sensitive to copper (45). They can also be distinguished from $P2X_4$ receptors by their sensitivity to extracellular calcium (IC_{50} ~5 mM) (39, 46). Single channel recordings indicate that this is in part due to ''fast'' block; that is, the kinetics of the individual blocking events are too fast to be resolved, leading to an apparent decrease in the unitary current amplitude (8, 47). Extracellular calcium also profoundly affects the time course of the currents evoked by ATP. As described above, whole-cell recordings of ATP-induced current show little or no decline during the applications of ATP that continue for several seconds. But in excised outside-out patches, the current declines with a time constant of approximately 100 ms. This decline is critically dependent on extracellular calcium, and it does not occur in calcium-free external solution (48). This inactivation of the current is strongly dependent on the concentrations of ATP and calcium. For ATP (in 1 mM calcium), the EC_{50} is ~20 μM, the Hill coefficient is close to 3, and the fastest time constant of inactivation is ~100 ms. For calcium (using 50 μM ATP), the EC_{50} is 1.3 mM, the Hill coefficient is close to 4, and the fastest time constant of inactivation (at 3 mM calcium) is 28 ms. These results indicate that the maintained activation of the channel is strongly inhibited both by extracellular calcium and by a diffusible messenger that is rapidly lost in

outside-out recordings. Other divalents (magnesium, barium, manganese) are considerably less effective than calcium. A splice variant of the $P2X_2$ receptor (or a fully processed mRNA?) that misses 69 amino acids in the intracellular C-terminus region inactivates more rapidly than the wild-type receptor (49–51); the effects of calcium and other ions on this difference have not been systematically studied.

$P2X_3$ Receptors

Homomeric $P2X_3$ receptors have a similar pharmacological profile to $P2X_1$ receptors as far as the agonists ATP and $\alpha\beta$meATP are concerned; Ap_3A is somewhat more effective at $P2X_3$ than $P2X_1$ receptors (33), whereas $\beta\gamma$meATP has the opposite selectivity (Table 1). The most useful discriminating antagonist is NF023, which is approximately 40 times less active at $P2X_3$ receptors than $P2X_1$ receptors (38), but suramin, PPADS, and TNP-ATP do not readily distinguish between these receptors (Table 1). The effects of calcium ions at $P2X_3$ receptors have been studied on HEK293 cells expressing $P2X_3$ receptors (and on rat dissociated trigeminal ganglion neurones, which project to tooth pulp) (52). Increasing the calcium concentration from 1 to 10 mM had no effect on the current elicited by a single application of ATP. However, exposure to a high-calcium solution between ATP applications much accelerated the rate of recovery from desensitization. So long as the rise in calcium concentration was of sufficient duration ($>$10 s), its presence was ''remembered'' by the cell for several minutes after washout. Convincing evidence was presented that this effect resulted from a direct action of calcium on the extracellular domain of the $P2X_3$ receptor; 10 μM gadolinium mimicked the effect of 10 mM calcium.

$P2X_4$ Receptors

Agonists Several groups have reported that 2-methylthioATP (2MeSATP) is 10- to 30-fold less potent than ATP in activating $P2X_4$ receptors [$rP2X_4$ (53–56), $hP2X_4$ (57)], and the receptors have little or no sensitivity to any of the Ap_nA compounds (33) (Table 1). Agonist actions at $P2X_4$ receptors are also unusual in that they are much potentiated by ivermectin (58). Ivermectin activates the glutamate-gated chloride channel of several invertebrates, including the nematode responsible for onchocerciasis, and it also allosterically modulates mammalian $GABA_A$ and nicotinic α7 receptors. Khakh et al (58) report that ivermectin (EC_{50} 250 nM) reversibly increases currents evoked by ATP in oocytes expressing $P2X_4$ receptors. The effect is use- and voltage-independent and fully reversible on washing; it is not seen in oocytes expressing homomeric $P2X_2$, $P2X_3$, or $P2X_7$ receptors or heteromeric $P2X_2/P2X_3$ receptors. Cibacron blue (3–30 μM) also increases ATP-evoked currents in HEK293 cells expressing $P2X_4$ receptors, but not in cells expressing $P2X_2$ receptors (59).

Antagonists $P2X_4$ receptors are also unusual with respect to antagonist sensitivity (Table 2). They are much less sensitive to suramin and NF023 than to other P2X receptors (38, 56, 60). The differences in suramin sensitivity between the human and rat $P2X_4$ receptor prompted experiments to determine the regions of the molecule that might be involved (57). Currents elicited by ATP (5 μM) in oocytes expressing $hP2X_4$ receptors are about 50% inhibited by suramin (200 μM). The $rP2X_4$ receptor with a single amino acid substitution (Q78K) has a much increased sensitivity to suramin and to NF023 (≈90% inhibition by 200 μM) (56, 57). PPADS is also a very weak antagonist at the $rP2X_4$ receptor (53, 56, 58, 60); however, a point mutation that provides the receptor with a lysine (E249K) at the equivalent position to that found in the $P2X_1$, $P2X_2$, and $P2X_3$ receptors restores the ability of PPADS to produce slowly reversible inhibition (70% by 10 μM) (60). The human receptor is more sensitive to PPADS than is the rat receptor, and the domain responsible for this difference was mapped to a 22–amino acid sequence beginning at Arg^{82} in $hP2X_4$ (57).

Ions With regard to ions (Table 3), the $P2X_4$ receptor seems to be among the most sensitive to potentiation by zinc (53, 56, 57, 61, 62), but the maximal degree of potentiation seen is less than that observed for the $P2X_2$ receptor (33). $P2X_4$ receptors are not inhibited by copper, and in this respect they differ from $P2X_2$ receptors (45).

$P2X_5$ Receptors

The currents elicited by ATP in cells expressing $P2X_5$ receptors are some 100-fold lower than those observed for $P2X_1$ through $P2X_4$, even when the procedures used for expression are very similar (20, 63). However, the agonist and antagonist profiles at the $P2X_5$ receptor appear to be similar to those reported for the $P2X_2$ receptor (20, 63); the effects of ions have not been systematically tested.

$P2X_7$ Receptors

Agonists The defining agonist pharmacology of $P2X_7$ receptors is that they are remarkably insensitive to ATP, but more sensitive to the analog 2′,3′-O-(4-benzoylbenzoyl)ATP (BzATP). BzATP is not specific for $P2X_7$ receptors; other P2X receptors are activated by BzATP, but at these it is equipotent with or less potent than ATP (e.g. 32). There are serious difficulties in making comparisons of agonist actions among studies, because the effects of agonists (and perhaps antagonists) at $P2X_7$ receptors are sensitive to the extracellular concentration of divalent cations (see below). In ‘‘normal’’ divalent concentrations (2 mM calcium, 1 mM magnesium) ions, the EC_{50}s for ATP and BzATP are about 300 μM and 8 μM, respectively, at the rat receptor (23); higher concentrations are required to activate the human $P2X_7$ receptor (64). Other agonists tested are either less effective than ATP (2MeSATP, ATPγS, ADP) or ineffective at 300–1000 μM (αβmeATP, βγmeATP, UTP, adenosine). The need to use such high ATP concen-

trations to activate the receptor can pose problems (for example, a 1 mM solution of ATP is acidic and can also contain significant concentrations of other nucleotides), and this has led to the extensive use of BzATP as the agonist of choice.

Antagonists At rat receptors, currents evoked by BzATP (30 μM) are antagonized only poorly by suramin (30% inhibition by 300 μM) and PPADS (IC_{50} ~50 μM) (Table 2) (23). Preincubation with 2′3′-dialdehyde-ATP (oxoATP) (100 μM) for 1–2 h irreversibly blocks currents induced by BzATP; this concentration also blocks ATP-evoked currents at $P2X_1$ and $P2X_2$ receptors by 60%, but at those receptors the inhibition is reversible by washing (32). TNP-ATP is a weak antagonist at rat $P2X_7$ receptors ($IC_{50} > 30$ μM) (36). Calmidazolium potently inhibits BzATP-activated currents in HEK293 cells expressing $P2X_7$ receptors (IC_{50} ~10 nM) (65). This action of calmidazolium seems unrelated to its more commonly studied use as an inhibitor of calmodulin; the effective concentrations are lower and the compound, which is cationic, acts from the extracellular aspect of the cell. Remarkably, calmidazolium has little or no effect on YOPRO-1 uptake into cells expressing $P2X_7$ receptors (65). This difference might represent the binding of calmidazolium to distinct conformations of the channel (i.e. the small cation permeable vs the large cation permeable states). On the other hand, the maximal inhibition of the current by calmidazolium was never more than 95%, so it is possible that the 5% of channels that remain unblocked provide a route for sufficient YOPRO-1 to enter to make the fluorescence signal appear undiminished.

The final group of compound used as $P2X_7$ receptor blockers are the isoquinolines related to KN-62 (1-[*N*,*O*-bis(5-isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine) and KN-04 (*N*-[1-[*N*-methyl-*p*-(5-isoquinolinesulfonyl)benzyl]-2-(4-phenylpiperazine)ethyl]-5-isoquinolinesulfonamide) (66). KN-62 inhibits currents evoked by BzATP in HEK293 cells expressing human $P2X_7$ receptors (IC_{50} 50 nM), but not those expressing rat receptors (66); a similar species selectivity had been first shown for the native P2Z receptor by Gargett & Wiley (67). It also inhibits currents in cells expressing a rat receptor in which the first 335 amino acids had been replaced with the human sequence (i.e. the entire subunit up to the beginning of the second transmembrane domain), indicating that parts of the large extracellular loop were involved in the KN-62 binding site. KN-04 had a similar effect; this indicates that inhibition of calmodulin-dependent kinase type II, for which these compounds were originally introduced, did not play any role (because KN-04 is inactive toward CaM kinase II). Essentially similar results were observed when ethidium uptake was measured (66).

Ions The concentrations of extracellular ions have marked effects on responses at $P2X_7$ receptors. In the case of the human receptor, removal of magnesium (from 1 to 0 mM) causes a six- to eightfold increase in the amplitude of the currents evoked by ATP or BzATP, with only a relatively small increase in potency (EC_{50}) (23, 64); in the case of the rat receptor, the increase is about fourfold. This potentiation by removal of magnesium (and/or calcium) is a hallmark of ATP actions

at the $P2X_7$ receptor and is one of the features that suggests that the receptor corresponds to the P2Z receptor of native cells (23). Because nucleotides bind divalent cations, removal of magnesium (and/or calcium) will change the relative concentrations of the different forms of ATP, particularly increasing the amount of ATP^{4-}. However, several arguments suggest that this is not the major reason for the increased effectiveness of ATP; a direct effect on the receptor of the altered divalent ion concentration is presumably responsible (65).

Systematic studies of the effects of cations at the rat $P2X_7$ receptor show that the concentrations causing half-maximal inhibition of the current evoked by BzATP (30 μM) are calcium 3000 μM, magnesium 500 μM, zinc 11 μM, hydrogen 1 μM (pH 6), and copper 0.5 μM (65). Broadly similar results have been reported for YOPRO-1 uptake (65). These experiments were carried out in normal extracellular sodium and in 2 mM calcium and 1 mM magnesium (except when those ions were being studied). Reducing the extracellular sodium concentration [N-methyl-D-glucamine (NMDG) substitution] increases the rate at which YOPRO-1 enters the cells after adding BzATP, indicating the external sodium is itself inhibitory to $P2X_7$ function (see below).

Because reduction of the divalent ion concentration has such a marked potentiating effect on agonist-induced currents at $P2X_7$ receptors, in some studies this has been used as a baseline condition. For example, the human $P2X_7$ receptor was studied in a magnesium-free, 0.5 mM calcium solution. It is more sensitive to inhibition by PPADS, with 50% inhibition by 1 μM (with 8 min preincubation); inhibition by suramin, KN-62, and calmidazolium are broadly as described above (68). In this low-divalent concentration, current measurements show that BzATP is approximately 30-fold more potent when chloride is replaced by glutamate, indicating a clear effect of the extracellular anion (69). YOPRO-1 uptake measurements showed that BzATP was 10-fold more potent in extracellular choline chloride, as compared with sodium chloride (potassium chloride was intermediate). Such an inhibitory effect of extracellular sodium ions on dye uptake has previously been shown for P2Z receptors in human lymphocytes (70, 71).

HETEROMERIC RECEPTORS

The subunit composition of native heteromeric receptors is not known. However, using epitope-tagged constructs, physical association can be shown between some pairs of P2X subunits in heterologous expression systems (26–29, 72, 73). $P2X_7$ subunits do not coimmunoprecipitate with any others, $P2X_5$ subunits coimmunoprecipitate with all others (except $P2X_7$), and the others have intermediate selectivities (72). Of those pairs that are now known to coimmunoprecipitate, some have also been studied functionally after coexpression.

$P2X_2/P2X_3$ Receptors

When $P2X_2$ and $P2X_3$ subunits are coexpressed, one must assume that $P2X_2$ homomers and $P2X_3$ homomers are formed in addition to one or more heteromeric channel species. Currents at homomeric $P2X_2$ receptors are not activated by αβmeATP; currents at homomeric $P2X_3$ recover from desensitization so slowly that they can be eliminated by repeated applications at relatively short intervals (≈2 min). These currents are readily antagonized by suramin and PPADS (25) (Table 2). They are also very sensitive to NF023 (38) and to TNP-ATP (36), implying that for both these antagonists, the presence of the $P2X_3$ subunit in the heteromer is sufficient to endow high sensitivity. On the other hand, the effect of pH changes is similar to that seen for homomeric $P2X_2$ receptors; acidification increases the currents at $P2X_2/P2X_3$ heteromers (39). In short, the $P2X_2/P2X_3$ heteromer (*a*) is activated by αβmeATP, (*b*) is blocked by TNP-ATP, (*c*) is potentiated by low pH, and (*d*) shows little or no desensitization. The first two properties are contributed by the $P2X_3$ subunit and the latter two by the $P2X_2$ subunit.

$P2X_1/P2X_5$ Receptors

There are two kinds of functional evidence for heteromeric channels formed from $P2X_1$ and $P2X_5$ subunits (27, 28), and these are analogous to the situation for $P2X_2/P2X_3$ heteromers described above (25). First, αβmeATP induces a sustained current, whereas with homomeric $P2X_1$ receptors the current desensitizes rapidly (<1 s) and with homomeric $P2X_5$ receptors αβmeATP has no effect. Second, currents evoked by αβmeATP at homomeric $P2X_1$ receptors exhibit marked ''run-down'' when the applications are repeated at intervals of less than several minutes; in the case of the heteromer, there is no such run-down even with applications every 10 s. The sensitivity of the heteromeric receptor to suramin, PPADS, and NF023 has not been reported; the antagonist TNP-ATP has an inhibitory effect similar to that observed at the $P2X_1$ receptor in the same study (28). Sensitivity to ions and protons has not been described.

$P2X_4/P2X_6$ Receptors

$P2X_4$ and $P2X_6$ subunits are extensively coexpressed throughout the central nervous system (20), and there is evidence for their heteropolymerization in *Xenopus* oocytes (29). Oocytes expressing the heteromeric channels gave larger currents (after 5 days) than those expressing homomeric $P2X_4$ receptors ($P2X_6$ alone gave no currents). The coinjected oocytes were also slightly more sensitive to 2MeSATP and αβmeATP than were oocytes injected only with the $P2X_4$ subunit cDNA. In the case of αβmeATP, a maximal concentration (300 μM) elicited a current that was about 13% of the current evoked by ATP (100 μM) in the $P2X_4/P2X_6$ oocytes, whereas this value was only about 6% in oocytes expressing $P2X_4$ alone (29, 58). Khakh et al (58) reported that the threshold concentration for αβmeATP was significantly lower (10 μM) in coinjected oocytes than in oocytes

expressing only $P2X_4$ receptors (300 μM), and this threshold was even lower (3 μM) in the presence of ivermectin. The coinjected ($P2X_4/P2X_6$) oocytes were more sensitive to inhibition by suramin and reactive blue than were singly injected oocytes ($P2X_4$) (29); there was no difference in the effects of zinc (10 μM; 80% potentiation) or protons (pH 6.5; 50% inhibition). One must assume in these experiments that the coinjected oocytes express a mixture of $P2X_4$ homomers and $P2X_4/P2X_6$ heteromers; the agonists used would be activating both sets of channels, and this makes experiments on antagonist sensitivity particularly difficult to interpret.

CONCLUDING REMARKS

There are several classes of ligand-gated ion channels. The first is the nicotinic superfamily—this includes both cation- and anion-selective channels, and channels activated by acetylcholine, 5-hydroxytryptamine, γ-aminobutyric acid, and glutamic acid. The molecular cloning of this family began (74) well after we had a thorough understanding of their agonist and antagonist pharmacology; indeed, it was also after the successful therapeutic exploitation of these receptors by drugs exemplified by tubocurare, hexamethonium, benzodiazepines, and ivermectin. The second family is the glutamate receptor family; the discovery of selective receptor agonists and antagonists (75) again predated the isolation of cDNAs and their heterologous expression (76–78); the tools were available with which to characterize the clones. In both these areas, much more highly subtype selective agonists and antagonists continue to be developed by using heterologously expressed receptors. P2X receptors form a third class of ligand-gated channels; it is to be hoped that the expression of cloned receptors will lead to the development of high-affinity and selective compounds, which are urgently required to probe their physiological role and to test for therapeutic potential.

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