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Effects of P2-Purinoceptor Antagonists on Degradation of Adenine Nucleotides by Ecto-Nucleotidases in Folliculated Oocytes of *Xenopus laevis*

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ABSTRACT. The aim of the present study was to examine the effects of a number of P2-purinoceptor antagonists on degradation of adenine nucleotides by *Xenopus laevis* oocyte ecto-nucleotidases. Folliculated oocytes readily metabolize all three naturally-occurring nucleotides, the order of preferential substrates being ATP > ADP > AMP. The degradation of ATP and ADP was decreased significantly in the presence of several P2X- and P2Y-purinoceptor antagonists, including suramin, PPADS, Cibacron blue, Coomassie Brilliant blue, Evans blue, Trypan blue, Congo red, and PIT (each compound was used at 100 μM). All these compounds inhibited the degradation of ATP by up to 60%, whereas the hydrolysis of ADP was inhibited by Congo red and PIT by 75–80%. In addition, DIDS (100 μM) and TNP-ATP (100 μM) selectively inhibited the breakdown of ATP, and sodium azide (10 mM) selectively inhibited the breakdown of ADP. The enzymatic breakdown of either ATP or ADP was unaffected by 8-*p*SPT (100 μM), an antagonist of P1-purinoceptors, or by oxidized ATP (100 μM), an antagonist of P2Z-purinoceptors. The degradation of AMP was prevented completely by PIT (100 μM) and inhibited significantly by Congo red (100 μM). In conclusion, the present study shows that most of currently available antagonists of P2-purinoceptors inhibit the enzymatic breakdown of extracellular ATP and ADP. The inhibitory effect on ecto-nucleotidase activity should be taken into account when these antagonists are used in pharmacological experiments. BIOCHEM PHARMACOL 51;7:897–901, 1996.

KEY WORDS. *Xenopus* oocytes; ecto-nucleotidase; P2-purinoceptor antagonists

Xenopus laevis oocytes are used widely in expression cloning studies of foreign membrane receptors and ion channels [1, 2], including several major subtypes of P2-purinoceptors at which ATP† is a principal endogenous ligand [3]. In addition, *Xenopus* oocytes possess native receptors for ATP (unidentified subtype) on their enveloping follicle cell layer [4]. Recently, we have characterized the properties of the oocyte ecto-ATPase [5, 6] an enzyme that serves to metabolize extracellular ATP and, therefore, limits the potency of agonists at either native or foreign P2-purinoceptors [7]. We showed that this ecto-enzyme is located mainly on the follicle cell layer enveloping oocytes, has a broad substrate specificity, and is highly dependent on extracellular Ca^{2+} and Mg^{2+} cations. We found that *Xenopus* oocytes represent a robust, effective, and simple system for analyzing the effect of compounds on ecto-nucleotidase activity. We have shown that oocyte ecto-ATPase is inhibited by the P2-purinoceptor antagonists, suramin, PIT, and TNP-ATP [6]. The inhibitory action of some P2-purinoceptor antagonists, namely suramin, DIDS, and Cibacron blue (Reactive blue 2) on degradation of extracellular ATP, but not of other adenine nucleotides, has been shown earlier on smooth muscle, endothelial, and hepatoma cells [8–10]. In the present study, we tested the effects of a number of structurally unrelated purinoceptor antagonists on the rate of enzymatic degradation of ATP, ADP, and AMP by *Xenopus* oocytes. The following compounds were studied: i) a series of P2-purinoceptor antagonists, including: suramin [11], PPADS [12], pyridoxal 5-phosphate [13], DIDS [14], Cibacron blue [15], Trypan blue [16], Evans blue [17], Coomassie Brilliant blue [18], Congo red [19], PIT [20], TNP-ATP [21], and oxidized ATP [22]; ii) a nonselective P1-purinoceptor antagonist, 8-*p*SPT; iii) an alkaline phosphatase inhibitor, β-glycerophosphate [23]; iv) an ATP-ADP diphosphohydrolase inhibitor, sodium azide [24]. The

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† *Abbreviations:* ATP, adenosine 5′-triphosphate; ADP, adenosine 5′-diphosphate; AMP, adenosine 5′-monophosphate; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulphonate; PIT, 2,2′-pyridylisatogen tosylate; PPADS, pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid; 8-*p*SPT, 8-*p*-sulphophenyltheophylline; TNP-ATP, trinitrophenyl-ATP.

effects of P2-purinoceptor antagonists have been tested at a concentration of 100 μM, at which the compounds cause clear antagonism at P2-purinoceptors [11–22]. The results from this survey of enzyme inhibitors reveal that the rate of breakdown of either ATP or ADP or AMP can be antagonized selectively by certain P2-purinoceptor antagonists and, thus, point the way towards synthesizing specific modulators of ecto-nucleotidases that influence the activity and potency of adenine nucleotides at purinoceptors. Part of this study has been communicated to the First European Congress of Pharmacology [25].

MATERIALS AND METHODS

Ovarian lobes were removed surgically from *Xenopus laevis* frogs, anaesthetized with tricaine (0.1% v/w), and follicular oocytes (stages V and VI) were separated mechanically from the inner ovarian epithelial layer and stored at 4°C for up to 7 days in a modified Barth's solution.

ATPase assay

ATPase assays were carried out at 20 ± 1°C in a modified Ringer's buffer solution containing (mM): 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (HEPES) 5, NaCl 110, KCl 2.5, $CaCl_2$ 1.8, $MgCl_2$ 1.8, and adjusted to pH 7.4. Oocytes were placed in 24-well dishes, three oocytes per well, in 300 μL buffer solution and prewashed for 15–20 min with continuous shaking. Prewash buffer was changed for 250 μL buffer containing ATP, ADP, or AMP (at 100 μM) and a given antagonist (100 μM) and cells were incubated in this buffer solution for 30 min. The reaction was halted by removing a sample (200 μL) of buffer and freezing it in liquid nitrogen. After freezing, samples were kept at −20°C until they were assayed for adenine nucleotides by high-performance liquid chromatography (HPLC).

HPLC Procedure

The chromatographic system comprised a Beckman 114M solvent delivery module coupled to a SA6500 UV/VIS absorbance detector (Savern Analytical). Runs with this system were performed with a Spherisorb ODS2 (25 cm length, 0.46 cm inner diameter) column from Hichrom. The column was equilibrated overnight with a mobile phase containing 0.2 M KH_2PO_4 and 3% (v/v) methanol, pH 6.0, flow rate 0.2 ml · min^{-1}. The separation of adenine nucleotides was carried out by reverse-phase chromatography at a flow rate of 1.5 ml · min^{-1}, setting the spectrophotometer at a wavelength of 260 nm. Under these conditions, the nucleotides eluted in the order: ATP, ADP, and AMP. The concentration of adenine nucleotides was calculated by comparing the height of peaks on chromatograph runs with the peaks of known standards.

Drugs

ATP, ADP, AMP, DIDS, Cibacron blue 3GA, Coomassie Brilliant blue, Evans blue, Trypan blue, Congo Red, oxidized ATP, and β-glycerophosphate were obtained from Sigma Chemical Co., U.K.; sodium azide was obtained by Fisons plc, U.K.; 8-*p*SPT was obtained from RBI (Semat), U.K.; TNP-ATP was obtained from Cambridge Bioscience, U.K.; suramin (Germanin) was generously donated from Bayer plc, U.K.; PIT was a gift from Dr. M. Spedding (Servier, France); PPADS was a gift from Professor G. Lambrecht (University of Frankfurt, Germany). PIT was dissolved in 0.1 N HCl for a stock solution of 30 mM. Other compounds were dissolved in distilled water to give stock solutions of 10 mM.

RESULTS AND DISCUSSION

Xenopus oocytes readily metabolized extracellular ATP, ADP, and AMP. Where ATP (100 μM) was used as the substrate for oocyte ecto-nucleotidase, the velocity of degradation was 116.9 ± 3.3 pmoles · min^{-1} · $oocyte^{-1}$ (n = 14). The velocity of enzymatic breakdown of ADP (100 μM) and AMP (100 μM) was 85.3 ± 7.8 pmoles · min^{-1} · $oocyte^{-1}$ and 67.5 ± 5.1 pmoles · min^{-1} · $oocyte^{-1}$ ($n = 14$), respectively. Thus, ATP showed the highest rate of enzymatic degradation among the naturally-occurring adenine nucleotides, confirming findings of an earlier study of oocyte ecto-ATPase [6], where the rate of dephosphorylation of nucleotides was assessed indirectly by the production of inorganic phosphate. The breakdown of all three adenine nucleotides was unaffected by β-glycerophosphate (10 mM), an inhibitor of alkaline phosphatase, indicating that their degradation involved ecto-enzymes other than a nonspecific pH-dependent phosphatase.

The inhibitory effects of some P2-purinoceptor antagonists on ecto-ATPase activity have been demonstrated in several tissues and cell lines [8–10, 26, 27], including *Xenopus* oocytes [6]. However, their ability to inhibit degradation of extracellular ATP has not been compared with their effects on the breakdown of other adenine nucleotides. In the present study, we found that all currently available P2X- and P2Y-purinoceptor antagonists (which include Congo red, Evans blue, suramin, PPADS, Cibacron blue, PIT, TNP-ATP, Trypan blue, Coomassie Brilliant blue, and DIDS) inhibited significantly and to approximately a similar extent the breakdown of ATP by oocytes (see Table 1). All these compounds are known to antagonise P2X- and/or P2Y-purinoceptor subtypes but, interestingly, oxidized ATP (an antagonist of P2Z-purinoceptors) and 8-*p*SPT (an antagonist of P1-purinoceptors) did not inhibit the breakdown of ATP, suggesting that only certain classes of purinoceptor antagonists can inhibit ecto-ATPase activity. The possibility has been raised that some purinoceptor subtypes, particularly the P2-purinoceptors on bovine aorta endothelial cells [10] and rat liver plasma membrane [28], may be ecto-nucleotidases themselves. Thus,

TABLE 1. Effects of purinoceptor antagonists and enzyme inhibitors on degradation of adenine nucleotides by ecto-enzymes of *Xenopus* oocytes. Data expressed as mean ± SEM of 4 to 8 experiments, except for controls where $n = 14$.

Compounds	Velocity of nucleotide degradation pmoles · min^{-1} · $oocyte^{-1}$* (% of control velocity)		
	ATP	ADP	AMP
Control	116.9 ± 3.3 (100)	85.3 ± 7.8 (100)	67.5 ± 5.1 (100)
P2-Purinoceptor antagonists (all at 100 μM)			
CR	50.3 ± 8.6 (43)‡	16.7 ± 5.1 (20)‡	41.4 ± 9.7 (61)†
EB	56.7 ± 11.4 (48)†	40.0 ± 4.4 (47)‡	70.3 ± 6.1 (104)
Suramin	56.7 ± 6.4 (49)‡	44.2 ± 9.2 (52)†	63.6 ± 10.8 (94)
PPADS	62.2 ± 5.8 (53)‡	50.8 ± 3.1 (60)†	83.1 ± 5.6 (123)
CB	60.8 ± 4.7 (52)‡	43.6 ± 3.9 (51)‡	106.7 ± 5.8 (158)†
PIT	66.9 ± 5.3 (57)‡	21.1 ± 6.1 (25)‡	0.0 ± 0.0 (0)‡
TNP-ATP	71.9 ± 8.3 (62)†	69.4 ± 12.2 (81)	63.6 ± 3.9 (94)
TB	72.7 ± 5.1 (62)‡	38.9 ± 4.2 (46)‡	69.4 ± 12.2 (103)
CBB	86.1 ± 9.7 (74)†	53.6 ± 4.7 (63)†	78.1 ± 11.4 (116)
DIDS	88.1 ± 5.0 (75)†	75.3 ± 5.0 (88)	80.3 ± 5.3 (119)
P5P	101.1 ± 6.1 (86)	90.6 ± 5.3 (106)	80.3 ± 14.7 (119)
o-ATP	116.4 ± 5.0 (100)	90.0 ± 7.8 (106)	67.5 ± 7.8 (100)
P1-Purinoceptor antagonist			
8-*p*SPT (100 μM)	113.1 ± 10.8 (97)	86.7 ± 7.5 (105)	74.2 ± 11.9 (110)
Enzyme inhibitors			
β-GP (10 mM)	105.8 ± 6.9 (91)	103.1 ± 11.7 (121)	63.9 ± 8.1 (95)
NaN_3 (10 mM)	104.2 ± 8.3 (89)	62.2 ± 3.9 (73)†	63.9 ± 7.5 (95)

Abbreviations: CR, Congo red; EB, Evans blue; PPADS, pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid; CB, Cibacron blue; PIT, 2,2'-pyridylisatogen tosylate; TNP-ATP, trinitrophenyl ATP; TB, Trypan blue; CBB, Coomassie Brilliant blue; DIDS, 4,4'-diisothiocyanatostilbene-2,2'-disulphonate; P5P, pyridoxal-5-phosphate; o-ATP, oxidized ATP; 8-*p*SPT, 8-*p*-sulphophenyltheophylline; β-GP, β-glycerophosphate.

* Each nucleotide was incubated separately with oocytes for 30 min (initial concentration 100 μM) and the remaining concentration of the nucleotide in buffer was measured by HPLC. †$P < 0.05$, ‡$P < 0.01$ against corresponding controls.

such purinoceptors would cleave nucleotides during receptor activation and, hence, purinoceptor occupancy by antagonists would appear to diminish enzyme activity. Because the subtype of native receptors for ATP on follicular *Xenopus* oocytes is not yet clearly identified [4], this hypothesis remains to be explored fully for these cells and was not resolved in the experiments reported here. However, such future experiments underscore the value of oocytes when they can be used to express specific P2-purinoceptor subtypes in isolation.

In some cases, the agonist potency of ADP and ATP on ionotropic (P2X family) and metabotropic (P2Y family) purinoceptors [3] is similar [29, 30] and, correspondingly, it is important from a functional perspective that ecto-enzymes degrade ADP as well as ATP. In the present study, we found that the degradation of ADP was also inhibited by most of the P2-purinoceptor antagonists tested, but some of the compounds proved to be exceptions. DIDS and TNP-ATP failed to inhibit the degradation of ADP (but not of ATP) and, on the other hand, sodium azide failed to inhibit the degradation of ATP (but not of ADP) (see Table 1). Although most antagonists inhibited the degradation of ATP and ADP by approximately the same extent, Congo red and PIT inhibited the hydrolysis of ADP much more effectively than that of ATP. Thus, the differential action of DIDS, TNP-ATP, Congo red, and PIT on the enzymatic degradation of ATP and ADP suggests that these two nucleotides are handled differently by an ecto-nucleotidase (e.g. different binding sites and/or occupancy number) or are handled separately by two different ecto-nucleotidases.

Sodium azide is used widely as an inhibitor of ATP-ADP diphosphohydrolase, which metabolizes ATP and ADP by cleaving two phosphates from the phosphate chain of these nucleotides [24], and it has been shown that this enzyme, but not ecto-ATPase, hydrolyzes extracellular ATP in some tissues [31]. However, the differential action of sodium azide on ADP and ATP breakdown was not readily explicable in terms of an inhibition of an oocyte ATP-ADP diphosphohydrolase. Instead, the results with sodium azide reinforce the view that ATP and ADP are handled differently by oocyte ecto-nucleotidase(s).

The enzymatic breakdown of AMP was abolished by PIT and inhibited significantly by Congo red, and Cibacron blue appeared to potentiate the rate of dephosphorylation (see Table 1). The actions of PIT and Congo red provide some evidence for a separate enzyme system handling AMP, distinct from the enzyme(s) handling the dephosphorylation of ATP and ADP. It is most likely that this enzyme is ecto-5'-nucleotidase, which converts AMP to adenosine, and the presence of which has been shown earlier on *Xenopus* oocytes [32]. The possibility that PIT (or structurally related analogues) could prove to be a selective inhibitor of ecto-5'-nucleotidase may lead to pharmacological tools that could discriminate between the direct actions of ATP/ADP and the indirect effects of ATP/ADP when degraded to adenosine, for example, in complex neural circuits.

Thus, *Xenopus* oocytes represent a robust and convenient system to study the effects of potential enzyme inhibitors on ecto-nucleotidase activity. A role for inhibitors of ecto-ATPases is now gaining recognition and importance be-

cause it remains true that P2-purinoceptor subtypes are characterized mainly by carefully-defined potency orders for adenine nucleotides and synthetic analogues of ATP. The viewpoint has been expressed that ecto-nucleotidases skew agonist potency at P2-purinoceptors in favor of more stable ATP analogues [30, 33] and, accordingly, the ever-increasing array of new pharmacologically defined purinoceptor subtypes might reflect the activity of ecto-ATPase at certain tissues more so than the affinity of nucleotides at their purinoceptors. So far, the only selective ecto-ATPase inhibitor described is FPL 67156 [34], which may become a useful pharmacological tool when it becomes widely available. Although the P2-purinoceptor antagonists tested here did not show much differential effect, especially between ATP and ADP breakdown, it will be most important to design selective inhibitors that are devoid of activity (agonistic or antagonistic) at the purinoceptors. Development of such compounds could provide a new generation of powerful pharmacological tools to investigate the activity and potency of adenine nucleotides in the most complex neural systems where purinergic transmission, neuromodulation, and trophism are critically important.

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