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Functional Expression of Adenosine A_{2b} Receptor in *Xenopus* Oocytes

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SUMMARY

RNA was transcribed *in vitro* from a cDNA clone (RFL9) that encodes the rat adenosine A_{2b} receptor. *Xenopus* oocytes that had been injected with this RNA several days earlier responded to adenosine (10 μM to 1 mM) with an inward current (45–750 nA) that peaked rapidly and then declined to a lower level; uninjected oocytes showed no effect of adenosine. The current reversed to outward at −25 mV and was blocked by intracellular injection of 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid. The action of adenosine (100 μM) was mimicked by 5′-*N*-ethylcarboxamidoadenosine (10 μM), but not by ATP, N^6-cyclohexyladenosine (10 or 100 μM), N^6-cyclopentyladenosine (10 μM), 1-deaza-2-chlorocyclopentyladenosine (50 μM), or CGS21680 (1 or 10 μM). It was substantially blocked by 8-cyclopentyl-1,3-dipropylxanthine (1 μM) and by 3,7-dimethyl-1-propargylxanthine (10 μM). The results indicate that activation of adenosine A_{2b} receptors increases a calcium-dependent chloride conductance in *Xenopus* oocytes, presumably by stimulating phospholipase C.

Adenosine and adenine nucleotides act at a variety of cell surface receptors to produce a range of physiological responses in different tissues (for reviews, see Refs. 1–3). Activation of A_1 receptors inhibits adenylyl cyclase, increases potassium conductance, and reduces calcium conductance, whereas the activation of A_2 receptors stimulates adenylyl cyclase (4, 5). A_2 receptors have been further divided into A_{2a} and A_{2b} subtypes (6), and molecular clones have been isolated that encode both of these subtypes (A_{2a}, Ref. 7; A_{2b}, Refs. 8 and 9). The A_{2a} and A_{2b} receptors have quite different tissue distributions, but heterologous expression shows that both readily couple to stimulation of adenylyl cyclase (7–9).

The ubiquitous coupling of A_2 receptors to stimulation of adenylyl cyclase has recently been questioned, based on the finding that adenosine and its analogs activate phospholipase C in rat basophilic leukemia cells (RBL-2H3) (10). The order of potency of agonists suggested that this resulted from activation of A_2 receptors. This action of adenosine was sensitive to both pertussis and cholera toxins, but there was no evidence for involvement of cAMP. However, in cells containing well defined A_{2b} receptors, there are no reports of adenosine mobilizing intracellular calcium.

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The *Xenopus* oocyte expression system is particularly suitable for the expression of receptors that couple to phospholipase C; the inositol trisphosphate that is formed liberates intracellular calcium and this can be detected by the inward current flowing through endogenous calcium-activated chloride channels (11). This robust response has been widely used for the functional identification of receptors (e.g., tachykinins and 5-HT_2). In the present study we have tested, by expression in *Xenopus* oocytes, the hypothesis that the A_{2b} receptor couples to phospholipase C.

Materials and Methods

The cloning of RFL9 (A_{2b} cDNA from rat brain) has been reported (8, 9). Capped RNA was synthesized *in vitro* as described previously (12). The harvesting, injection, and incubation of oocytes (stage V-VI) from *Xenopus laevis* were as described previously (12). Oocytes were injected 7–17 days before electrical recordings were made. Two electrodes (filled with 3 M KCl, resistance of ≤1 MΩ) were used to measure membrane currents, with a voltage-clamp amplifier. Unless otherwise mentioned, oocytes were held at −60 mV. The oocytes were continually bathed in a solution of composition (in mM) 96 NaCl, 2 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, and 5 Na-HEPES (pH 7.5), to which adenosine and other compounds could be added.

The responses to adenosine declined during applications lasting for 2 min (which was used in most experiments), and second applications usually evoked smaller responses when applied less than 15 min after

ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; CGS21680, 2-[*p*-(carboxyethyl)phenylethylamino]-5′-*N*-ethylcarboxamidoadenosine; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; DMPX, 3,7-dimethyl-1-propargylxanthine; NECA, 5′-*N*-ethylcarboxamidoadenosine; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

the first. However, even when the interval was held at 15 min, the amplitude of the response to adenosine often decreased progressively during the first several applications. The data were, therefore, normalized as follows. The response to a 'test' concentration of adenosine (10 μM to 3 mM) is expressed relative to that to 'control' concentrations applied to the same oocyte, where the control response was the average current evoked by 100 μM adenosine applied both before and after the test concentration.

Drugs used were purchased from Research Biochemicals, Inc., except for BAPTA and ATP (magnesium salt), which were purchased from Sigma. BAPTA was injected into oocytes with a third microelectrode (50 nl of a 50 mM solution; 2.5 nmol). Drugs were diluted into the perfusing saline from aqueous stock solutions, except for DPCPX. DPCPX was prepared in ethanol stock solution, which gave a final ethanol concentration in the saline of 0.1%; this concentration of ethanol had no effect on the response to adenosine. Numerical values are given as mean ± standard error.

Results

Adenosine activates a calcium-dependent chloride current. Adenosine (30 μM to 3 mM) evoked an inward current in 86% (n = 43) of oocytes that had been injected with RFL9 RNA (Figs. 1 and 2). The current developed rapidly, reached its peak amplitude within a few seconds, and then declined to lower amplitude (Fig. 1). In a minority of cells there was a delay of several seconds before the onset of the response, which usually was oscillatory (e.g., Fig. 2A). Adenosine never elicited a response in uninjected oocytes from the same donor frogs (n = 21).

The maximum response was observed with an adenosine concentration of 300 μM; the response to 1 mM was 90 ± 2% (n = 4) of the amplitude of the response to 300 μM. From the normalized dose-response curve (Fig. 1B; see Materials and Methods), the concentration producing a half-maximal response was approximately 50 μM.

The inward current evoked by adenosine became larger at hyperpolarized potentials and smaller at depolarized potentials; it reversed polarity at −25 ± 2 mV (n = 7), which is close to the equilibrium potential for chloride (13, 14). No response to adenosine was observed in oocytes that had been injected with BAPTA (2.5 nmol; n = 3) (see Materials and Methods) (Fig. 2A), even though the same oocytes provided a typical response before BAPTA injection.

Pharmacological properties indicate the A_{2b} receptor. NECA, which has been suggested to be selective for A_2 rather than A_1 adenosine receptors (6), was approximately 10 times more effective than adenosine. The inward current caused by 10 μM NECA was 95 ± 4% (n = 3) of that evoked by 100 μM adenosine in the same oocytes. No effects were observed with the A_1-selective agonists N^6-cyclohexyladenosine (10 or 100 μM; n = 4), N^6-cyclopentyladenosine (10 μM; n = 5), or 1-deaza-2-chlorocyclopentyladenosine (50 μM; n = 3), with the A_{2a}-selective agonist CGS21680 (1 or 10 μM; n = 4), or with ATP (100 μM; n = 5).

DPCPX is a more selective antagonist of the A_1 adenosine receptor but also blocks A_2 receptors in the micromolar range (15, 16). At 1 μM DPCPX reversibly reduced the amplitude of the adenosine response by 95 ± 4% (n = 6; Fig. 2B), whereas at 100 nM the amplitude was reduced by only 27 ± 3% (n = 5). DMPX is more selective for the A_2 versus the A_1 adenosine receptors (17); at 10 μM, DMPX reversibly reduced the amplitude of the adenosine response by 75 ± 5% (n = 4).

Discussion

These results show that the adenosine A_{2b} receptor readily couples to phospholipase C activation in *Xenopus* oocytes. The properties of the inward currents evoked by adenosine (desensitization, reversal at −25 mV, and block by intracellular BAPTA) are characteristic of those evoked by agonists at receptors that stimulate phospholipase C, such as M_1 muscarinic (18), 5-HT_2 (19), and tachykinin (20) receptors. The relative ease with which the inward current can be elicited allowed us to determine some pharmacological properties of the adenosine-activated current. These were consistent with the previously reported pharmacological profile for the A_{2b} receptor (6, 9). Thus, A_1-selective adenosine receptor agonists never elicited any response, whereas NECA was effective and about 10 times more potent than adenosine. CGS21680, which has been widely used as an A_2 receptor agonist, did not elicit any response at a concentration up to 10 μM. This is also consistent with the A_{2b} receptor, because A_{2a} receptors have a much higher affinity for CGS21680 than do A_{2b} receptors (9, 21, 22). Both DMPX (10 μM) and DPCPX (1 μM) caused >50% inhibition of the response to adenosine. Quantitative estimates of the

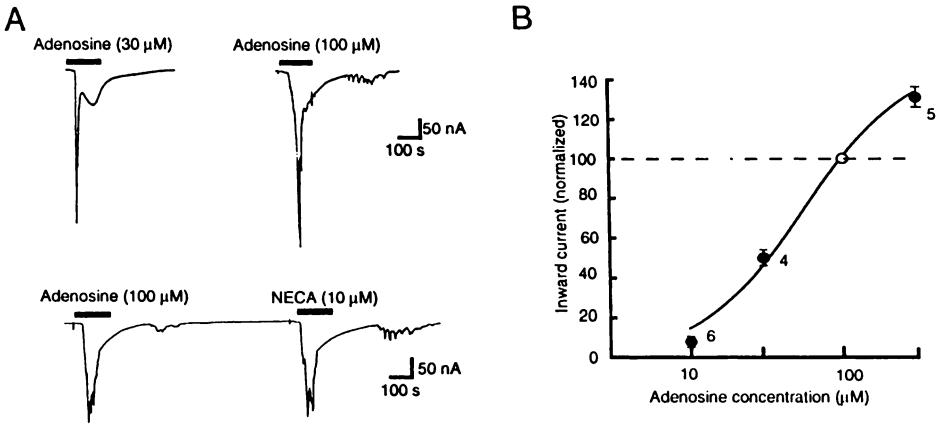

Fig. 1. Inward currents evoked by adenosine and NECA. A, *Upper*, examples of the effects of adenosine (30 and 100 μM) in the same oocyte; *lower*, effect of adenosine (100 μM) compared with effect of NECA (10 μM) in the same oocyte. B, Currents produced by different concentrations of adenosine, normalized with respect to the current caused by 100 μM adenosine in the same oocyte (see Materials and Methods). In this group of oocytes, the current evoked by 100 μM adenosine was 334 ± 78 nA (n = 11).

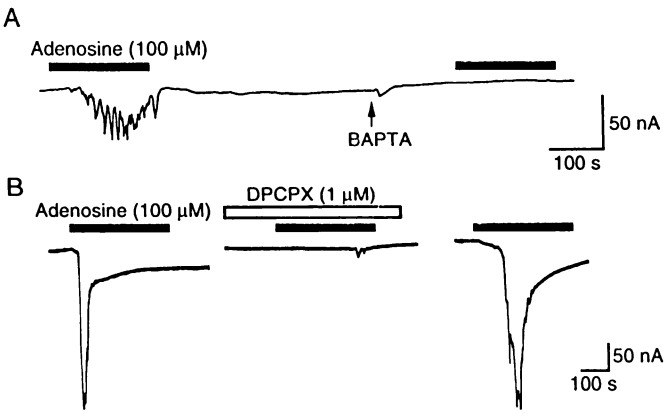

Fig. 2. A, Response to adenosine is blocked by intracellular injection of BAPTA. B, Response to adenosine is reversibly inhibited by the antagonist DPCPX (1 μM).

dissociation constants have not been made and are difficult because of the variability of the adenosine action over time. However, the effective concentrations are consistent with those required at the A_2 receptor. DMPX is only weakly A_2 selective (17); DPCPX is more strongly A_1 selective, but the concentrations required in the present experiments are >100 times greater than its dissociation constant at the A_1 receptor (15, 23).

Others have reported that oocytes that have not been injected with any receptor RNA can show responses to adenosine (24, 25); however, in defolliculated oocytes such as we have used, the inward chloride current is caused by ATP and not by adenosine (24, 25). We did not observe any responses to ATP in the present experiments, and adenosine also had no effect on oocytes that had not been injected with A_{2b} receptor RNA.

Previous work (8, 9) has shown that the A_{2b} receptor couples to adenylyl cyclase when transfected into COS-6M and Chinese hamster ovary cells and in untransfected VA 13 cells (a fibroblast line from human lung transformed by simian virus 40). This raises the possibility that the chloride current in the oocytes also results from elevation in cAMP levels. However, this is unlikely because 8-bromo-cAMP (100 μM) does not cause any inward current in *Xenopus* oocytes,[1] and neither does intracellular injection of cAMP or the catalytic subunit of protein kinase A (26, 27). The simplest interpretation is that the A_{2b} receptor couples to phospholipase C when expressed in *Xenopus* oocytes. Stimulation of phosphatidylinositol hydrolysis has previously been reported to follow activation of adenosine A_1 receptors (28–31; see Ref. 32) and A_2 receptors (10, 29). Although the receptor in RBL-2H3 cells (10) is somewhat atypical (33), there are several similarities between the effects seen in those cells and in *Xenopus* oocytes; the response to NECA desensitized rapidly, NECA was maximally effective at 10 μM, NECA was more potent than adenosine, and ATP was ineffective. Probes based on the A_{2b} receptor nucleotide sequence could be used to determine under what conditions the RNA is present in RBL-2H3 cells.

The expression of the A_{2b} adenosine receptor in frog oocytes should be convenient for studies of structure-function relations. For example, the response undergoes desensitization (see above). The carboxyl terminus of this receptor contains several serine and threonine residues that are potential phosphorylation sites (8, 9). Moreover, the observation that the expressed receptor couples so readily to both adenylyl cyclase and phospholipase C stimulation may provide a useful system for the identification of those parts of the molecule, particularly the membrane-proximal regions of the third cytoplasmic loop, that confer this coupling specificity.

[1] J. Yakel, Unpublished observations.

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