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Adenine Nucleotide-Induced Activation of Adenosine A_{2B} Receptors Expressed in *Xenopus laevis* Oocytes: Involvement of a Rapid and Localized Adenosine Formation by Ectonucleotidases

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ABSTRACT

We recently demonstrated that extracellular ATP effectively activates adenosine (Ade) A_{2B} receptors indirectly through a localized rapid conversion to Ade by ectonucleotidases on the membrane surface of C6Bu-1 rat glioma cells. These responses were observed even in the presence of adenosine deaminase (ADA). Here, we demonstrate that such responses indeed occur in A_{2B} receptor-expressing *Xenopus laevis* oocytes, which possess endogenous ectonucleotidase activity. In oocytes coexpressing the A_{2B} receptor and cystic fibrosis transmembrane conductance regulator (CFTR), Ade induced a concentration-dependent increase in a cyclic AMP-activated CFTR current, a response that was inhibited by the P1 antagonist xanthine-amine congener (XAC). A brief application of ATP and β,γ-methylene ATP (β,γ-MeATP) also induced the CFTR current in a manner similar to that seen with Ade. Among several nucleotide agonists, ADP, AMP, and adenosine-5′-*O*-(3-thio)triphosphate induced the CFTR current. Although adenine nucleotide-induced CFTR currents were inhibited by XAC, they were highly resistant to ADA treatment; 5 U/ml ADA was required for inhibition of adenine nucleotide-induced CFTR current, whereas 1 U/ml ADA was sufficient to abolish the Ade-induced response. In addition, the ecto-5′-nucleotidase inhibitor α,β-methylene ADP markedly inhibited the β,γ-MeATP-induced response but not the Ade-induced one. These results support our hypothesis that adenine nucleotides are rapidly and locally converted into Ade on the membrane surface, resulting in the activation of A_{2B} receptors.

Extracellular adenosine (Ade) and adenine nucleotides induce various cellular responses through the activation of specific receptors termed P1 and P2 receptors (Abbracchio and Burnstock, 1998). P1 receptors preferentially interact with Ade, and four different G protein-coupled receptors (A_1, A_{2A}, A_{2B}, and A_3 subtypes) have been identified (Fredholm et al., 1997; Ralevic and Burnstock, 1998). However, P2 receptors are activated by adenine and/or uridine nucleotides and are classified into the ionotropic P2X ($P2X_{1-7}$) and the G protein-coupled P2Y ($P2Y_{1, 2, 4, 6, 11,}$ and $_{12}$) receptors (Harden et al., 1995; Ralevic and Burnstock, 1998; Hollopeter et al., 2001).

Despite molecular cloning of the ATP receptor subtypes, the pharmacological characteristics observed in tissues, especially in the central (Anwar et al., 1999; Bennett and Boarder, 2000; Mendoza-Fernandez et al., 2000) and peripheral nervous systems (Shinozuka et al., 1988, 1990; Forsyth et al., 1991; Barajas et al., 1995), are diverse and differ considerably from those described for individual recombinant receptor subtypes. For example, the modulating effects of adenine nucleotides and their derivatives on the evoked release of neurotransmitters exhibit atypical agonist selectivity and are inhibited by α,β-methylene ATP (α,β-MeATP) and P1 antagonist methylxanthines (Shinozuka et al., 1988; Forsyth et al., 1991). In those studies, the possible involvement of metabolically generated Ade was excluded, because the responses were not inhibited by adenosine deaminase (ADA) (Barajas et al., 1995; Anwar et al., 1999; Bennett and

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ABBREVIATIONS: Ade, adenosine; ADA, adenosine deaminase; α,β-MeATP, α,β-methylene ATP; MBS, modified Barth's solution; XAC, xanthine-amine congener (8-[4-[[[[(2-aminoethyl)amino]carbonyl] methyl]oxy]phenyl]-1,3-dipropylxanthine); PPADS, pyridoxalphosphate-6-azophenyl-2′, 4′-disulfonic acid; β,γ-MeATP, β,γ-methylene ATP; HPLC, high-performance liquid chromatography; α,β-MeADP, α,β-methylene ADP; ATPγS, adenosine-5′-*O*-(3-thio)triphosphate; 2MeS-ATP, 2-methylthio ATP; CFTR, cystic fibrosis transmembrane conductance regulator; Epi, epinephrine; PCR, polymerase chain reaction.

Boarder, 2000; Mendoza-Fernandez et al., 2000) or were not augmented by Ade uptake inhibitors (Shinozuka et al., 1988; Forsyth et al., 1991). In addition, ATP analogs, which are metabolically more stable than ATP, were as potent as ATP (Shinozuka et al., 1988; Forsyth et al., 1991; Bennett and Boarder, 2000). Similar pharmacological characteristics were demonstrated with respect to ATP-induced effects in the peripheral tissues (Hourani et al., 1991; Tada et al., 1992; Cote et al., 1993; King et al., 1996). These results suggest the existence of novel purinoceptors, termed the P3 receptor (Shinozuka et al., 1988) or ATP-sensitive P1 receptors (Hourani et al., 1991; King et al., 1996).

In contrast, Sebastião et al. (1999) suggested that presynaptic inhibition of neurotransmitter release by adenine nucleotides is mediated by Ade, which is produced by the ectonucleotidase cascade. In agreement with this idea, Dunwiddie et al. (1997) and Cunha et al. (1998) demonstrated that in the rat hippocampus, extracellular ATP and adenine nucleotides are rapidly and locally hydrolyzed to Ade by ectonucleotidases, thereby inducing a response through the activation of P_1 Ade receptors. The responses described for the rat hippocampus showed agonist selectivity that was very similar to those reported for peripheral tissues, but they exhibited a significant decline in the presence of ADA and a marked enhancement in the presence of Ade uptake inhibitors (Cunha et al., 1998; Sebastião et al., 1999), which is somewhat inconsistent with the existence of a novel purinoceptor.

We recently demonstrated that a role for metabolically generated Ade cannot be excluded, even in responses which are highly resistant to ADA and are insensitive to Ade uptake inhibitors. Specifically, in C6Bu-1 rat glioma cells, the stable ATP analog β,γ-methylene ATP (β,γ-MeATP) increased cAMP formation, and this response was inhibited by both P1 and P2 receptor antagonists (Ohkubo et al., 2001). Although this response was resistant to ADA, an analysis of extracellular nucleotide metabolism suggested the involvement of local Ade formation and subsequent activation of Ade A_{2B} receptors. Similar results were obtained with the neuroblastoma x glioma hybrids NG108–15 cells in which ATP also induces a P1 antagonist-sensitive cyclic AMP accumulation, which is insensitive to ADA and Ade uptake inhibitors (Matsuoka et al., 1995; Ohkubo et al., 2000a,c,d). However, in those cell lines, Ade receptors are coexpressed with several ATP receptor subtypes, making it difficult to distinguish clearly between the responses mediated by Ade receptors and those mediated by P2 receptors (Matsuoka et al., 1995; Kaiho et al., 1996, 1998). To address this problem, we examined the pharmacological profile of A_{2B} receptors expressed in *Xenopus laevis* oocytes, which possess ectonucleotidase activity (Ziganshin et al., 1995).

Materials and Methods

Preparation of cRNA. cRNAs for the human cystic fibrosis transmembrane conductance regulator (CFTR) and the β_2 adrenoceptor were prepared as described previously (Uezono et al., 1993, 1997). cDNA encoding the rat Ade A2B receptor was obtained by reverse transcriptase-polymerase chain reaction amplification of the rat brain cDNA (Ohkubo et al., 2001). PCR primers were designed to amplify the complete coding sequence of the rat Ade A_{2B} receptor (GenBank accession no. M91466). The nucleotide sequences and the locations in the cDNA are 5′-CACCTTAGCGGCTGTCCTGA-3′ (sense, 39–58) and 5′-GGGCCACATGCTTGAGAGGGTA-3′ (antisense, 1211–1190). PCR was carried out in 50 μl of a solution containing 25 U/ml Klen *Taq* DNA polymerase (CLONTECH, Palo Alto, CA). The conditions were 94°C for 1 min, followed by 30 cycles of 30 s at 94°C, 30 s at 58°C, and 2 min at 72°C, with final extension at 72°C for 5 min. A single PCR product having the expected length of 1173 base pairs was directly subcloned into pGEM-T vector (Promega, Madison, WI). The full-length rat Ade A_{2B} receptor cDNA was then isolated after digesting with *Not*I and *Spe*I and ligated into pcDNA 3.1+ (Invitrogen, Carlsbad, CA), which had been digested with *Not*I and *Xba*I. The resultant DNA construct was sequenced using an automated DNA sequencer (ABI Prism; Applied Biosystems, Tokyo, Japan). The Ade A_{2B} receptor cDNA was linearized with *Stu*I; cRNA was then transcribed using a T7 RNA polymerase kit (mMESSAGE mMACHINE T7 Kit; Ambion Inc., Austin, TX). After DNase treatment, cRNAs were purified by phenol/chloroform extraction, and precipitation was produced with ethanol. The cRNAs were then dissolved in RNase-free water.

Preparation and Injection of Oocytes. *X. laevis* ovaries were obtained from COPACETIC (Aomori, Japan). Ovaries were dissected into small pieces containing approximately 20 to 50 cells. They were washed with modified Barth's solution (MBS), consisting of 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.91 mM $CaCl_2$, 0.33 mM $CaNO_3$, 0.82 mM $MgSO_4$, 2.5 mM sodium pyruvate, 10 mM HEPES, pH 7.5, 100 μg/ml streptomycin, and 100 U/ml penicillin. They were then washed twice in Ca^{2+}- and Mg^{2+}-free MBS and incubated for 60 min with 1.5 mg/ml dispase II (Roche Molecular Biochemicals, Tokyo, Japan) changing the solution every 20 min. Dissociated oocytes were washed 4 to 5 times with Ca^{2+}- and Mg^{2+}-free MBS and then twice with normal MBS. Defolliculated healthy oocytes in stages V and VI were collected under a microscope and maintained at 18°C overnight. Healthy oocytes were selected again and injected with cRNAs (1 ng CFTR, 0.5 ng β_2 adrenoceptor, and/or 1 ng rat A_{2B} receptor) or sterile water (as a control) in a final volume of 50 nl using an automatic oocyte injector (Nanoject; Drummond Scientific Co., Broomall, PA). Oocytes were incubated at 18°C in MBS. The medium was changed every day, and the oocytes were used 3 to 10 days after injection.

Recordings. Electrophysiological recordings were performed at room temperature with the two-electrode voltage-clamp method using a TEV-200 Voltage Clamp System (Dagan, Minneapolis, MN). An oocyte was placed in a 100-μl chamber containing ND96 solution composed of 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM HEPES, pH 7.6. Two microelectrodes with tip resistances of 0.2 to 1.0 MΩ filled with 3 M KCl were inserted. Membrane potential was then held at −60 mV. Oocytes were continuously superfused at a flow rate of 4 ml/min with ND96 solution. All test compounds were added to the ND96 superfusion solution. The duration of agonist application was 10 s. When the effects of inhibitors were examined, oocytes were perfused with the solution containing inhibitor for 1 min and then stimulated by agonists for 10 s in the presence of inhibitors. After stimulation by agonists, the inhibitor solution was applied for another 1 min. When the effects of ADA were examined, oocytes were continuously perfused with the solution containing ADA, and agonists were applied with a separate line for 10 s. The current-voltage relation was obtained by ramp pulses using a function generator (NF-121B; NF Corp, Yokohama, Japan). Currents were continuously recorded and analyzed using MacLab (ADInstruments Pty Ltd., Castle Hill, Australia).

Measurement of Nucleotide Hydrolysis. Oocytes in a 48-well plate (5 cells/well) were washed twice with ND96 solution and preincubated for 2 min in the presence or absence of inhibitors [α,β-MeADP, xanthine-amine congener (XAC), or pyridoxalphosphate-6-azophenyl-2′, 4′-disulphonic acid (PPADS)] in 200 μl of ND96 solution. Oocytes were then mixed with an equal volume of solution containing ATP or β,γ-MeATP and incubated at room temperature (approximately 22°C) for 10 min. In preliminary experiments, nucleotide hydrolysis increased linearly for at least 30 min. The incuba-

tion was terminated by the addition of EDTA to a final concentration of 10 mM. The supernatants were collected and stored at −20°C. Adenine nucleotides and their metabolites were measured by reverse-phase HPLC on an analytical C18 column (100 × 4.6 mm; YMC Co. Ltd., Kyoto, Japan) using 50 mM NaH_2PO_4 (pH 5.5) as the solvent at a flow rate of 1 ml/min (Matsuoka et al., 1995). Absorbance at 258 nm was monitored online with a UV detector (Nihon Bunko, Tokyo, Japan).

For the measurement of [^{3}H]Ade formation from [^{3}H]AMP on the membrane surface, oocytes in a 0.5-ml microcentrifuge tube (5 cells/tube) were washed twice and were incubated with ND96 solution containing ADA (1 U/ml) for 2 min. Cells were then pretreated with ATP, β,γ-MeATP, AMP, or α,β-MeADP for 30 s, followed by the addition of [^{3}H]AMP (5 μCi/tube, to a final concentration of 2.5 μM) and incubation for 1 min. To investigate whether [^{3}H]Ade existed on the cell surface, the reaction was stopped by aspirating the medium, followed immediately by adding 100 μl of 2.5% perchloric acid. The acid-extract (90 μl) was mixed with 9 μl of 4.2 M KOH to neutralize and deposit potassium perchlorate and was stored at −20°C until analysis by the use of HPLC. The sample was mixed with an equal volume of 100 μM Ade as a standard marker and [^{3}H]Ade in 150 μl of mixture was separated by HPLC. Ade was monitored by UV detection, and the fraction containing [^{3}H]Ade was collected and measured by a liquid-scintillation counter.

Statistics. All experiments were repeated at least three times, and similar results were obtained. Statistical analyses of the data were performed by the paired Student's *t* test for two data comparison and one-way analysis of variance with the Dunnett two-tailed test for multiple data comparison. *P* values of less than 0.05 were considered to be statistically significant.

Drugs. ADA, ATP, ADP, AMP, adenosine-5′-*O*-(3-thio)triphosphate (ATPγS), α,β-MeADP, and UTP were obtained from Sigma Chemical Co. (St. Louis, MO). β,γ-MeATP was purchased from Nacalai Tesque Inc. (Kyoto, Japan). 2-Methylthio ATP (2MeS-ATP), XAC, and PPADS were obtained from Sigma/RBI (Natick, MA). [2,8-^{3}H]AMP (20 Ci/mmol, 1 mCi/ml) was obtained from Moravek Biochemicals (Brea, CA). All other chemicals were reagent grade or the highest quality available.

Results

Functional Expression of A_{2B} Receptors in *X. laevis* Oocytes. We first examined the effects of Ade and ATP on the electrophysiological response of noninjected oocytes. Ade and ATP (both at 10 μM) had no effect on membrane current observed with a holding potential at −60 mV (Fig. 1A). In oocytes coexpressing β_2 adrenoceptor and CFTR, epinephrine (Epi, 1 μM) induced a slowly developing Cl^- current, a response elicited by β_2 receptor-mediated increase in cyclic AMP, which activates CFTR by cyclic AMP-dependent phosphorylation (Bear et al., 1991). Exposure of these oocytes to Ade or ATP did not induce any current (Fig. 1 B), indicating that oocytes do not possess endogenous adenylyl cyclase-linked purinoceptors. In oocytes injected with the A_{2B} receptor cRNA alone, Ade and ATP failed to induce any membrane current (data not shown). When A_{2B} receptor cRNA was coinjected with β_2 adrenoceptor and CFTR cRNAs, the brief application of Ade (10 μM) for 10 s induced a membrane current (Fig. 1C). The Ade receptor antagonist XAC (1 μM) inhibited the currents induced by 10 μM Ade without affecting the Epi-induced current (Fig. 1C). In contrast, the β receptor antagonist propranolol (10 μM) inhibited the Epi-induced current, but it had no effect on the current induced by 10 μM Ade (Fig. 1C). These results suggest that the A_{2B} receptor is functionally expressed in cRNA-injected *X. laevis* oocytes.

Effects of Nucleotide Agonists. In oocytes coexpressing the β_2 and A_{2B} receptors together with CFTR, exposure to ATP (10 μM) for 10 s induced a current (Fig. 2 A). This response to ATP was similar to those to Epi (1 μM) and Ade (10 μM) with respect to the time course of development and magnitude of the current (Fig. 2). β,γ-MeATP, an ATP analog that is metabolically more stable than ATP, also induced a current (Fig. 2). We also examined the effects of several nucleotide agonists, such as ADP, AMP, UTP, α,β-MeATP, ATPγS, and 2MeS-ATP at a concentration of 10 μM each. Among them, ADP, AMP, and ATPγS induced the current, whereas UTP, α,β-MeATP, and 2MeS-ATP essentially had no effect (Fig. 2). The current induced by nucleotide agonists exhibited characteristics of the Cl^- current through CFTR, as determined from the reversal potential and its shift by changing the external concentrations of Cl^- (data not shown; Uezono et al., 1993). In additional experiments, we used β,γ-MeATP as the nucleotide agonist.

Comparison of the Effects of Ade, AMP, ATP and β,γ-MeATP. Fig. 3 A shows a representative chart recording of the effects of different concentrations of Ade and β,γ-MeATP on the membrane current in an oocyte coexpressing the CFTR and A_{2B} receptors. Brief applications (10 s) of increasing concentrations of Ade or β,γ-MeATP induced the CFTR current in a concentration-dependent manner. A similar concentration-dependent increase in the CFTR current was observed with AMP and ATP. Average values of current amplitude in five to six different oocytes from four different batches are shown in Fig. 3B. EC_{50} values for Ade, β,γ-MeATP, ATP, and AMP were 4.3 ± 1.4 (n = 8), 7.8 ± 1.6 (n = 8), 7.4 ± 1.8 (n = 5), and 6.2 ± 1.2 μM (n = 5), respectively.

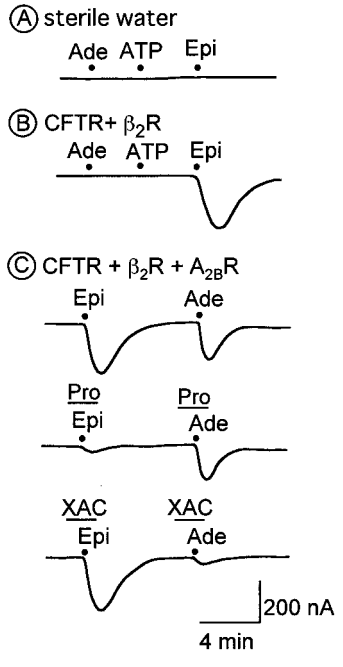

Fig. 1. Membrane current in oocytes expressing the A_{2B} receptor and CFTR. Oocytes injected with sterile water (A), with β_2 adrenoceptor (β_2R) cRNA and CFTR cRNA (B), and with β_2R, A_{2B} receptor (A_{2B}R), and CFTR cRNAs (C) were voltage clamped at −60 mV. Epinephrine (Epi, 1 μM), ATP (10 μM), or Ade (10 μM) were superfused for 10 s as indicated (●). C, middle and bottom, the oocyte was pretreated with propranolol (Pro, 10 μM) and XAC (1 μM), respectively, for 1 min before agonist application. The antagonists were present during and after agonist application for 1 more min. Similar traces were obtained with three to eight oocytes.

Effects of ADA and Purinoceptor Antagonists. When oocytes were perfused with ADA (1 U/ml) containing medium, Ade (20 μM) applied by a separate line for 10 s failed to induce the CFTR current (Fig. 4A). Under the same condition, there was still an effect of β,γ-MeATP, although it was smaller than in the absence of ADA. When the ADA concentration in the perfusing medium was increased up to 5 U/ml, β,γ-MeATP-induced current was inhibited (Fig. 4A). This inhibition by 5 U/ml ADA was not nonspecific effects, because such treatment did not affect the CFTR current induced by

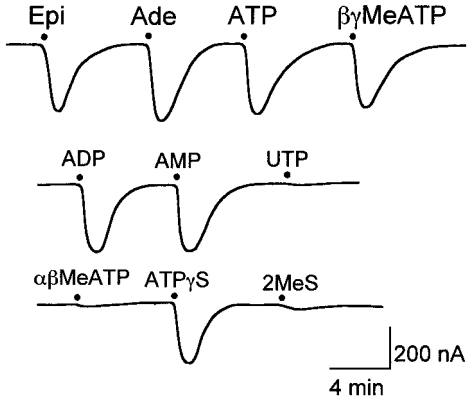

Fig. 2. Effects of nucleotide agonists on membrane current in oocytes expressing β_2 adrenoceptor, A_{2B} receptor, and CFTR. Oocytes injected with β_2 adrenoceptor, A_{2B} receptor, and CFTR cRNAs were voltage clamped at −60 mV and exposed for 10 s to epinephrine (Epi, 1 μM), Ade (10 μM), and several nucleotide agonists at the concentration of 10 μM each. Similar traces were obtained with four oocytes. 2 MeS, 2MeS-ATP.

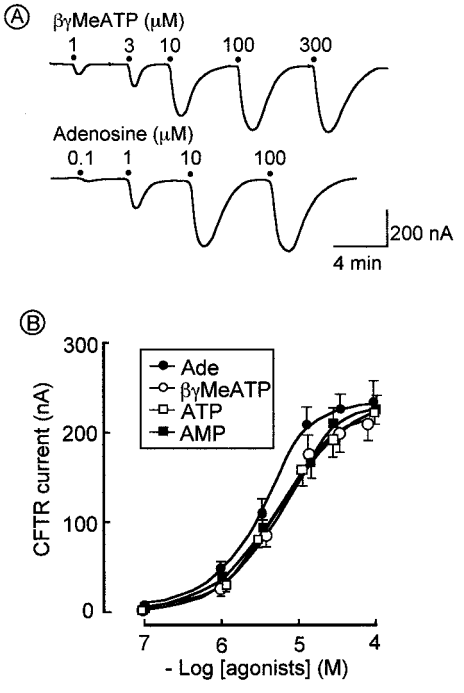

Fig. 3. Comparison of concentration-dependent effects of Ade, β,γ-MeATP, ATP, and AMP on membrane current in oocytes expressing the A_{2B} receptor and CFTR. A, oocytes injected with A_{2B} receptor and CFTR cRNAs were voltage clamped at −60 mV and stimulated by different concentrations of adenosine (top) or β,γ-MeATP (bottom) for 10 s. Similar traces were obtained with 4 oocytes. B, concentration-response curves of CFTR current induced by Ade, β,γ-MeATP, ATP, and AMP. The peak current amplitudes are shown as the mean ± S.E.M. from four to six different experiments.

N-ethylcarboxamide adenosine, an ADA-resistant Ade analog (data not shown). Similar results showing a resistance to ADA (inhibited by 5 U/ml but not by 1 U/ml) were obtained with ATP and AMP (data not shown). We examined next the effects of purinoceptor antagonists on Ade- and β,γ-MeATP-induced currents. The Ade-induced current was inhibited by XAC, as shown in Fig. 1C. The P2 receptor antagonist PPADS (1 mM) had no effect on the response to 10 μM Ade (Fig. 4B). In contrast, the current induced by β,γ-MeATP (10 μM) was inhibited by both 1 mM PPADS and 1 μM XAC (Fig. 4). We previously showed that these antagonists at the same concentrations effectively inhibited a P1 antagonist-sensitive ATP response in C6Bu-1 rat glioma cells, in which XAC inhibited the response directly by blocking agonist-receptor interaction, whereas PPADS inhibited the response indirectly by preventing extracellular adenine nucleotide metabolism (Ohkubo et al., 2001).

Role of Metabolically Generated Ade in Nucleotide-Induced A_{2B} Receptor Activation. To examine whether β,γ-MeATP activates A_{2B} receptors directly or indirectly through metabolically generated Ade, we determined the effects of α,β-MeADP, a potent inhibitor of ecto-5′-nucleotidase (Bruns, 1980), on the β,γ-MeATP-induced current. α,β-

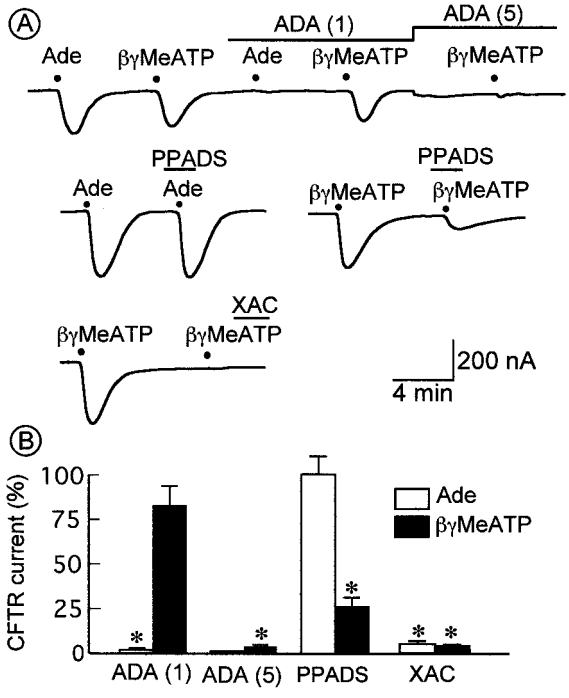

Fig. 4. Effects of ADA and purinoceptor antagonists on CFTR current induced by Ade or β,γ-MeATP. A, oocytes injected with A_{2B} receptor and CFTR cRNAs were voltage clamped at −60 mV and stimulated by β,γ-MeATP or Ade for 10 s as indicated (●). In the experiments with ADA (top), agonists and ADA were delivered by separate lines. The effects of Ade (20 μM) and β,γ-MeATP (20 μM) were tested in the absence or presence of ADA, which was first applied at 1 U/ml and then at 5 U/ml, as shown. PPADS (1 mM, middle) and XAC (1 μM, bottom) were applied 1 min before agonist stimulation. Antagonists were present for another 1 min, as indicated by the bars, during which time agonists were applied for 10 s at the concentration of 10 μM each. Representative current traces from six oocytes are shown. B, the peak current amplitudes induced by Ade (□) or β,γ-MeATP (■) in the presence of ADA (1 U and 5 U/ml), PPADS (1 mM), or XAC (1 μM) were averaged from four to six experiments. Results are expressed as a percentage of the control response. Data shown are the mean ± S.E.M. *, $P < 0.05$ compared with the control response by Student's *t* test.

MeADP alone at the concentration tested (250 μM) had no effect on membrane current. Pretreatment of oocytes for 1 min with α,β-MeADP significantly inhibited the β,γ-MeATP-induced current without affecting the Ade-induced current (Fig. 5). Similar results were obtained with the ATP- and AMP-induced response (data not shown).

We next examined whether oocytes could convert ATP or β,γ-MeATP into Ade. After a 10-min incubation, ATP (100 μM) was decreased by 57.7 ± 5.2% and converted into ADP, AMP, and Ade [24.5 ± 2.6, 9.2 ± 0.7, and 15.2 ± 2.2% (n = 5), respectively]. β,γ-MeATP (100 μM) was very resistant to hydrolysis, being decreased by 4.7 ± 1.6% and converted mainly to Ade 1.1 ± 0.2% (n = 5). ADP and AMP production from β,γ-MeATP was less than 0.5%. These results demonstrate that oocytes possess an ectonucleotidase cascade that can produce Ade from ATP and β,γ-MeATP. We next examined the effects of α,β-MeADP, PPADS, and XAC on Ade production from ATP and β,γ-MeATP (Fig. 6, A and B). Although the rate of hydrolysis of ATP in oocytes was largely different from that of β,γ-MeATP, Ade production from these nucleotides were affected similarly by inhibitors. α,β-MeADP (250 μM) and PPADS (1 mM) significantly inhibited Ade production from ATP and β,γ-MeATP, whereas XAC (1 μM) had no effect. These results indicate that the inhibitory effects of α,β-MeADP and PPADS on the CFTR current induced by ATP and β,γ-MeATP are caused by the inhibition of Ade production and that XAC acts as an A_{2B} receptor antagonist.

Local Ade Formation from Adenine Nucleotides on Oocyte Membrane Surface. The results above suggest that ATP and β,γ-MeATP both activate the A_{2B} receptors after conversion into Ade. However, the Ade production from β,γ-MeATP was quite low compared with that from ATP, whereas these agonists induced nearly identical time- and dose-dependent responses, which were also similar to the Ade-induced response. A possible explanation for these observations is that the ectonucleotidase cascade led to a rapid and localized Ade formation from ATP and β,γ-MeATP to a similar extent on the membrane surface, where the Ade concentration is much higher than that measured in the bulk bath volume. To explore this possibility, we first examined [^{3}H]Ade formation from [^{3}H]AMP on the oocyte membrane surface. When oocytes were incubated with [^{3}H]AMP (5 μCi/tube, to a final concentration of 2.5 μM) in the presence of ADA (1 U/ml), levels of membrane-associated [^{3}H]Ade, which was detected in an acid-cell extract prepared after aspirating the incubation medium, was rapidly increased within 1 min (Fig. 7). This increase in [^{3}H]Ade was inhibited by α,β-MeADP (250 μM), suggesting that the Ade formation is mediated by ecto-5′-nucleotidase and that the generated Ade is retained in a membrane surface microenvironment even in the presence of ADA. If the conversions of ATP and β,γ-MeATP into Ade occur in the same environment, an excess amount of ATP and β,γ-MeATP should decrease the conversion of [^{3}H]AMP into [^{3}H]Ade through a competition with unlabeled AMP derived from ATP and β,γ-MeATP. As shown in Fig. 7, preincubation of oocytes with 250 μM ATP or β,γ-MeATP for 30 s significantly decreased [^{3}H]Ade production to a similar extent, which was equivalent to the effects of

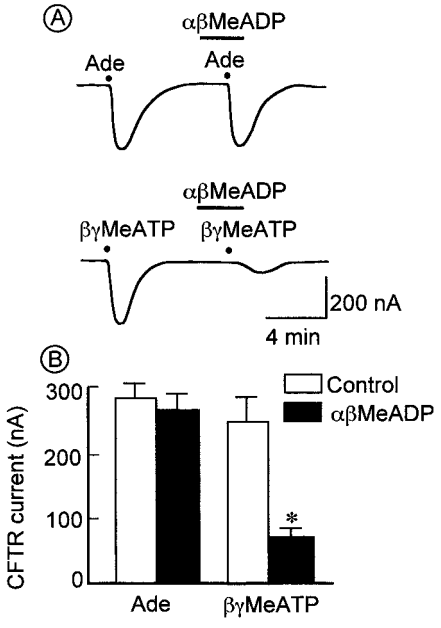

Fig. 5. Effects of α,β-MeADP on CFTR current induced by β,γ-MeATP or Ade. A, oocytes injected with A_{2B} receptor and CFTR cRNAs were voltage clamped at −60 mV. Ade (10 μM, top) or β,γ-MeATP (10 μM, bottom) was applied for 10 s as indicated (●). α,β-MeADP (250 μM) was applied 1 min before agonist stimulation and was present for another 1 min, as indicated by the bars, during which time the agonist was applied for 10 s. Representative current traces from 6 oocytes are shown. B, the peak current amplitudes induced by Ade (10 μM) or β,γ-MeATP (10 μM) in the presence (■) or absence (□) of α,β-MeADP (250 μM) were averaged from six experiments. Data shown are the mean ± S.E.M. *, $P < 0.05$ compared with the control response by the paired Student's t test

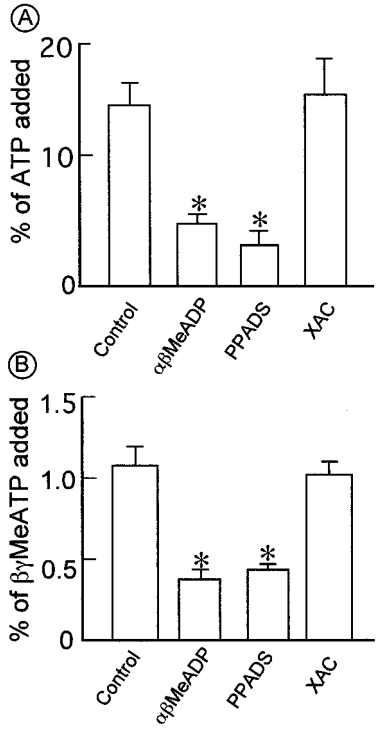

Fig. 6. Effects of α,β-MeADP, PPADS, and XAC on Ade production from ATP and β,γ-MeATP in oocytes. Oocytes were preincubated in the presence or absence of α,β-MeADP (250 μM), PPADS (1 mM), or XAC (1 μM) for 5 min and then incubated with ATP (100 μM, A) or β,γ-MeATP (100 μM, B) for 10 min. Metabolites in the extracellular medium were separated by HPLC, and Ade production is expressed as the percentage of the initial substrate added. Data shown are the mean ± S.E.M. from five experiments. *, $P < 0.05$ compared with the control by Dunnett's test.

250 μM AMP. These results suggest that at the membrane surface, ATP, β,γ-MeATP, and AMP are equally converted into Ade.

Discussion

In this study, we succeeded in having functional A_{2B} receptors expressed in *X. laevis* oocytes by injecting them with cRNA from the rat A_{2B} receptor. Although oocytes injected with the A_{2B} receptor cRNA alone did not have an electrophysiological response to Ade, oocytes coexpressing the A_{2B} receptor and CFTR carried out A_{2B} receptor-mediated cyclic AMP formation through an activation of CFTR current. Cyclic AMP formation through CFTR current has been demonstrated in oocytes coexpressing CFTR with several Gs-coupled receptors, such as adrenergic β_2 (Uezono et al., 1993), vasoactive intestinal peptide, and pituitary adenylyl cyclase-activating peptide receptors (Uezono et al., 1997). In oocytes coexpressing A_{2B} and β_2 receptors together with CFTR, the Ade receptor antagonist XAC selectively inhibited Ade-induced CFTR current without affecting the Epi-induced current. In contrast, the β receptor antagonist propranolol inhibited the CFTR current induced by Epi, but not that induced by Ade. Therefore, we concluded that the Ade-induced response is mediated by A_{2B} receptors.

Using oocytes expressing the A_{2B} receptors, we found that ATP and β,γ-MeATP stimulated the CFTR current. The following results indicate that currents induced by ATP and β,γ-MeATP are CFTR currents mediated by an activation of A_{2B} receptors. First, the adenine nucleotides did not induce a current in oocytes injected with water or CFTR cRNA alone, indicating that the responses are dependent on the A_{2B} receptor expression. Second, the reversal potentials of the current at different Cl^- concentrations were consistent with the CFTR Cl^- current. Finally, the P1 antagonist XAC inhibited the adenine nucleotide-induced currents. β,γ-MeATP is suggested to stimulate a certain class of Ade receptors directly

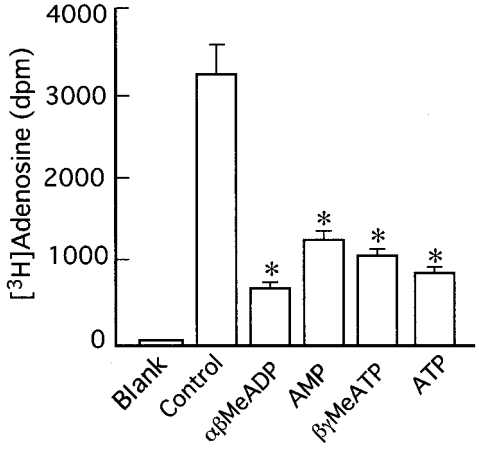

Fig. 7. Detection of locally produced [^{3}H]Ade from [^{3}H]AMP in oocytes. Oocytes (five cells) were preincubated with ADA (1 U/ml) for 2 min. After treatment with buffer (control), α,β-MeATP, ATP, β,γ-MeATP, or AMP (each 250 μM) for 30 s, [^{3}H]AMP (5 μCi/tube, to a final volume of 2.5 μM) was applied and incubated for 1 min. Reactions were terminated by aspirating the incubation medium, and cells were extracted with 2.5% perchloric acid. [^{3}H]Ade in the acid-extracts were determined after separation by HPLC. Data shown are the mean ± S.E.M. from the experiments. Blank shows the [^{3}H]Ade level in the reaction mixture after incubating without oocytes. *, $P < 0.05$ compared with the control by Dunnett's test.

(Hourani et al., 1991). However, the activation of A_{2B} receptors in oocytes by β,γ-MeATP clearly required its conversion into Ade, because an ecto-5′-nucleotidase inhibitor, α,β-MeADP, blocked the β,γ-MeATP-induced current without affecting the Ade-induced current. These observations indicate that α,β-MeADP does not have an antagonist effect on the A_{2B} receptor and that the inhibitory effect on β,γ-MeATP-induced current is caused by a blockade of Ade formation. Correspondingly, the conversion of ATP and β,γ-MeATP into adenosine was inhibited by α,β-MeADP in oocytes. Furthermore, PPADS also inhibited Ade production from ATP and β,γ-MeATP in oocytes. Although PPADS is widely used as a P2 antagonist, it also inhibits the extracellular adenine nucleotide metabolism (Grobben et al., 1999). Our data in oocytes suggest that PPADS inhibits the adenine nucleotide-induced CFTR current as an ectonucleotidase inhibitor rather than as a P2 antagonist.

The involvement of adenosine in the effects of ATP and β,γ-MeATP was also supported by experiments with ADA. The CFTR current induced by ATP and β,γ-MeATP were inhibited by exogenously added ADA. However, this inhibition required high concentration of ADA (5 U/ml), because ATP- and β,γ-MeATP-induced responses were little affected by ADA at 1 U/ml, a concentration which abolished Ade-induced effects. Similar results were reported in the rat hippocampal preparation, in which 5 to 10 U/ml ADA is necessary for the removal of the membrane-associated Ade (Cunha et al., 1996). Studies from other laboratories demonstrated the P1 antagonist-sensitive ATP responses in the rat hippocampus (Dunwiddie et al., 1997; Cunha et al., 1998).

β,γ-MeATP has been considered to be a metabolically stable P2 agonist (Hourani et al., 1991). However, the present results suggest that β,γ-MeATP is immediately converted into Ade on the oocyte membrane surface, thereby causing the CFTR current, which was very similar to the ATP- and Ade-induced responses concerning the time course, the maximal current amplitude, and the effective concentration range of the agonists. This is somewhat surprising because only 1% of an added dose of β,γ-MeATP was converted into Ade in the bulk incubation medium within 10 min. However, a lack of correlation between the Ade formation and the induced response was reported in the inhibition of neurotransmitter release by ATP and β,γ-MeATP in the rat hippocampal slice (Cunha et al., 1998). We also reported that β,γ-MeATP induced a nearly identical response to ATP or AMP in a manner that was dependent on Ade formation in C6Bu-1 and NG108–15 cells, despite their marked difference in the rates of hydrolysis in the bulk medium (Ohkubo et al., 2000a, 2001). In those cells, we showed previously that [^{3}H]Ade converted from [^{3}H]ATP or [^{3}H]AMP was differently distributed in the cell surface and bulk incubation medium, especially in the presence of ADA (Ohkubo et al., 2000a, 2001). Using a similar protocol, we demonstrated a rapid increase in [^{3}H]Ade level within 1 min on the oocyte membrane surface during incubation with [^{3}H]AMP in the presence of ADA. This [^{3}H]Ade formation was inhibited by α,β-MeADP, suggesting that the Ade formation is mediated by ecto-5′-nucleotidase. If this [^{3}H]Ade accumulation by [^{3}H]AMP hydrolysis reflected the rapid activation of A_{2B} receptors by AMP, the hydrolysis of ATP or β,γ-MeATP would occur in the same environment on the membrane. Indeed, we observed that a brief treatment of oocytes with an

excess amount of ATP or β,γ-MeATP markedly reduced the following [^{3}H]Ade accumulation by [^{3}H]AMP hydrolysis. These results suggest that ATP and β,γ-MeATP are rapidly and efficiently hydrolyzed into Ade on the membrane surface, leading to the A_{2B} receptor activation similar to a direct effect of Ade.

The present results support a hypothesis proposed in our previous study with C6Bu-1 cells, stating that cyclic AMP formation induced by ATP and β,γ-MeATP are mediated by locally generated Ade on the membrane surface and subsequent activation of A_{2B} receptors (Ohkubo et al., 2001). In C6Bu-1 cells, the effects of ATP and β,γ-MeATP were not affected by ADA treatment, but were inhibited by XAC, PPADS, and α,β-MeADP. ADP, AMP, and ATPγS mimicked those responses, whereas typical P2 receptor agonists, such as α,β-MeATP, 2MeS-ATP, UTP, and UDP, had no effect. In this study, we found that such characteristics were all reproduced in oocytes expressing the A_{2B} receptor. Therefore, we suggest that the ATP-induced response having the characteristics described above might occur in native tissues and cells through a combination of ectonucleotidase-dependent Ade formation and subsequent activation of Ade receptors. Although we demonstrated adenine nucleotide responses with the A_{2B} receptor, such responses would also occur with other Ade receptor subtypes. Indeed, ATP-induced inhibition of neurotransmitter release in the rat hippocampus (Dunwiddie et al., 1997; Cunha et al., 1998) and neuromuscular junctions (Sebastião et al., 1999) are likely to involve the A_1 receptor subtype. Furthermore, we demonstrated recently that ATP-induced cyclic AMP accumulation in a neuroblastoma cell line is mediated by the A_{2A} receptor subtype, in which Ade formation was catalyzed by ectoalkaline phosphatase instead of ecto-5′-nucleotidase (Ohkubo et al., 2000b).

Recently, several ectoenzymes involved in adenine nucleotide metabolism have been identified and characterized (Zimmermann and Braun, 1999). These include the ectonucleotide triphosphatase family, which hydrolyzes either nucleoside 5′-triphosphates or both nucleoside 5′-tri- and diphosphates; the ectophosphodiesterase/pyrophosphatase family, which hydrolyzes nucleoside 5′-triphosphates directly to nucleoside 5′-monophosphates; and the enzymes catalyzing the conversion of nucleoside 5′-monophosphates to the corresponding nucleosides, including ecto-5′-nucleotidase and alkaline phosphatase. Ectonucleotidase activity in *X. laevis* oocytes has been examined extensively by Ziganshine et al. (1995, 1996a,b). They demonstrated that although the enzyme activity in oocytes is localized mainly in the follicle cell layer, defolliculated oocytes also possess moderate enzyme activity (Ziganshin et al., 1995, 1996b). However, the enzymes involved in nucleotide hydrolysis in oocytes have not yet been identified. Previously, we showed that C6Bu-1 cells express ectophosphodiesterase/pyrophosphatase1 and ecto-5′-nucleotidase, and these two enzymes seem to be sufficient for producing Ade from β,γ-MeATP (Ohkubo et al., 2001). In addition, PPADS was demonstrated to inhibit ectophosphodiesterase/pyrophosphatase activity in C6 glioma cells (Grobben et al., 1999). Therefore, it seems likely that oocytes express at least two different enzymes: ecto-5′-nucleotidase and the PPADS-sensitive ectophosphodiesterase/pyrophosphatase1-like enzyme.

There are many reports that methylxanthine derivatives block the P2 receptor agonist-induced response in several tissue preparations. Different purinoceptors are generally expressed in the same tissue and even in the same cells, making it difficult to elucidate the mechanism of action of ATP. In this study, using the expression of A_{2B} receptors in *X. laevis* oocytes, we showed that ATP and several adenine nucleotides can stimulate A_{2B} receptors after their conversion to Ade. These findings suggest that the close association of P1 receptors to ectonucleotidases may constitute a functional receptor for ATP and that some important physiological responses to ATP may occur through this mechanism.

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