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Functional characterization of an endogenous *Xenopus* oocyte adenosine receptor

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1 To investigate the effects of adenosine on endogenous *Xenopus* oocyte receptors, we analysed defolliculated oocytes injected with mRNAs for the G protein-activated inwardly rectifying K^+ (GIRK) channels.

2 In oocytes injected with mRNAs for either GIRK1/GIRK2 or GIRK1/GIRK4 subunits, application of adenosine or ATP reversibly induced inward K^+ currents, although ATP was less potent than adenosine. The responses were attenuated by caffeine, a non-selective adenosine receptor antagonist. Furthermore, in uninjected oocytes from the same donor, adenosine produced no significant current.

3 The endogenous receptor was activated by two selective A_1 adenosine receptor agonists, N^6-cyclopentyladenosine (CPA) and N^6-cyclohexyladenosine (CHA), and antagonized by a selective A_1 adenosine receptor antagonist, 1,3-dipropyl-8-cyclopenylxanthine (DPCPX) at moderate nanomolar concentrations, but insensitive to micromolar concentrations of selective A_{2A} and A_3 adenosine receptor agonists, 2-[*p*-(2-carbonyl-ethyl)-phenylethylamino]-5′-N-ethylcarboxamidoadenosine (CGS21680) and N^6-(3-iodobenzyl)-5′-(N-methylcarbamoyl)adenosine (IB-MECA), respectively. However, the pharmacological characteristics of the receptor were different from those of the cloned *Xenopus* A_1 adenosine receptor and previously proposed adenosine receptors.

4 The adenosine-induced GIRK currents were abolished by injection of pertussis toxin and CPA inhibited forskolin-stimulated cyclic AMP accumulation.

5 We conclude that an adenosine receptor on the *Xenopus* oocyte membrane can activate GIRK channels and inhibit adenylyl cyclase *via* $G_{i/o}$ proteins. Moreover, our results suggest the existence of an endogenous adenosine receptor with the unique pharmacological characteristics. As the receptor was activated by nanomolar concentrations of adenosine, which is a normal constituent of extracellular fluid, the receptor may be involved in some effects through the $G_{i/o}$ protein signalling pathways in ovarian physiology.

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Keywords: Adenosine receptor; $G_{i/o}$ protein; G protein-activated inwardly rectifying K^+ (GIRK) channel; *Xenopus* oocyte

Abbreviations: Ado, adenosine; CHA, N^6-cyclohexyladenosine; CGS21680, 2-[*p*-(2-carbonyl-ethyl)-phenylethylamino]-5′-N-ethylcarboxamidoadenosine; CPA, N^6-cyclopentyladenosine; DMSO, dimethyl sulphoxide; DPCPX, 1,3-dipropyl-8-cyclopenylxanthine; E_K, K^+ equilibrium potential; $G\beta\gamma$, G protein $\beta\gamma$-subunits; $G_{i/o}$, G_i and G_o; GIRK, G protein-activated inwardly rectifying K^+ channel; GPCR, G protein-coupled receptor; hK, high-potassium; IB-MECA, N^6-(3-iodobenzyl)-5′-(N-methylcarbamoyl)adenosine; ND98, K^+-free high-sodium; NECA, 5′-(*N*-ethylcarboxaminido)adenosine; n_H, Hill coefficient; PCR, polymerase chain reaction; PTX, pertussis toxin; XA_1, *Xenopus* A_1 adenosine receptor; XIR, endogenous *Xenopus* oocyte GIRK-related polypeptides

Introduction

Xenopus ovarian follicles consist of an oocyte surrounded by a vitelline envelope, follicle cells and thecal tissues (Fraser & Djamgoz, 1992). Mature follicular oocytes (stage V and VI) are known to possess receptors for adenosine and ATP as well as for acetylcholine, serotonin, dopamine, prostaglandins and progesterone (Dascal, 1987; Fraser & Djamgoz, 1992). The maturation phenomenon of *Xenopus* oocytes serves as a useful experimental model for studying its complicated mechanism, and is affected by adenosine (Gelerstein *et al*., 1988). Oocytes of *Xenopus laevis* are also used widely in studies of mammalian neurotransmitter/hormone receptors and ion channels, which can be expressed in the oocyte membrane by microinjection of foreign RNAs. Examining receptor systems and channels in naive oocytes is therefore important in terms of ovarian physiology and for interpreting the functions of foreign receptors and channels when expressed in oocytes.

Adenosine is a normal constituent of intra- and extracellular fluids (Fredholm, 1995). Adenosine is released from cells that are metabolically active or stressed, and modulates the activity of various cells via the adenosine receptors (Collis & Hourani, 1993; Fredholm, 1995). To date, four adenosine receptor subtypes, A_1, A_{2A}, A_{2B} and A_3, have been identified

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and pharmacologically characterized (Collis & Hourani, 1993; Poulsen & Quinn, 1998; Ralevic & Burnstock, 1998). All of these subtypes belong to the superfamily of G protein-coupled receptors (GPCRs), with the A_1 and A_3 adenosine receptors interacting with pertussis toxin (PTX)-sensitive G proteins, including G_i and G_o ($G_{i/o}$) proteins, and the A_{2A} and A_{2B} adenosine receptors interacting with G_s protein. Also, the A_{2B} and A_3 adenosine receptors are coupled to G_q protein. Furthermore, physiological, pharmacological and biochemical studies have suggested the existence of additional adenosine receptors that have not yet been identified. In follicular oocytes of *Xenopus laevis*, adenosine activated adenylyl cyclase and elicited cyclic AMP-mediated outward K^+ currents (Lotan *et al*., 1982; 1985) but the effects were either abolished or markedly reduced by defolliculation, indicating that the receptors for adenosine are located in the follicle cells (Miledi & Woodward, 1989; Greenfield *et al*., 1990a,b; King *et al*., 1996). However, Gelerstein *et al*. (1988) showed that adenosine elevated cyclic AMP levels in defolliculated oocytes to the same extent as in follicular oocytes, suggesting that an adenosine receptor is also located on the oocyte membrane. In contrast, adenosine at high micromolar concentrations inhibited adenylyl cyclase activity in the defolliculated oocyte membrane fraction (Finidori *et al*., 1982). In addition, previous studies using a variety of mammalian tissue preparations have suggested the existence of putatively additional subtypes of adenosine receptors (Shinozuka *et al*., 1988; Daut *et al*., 1990; Kirsch *et al*., 1990; Forsyth *et al*., 1991; Cornfield *et al*., 1992; Abebe *et al*., 1994). These findings suggest the possibility that there are additional endogenous adenosine receptors on the oocyte membrane. Although extensive studies using molecular biological techniques have elucidated the functional roles of the adenosine receptor subtypes (Nyce, 1999), the molecular and cellular mechanisms of action of the adenosine receptors in *Xenopus* oocytes remain poorly understood.

Various GPCRs, such as M_2 muscarinic, α_2 adrenergic, D_2 dopamine, opioid, nociceptin/orphanin FQ receptors and A_1 adenosine receptor, functionally couple to G protein-activated inwardly rectifying K^+ (GIRK) channels *via* interaction with $G_{i/o}$ proteins (North, 1989; Brown & Birnbaumer, 1990; Ikeda *et al*., 1995; 1996; 1997; Pfaff & Karschin, 1997). Taken together with inhibition of adenylyl cyclase by adenosine in the oocyte membrane (Finidori *et al*., 1982), unknown oocyte receptors for adenosine may also be able to activate GIRK channels as a signalling pathway. Hence, we examined the responses of endogenous *Xenopus* oocyte receptors to adenosine in defolliculated oocytes expressing GIRK channels. Here we demonstrate that an endogenous adenosine receptor on the *Xenopus* oocyte membrane can activate GIRK channels *via* $G_{i/o}$ proteins and inhibit forskolin-stimulated cyclic AMP accumulation and that the receptor shows unique pharmacological characteristics for adenosine receptor ligands.

Methods

Molecular biology

Plasmids containing the entire coding sequences for mouse GIRK1, GIRK2 and GIRK4 channel subunits were obtained using the polymerase chain reaction (PCR) method as described previously (Kobayashi *et al*., 1995; 2000). Based on the cDNA sequence for the *Xenopus* A_1 adenosine (XA_1) receptor (GenBank/EMBL/DDBJ databases: accession number AJ249842), a pair of oligonucleotide primers corresponding to the regions containing either a translational initiation codon or a stop codon were synthesized. Primers for the XA_1 receptor were 5′-GCCCATGGGAATCCCAGCCTCGCTTGCT-3′ and 5′-GCTCTAGAGGACATTTTTAGAACTCTCAC-3′. The template cDNA was synthesized using mRNA prepared from *Xenopus laevis* oocytes and surrounding tissues and the 1st-Strand cDNA Synthesis Kit (Clontech) as described previously (Ikeda *et al*., 2001). PCR was carried out using *Pfu* DNA polymerase (Stratagene) as described previously (Ikeda *et al*., 1995). The PCR product was inserted into the plasmid pSP35T to yield the plasmid pSPXA1 and the cDNA insert was sequenced using an automated DNA sequencer model 373S (Applied Biosystems Inc.). The specific mRNAs were synthesized *in vitro* from the linearized plasmids using the mMESSAGE mMACHINE™ *In Vitro* Transcription Kit (Ambion).

Electrophysiological analyses

Adult female *Xenopus laevis* frogs were purchased from Copacetic (Soma, Aomori, Japan) and maintained in the laboratory until use. Frogs were anaesthetized by immersion in water including 0.15% tricaine (Sigma Chemical Co.). A small incision was made on the abdomen to remove several ovarian lobes from the frogs that were humanely killed after the final collection. Oocytes (Stage V and VI) were isolated manually from the ovary and maintained in Barth's solution (composition in mM: NaCl 88, KCl 1, $Ca(NO_3)_2$ 0.33, $CaCl_2$ 0.41, $MgSO_4$ 0.82, $NaHCO_3$ 2.4, Tris-HCl 7.5 (pH 7.4), and 0.1 mg ml^{-1} gentamicin sulphate; Wako Pure Chemical Industries). Oocytes were injected with either GIRK1 and GIRK2 mRNAs or GIRK1 and GIRK4 mRNAs ($\sim$0.6 ng of each mRNA per oocyte) and/or XA_1 receptor mRNA ($\sim$10 ng per oocyte). The oocytes were incubated at 19°C in Barth's solution, and defolliculated by manual dissection after treatment with 0.8 mg ml^{-1} collagenase (Wako Pure Chemical Industries) for 1 h (Kobayashi *et al*., 1998). Whole-cell currents of the oocytes were recorded at 18–19°C from 2 to 7 days after injection with a conventional two-electrode voltage clamp (Kobayashi *et al*., 2000). The membrane potential was held at -70 mV, unless otherwise specified. Microelectrodes were filled with 3M KCl. The oocytes were placed in a 0.05 ml narrow chamber and superfused continuously with a high-potassium (hK) solution (composition in mM: KCl 96, NaCl 2, $MgCl_2$ 1 and $CaCl_2$ 1.5) or a K^+-free high-sodium (ND98) solution (composition in mM: NaCl 98, $MgCl_2$ 1 and $CaCl_2$ 1.5) at a flow rate of 2.5 ml min^{-1}. In the hK solution, the K^+ equilibrium potential (E_K) is close to 0 mV and enables K^+ inward current flow through inward-rectifier K^+ channels at negative holding potentials. Agonist effect was expressed as a ratio of maximal effect induced by adenosine. Antagonist effect was quantified by dividing amplitude of adenosine-induced current in the presence of an antagonist by amplitude of adenosine-induced current without the antagonist. Data were fitted to a standard logistic equation using SigmaPlot (Jandel Scientific) to compute the EC_{50}, the IC_{50} and the Hill

coefficient (n_H) in analysis of concentration-response relationships.

Pertussis toxin treatment

For PTX (Sigma Chemical Co.) experiments (Kobayashi *et al*., 1999), ~1.38 ng of PTX (0.1 μg μl^{-1} in distilled water) per oocyte was injected 7 h before recording using a Nanoliter injector (World Precision Instruments). Oocytes of the control group were prepared from the same donor and sham injected with the same volume of distilled water.

Cyclic AMP assay

Cyclic AMP levels in *Xenopus* oocytes were determined using the Biotrak™ cAMP enzymeimmunoassay system (Amersham Pharmacia Biotech). Groups of 25 defolliculated oocytes carefully selected from the same donor on the basis of morphological similarity were incubated at room temperature for 30 min in Barth's solution containing N^6-cyclopentyladenosine (CPA) or forskolin (Wako Pure Chemical Industries). The incubation solution was replaced with a lysis reagent buffer containing 0.25% dodecyltrimethylammonium bromide in the kit, homogenized and centrifuged at 10,000 × *g* for 10 min at 4°C. The measurements of cyclic AMP contents in the diluted supernatants were conducted according to the manufacture's procedure.

Statistical analysis of results

The values obtained are expressed as mean ± s.e.mean, and n is the number of oocytes tested. Statistical analysis of differences between groups was carried out using Student's t-test. A probability of 0.05 was taken as the level of statistical significance.

Compounds

All the compounds used were purchased from Research Biochemicals International. N^6-(3-iodobenzyl)-5′-(N-methylcarbamoyl)adenosine (IB-MECA) and 1,3-dipropyl-8-cyclopenylxanthine (DPCPX) were dissolved in dimethyl sulphoxide (DMSO). Other compounds were dissolved in distilled water. The stock solutions of all compounds were stored at −20°C until use. Each compound was added to the perfusion solution in appropriate amounts immediately before the experiments.

Results

Activation of GIRK channels by an endogenous adenosine receptor in Xenopus *oocytes*

Using the *Xenopus* oocyte system expressing GIRK channels (Kobayashi *et al*., 1999), we investigated whether endogenous adenosine receptors in defolliculated oocytes activate GIRK channels. In oocytes injected with mRNAs for GIRK 1 and GIRK2 subunits, application of adenosine reversibly induced inward currents in a concentration-dependent manner in hK perfusion solution containing 96 mM K^+ and 2 mM Na^+ (Figure 1a, b). The EC_{50} and n_H values obtained from the concentration-response relationships were 917.3 ± 69.4 nM and 0.88 ± 0.02 (n = 10), respectively (Figure 1b and Table 1). The current responses to 3 μM adenosine were attenuated by caffeine, a non-selective adenosine receptor antagonist of methylxanthines, with the IC_{50} value of 245.5 ± 18.5 μM and n_H value of 0.72 ± 0.04 (n = 8) (Figures 1a and 2d). Although micromolar concentrations of ATP also induced inward currents, which were sensitive to caffeine, ATP was much less potent than adenosine (Figure 1b). These results suggest the existence of an endogenous receptor for adenosine in defolliculated oocytes. Moreover, the current responses to adenosine were completely abolished in the presence of 3 mM Ba^{2+}, a GIRK channel blocker (Dascal *et al*., 1993), (n = 8, Figure 1a) which also blocked basally active GIRK currents (IC_{50} value of 170.7 ± 12.2 μM, n = 6; Kobayashi *et al*., 2000). In contrast, in uninjected oocytes from the same donor, no significant current response was observed by application of 10 μM adenosine, 10 μM ATP or 3 mM Ba^{2+} (1.9 ± 0.7 nA, n = 6; 0.9 ± 0.6 nA, n = 6; 5.0 ± 1.3 nA, n = 10, respectively; Figure 1a). These results suggest that the adenosine-induced currents are mediated by GIRK channels. In addition, responses to adenosine were not observed in ND98 solution containing 98 mM Na^+ and no K^+ instead of the hK solution (n = 8; data not shown), suggesting that the currents show K^+ selectivity. The current-voltage relationship of the response to 3 μM adenosine showed strong inward rectification (Figure 1c), typical of a current response mediated by GIRK channels. In addition, similar results were obtained in oocytes injected with mRNAs for GIRK 1 and GIRK4 subunits (data not shown). These results suggest that an endogenous adenosine receptor can functionally couple to GIRK channels.

In the present study, although basally active GIRK currents sensitive to Ba^{2+} were observed in all oocyte batches injected with GIRK mRNAs, the successful responses to adenosine were observed in oocyte batches prepared from 14 of 56 donors (25.0%). The GIRK currents induced by 3 μM adenosine were variable among oocytes prepared from the same donor, and amplitudes of responses to adenosine were not significantly correlated with amplitudes of the 3 mM Ba^{2+}-sensitive current components (Figure 1d). Also, similar results were obtained in other oocyte batches from different donors (data not shown). In contrast, the unsuccessful responses to adenosine in oocytes prepared from most donors showed little or no inward current (11.9 ± 1.3 nA at 3 μM, n = 304), although amplitudes of the Ba^{2+}-sensitive current components, which are related to expression levels of GIRK channels (Kobayashi *et al*., 2000), were large enough in the same oocytes (821.5 ± 58.7 nA, n = 304). Taken together, these results indicate that the expression level of an endogenous receptor for adenosine in defolliculated oocytes may be dependent on the oocyte batch from donors and may be variable from cell to cell among oocyte batches.

Pharmacological characteristics of a Xenopus *oocyte adenosine receptor*

To investigate the pharmacological characteristics of the *Xenopus* oocyte adenosine receptor found in the present study, we examined the effects of various adenosine receptor ligands in oocytes expressing GIRK channels. Two selective A_1 adenosine receptor agonists, CPA and N^6-cyclohexyladenosine (CHA), and a non-selective adenosine receptor agonist, 5′-(*N*-ethylcar-

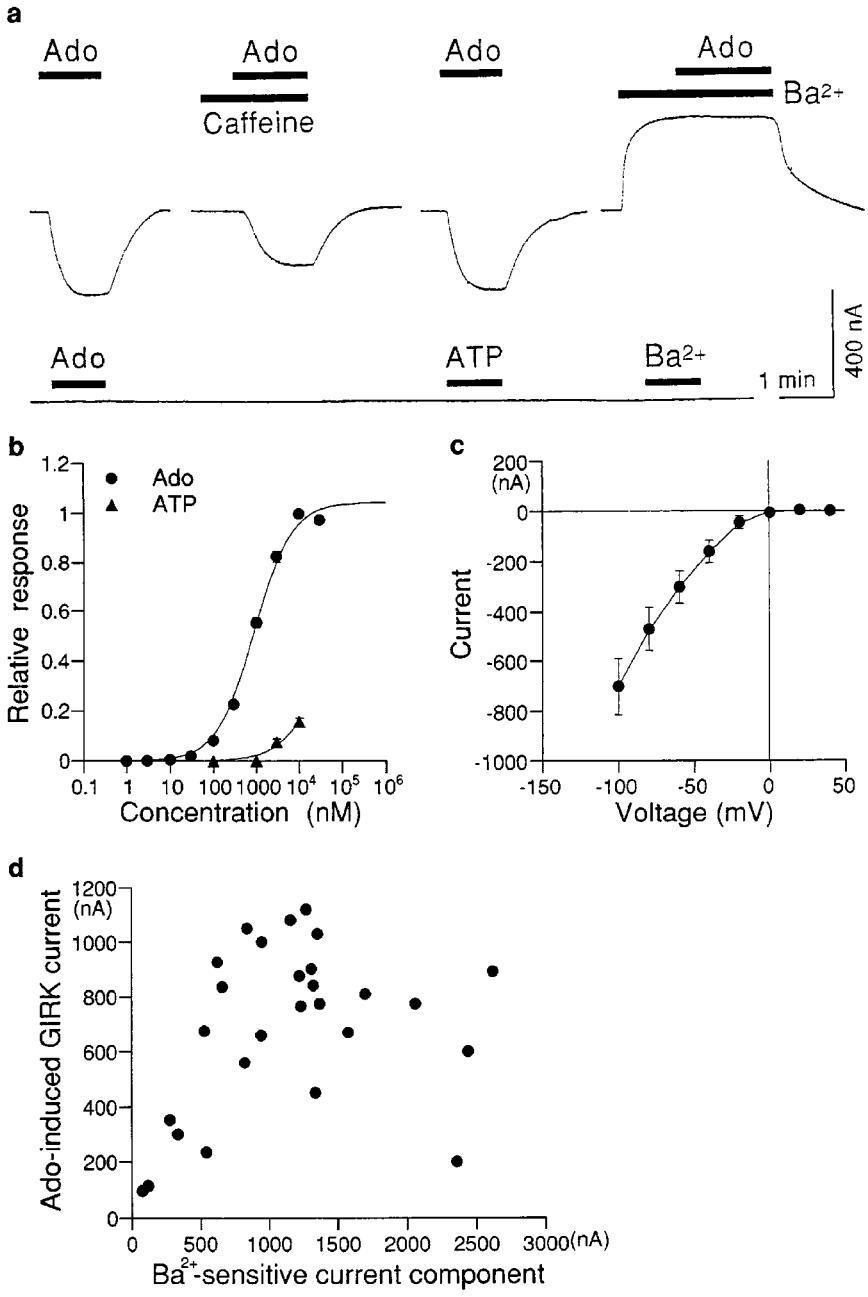

Figure 1 Effects of adenosine in *Xenopus* oocytes expressing GIRK channels. (a) Upper row, in a defolliculated oocyte injected with GIRK1 mRNA and GIRK2 mRNA, current responses to adenosine (Ado), Ado in the presence of 100 μM caffeine, Ado and Ado in the presence of 3 mM Ba^{2+} are shown. The concentration of Ado used was 3 μM. Lower row, in an uninjected oocyte, current responses to 10 μM Ado, 10 μM ATP and 3 mM Ba^{2+} are shown. Current responses were measured at a membrane potential of -70 mV in a high-potassium solution containing 96 mM K^+. Bars show the duration of application. (b) Concentration-response relationships for GIRK channel activation induced by Ado or ATP. Effects of these drugs were normalized to the magnitudes of 10 μM Ado-induced GIRK currents, which were 556.4 ± 64.0 nA ($n=20$). Each point and error bar represents the mean and s.e.mean of the relative responses obtained from 10 oocytes. Data points were fitted using a logistic equation. (c) Current-voltage relationship of 3 μM Ado-induced GIRK currents in oocytes expressing GIRK1/2 channels ($n=8$). (d) Correlation between the amplitudes of current response to 3 μM Ado and of the 3 mM Ba^{2+}-sensitive current component in oocytes prepared from the same donor. The correlation coefficient was 0.317 ($P>0.1$, $n=27$; correlation analysis).

boxaminido)adenosine (NECA), concentration-dependently induced inward current responses at nanomolar concentrations (Figure 2a,c and Table 1), whereas at the highest concentration used they induced no current response in uninjected oocytes ($n=5$; data not shown). The rank order of potency for these adenosine receptor agonists was CHA $\geqslant$ CPA > NECA > adenosine, whereas the rank order of efficacy for these agonists was NECA > adenosine > CPA > CHA (Figure 2c and Table 1). The n_H values for the these adenosine receptor agonists were not significantly different from 1 (Student's t-test, $P>0.05$), suggesting a single site of action for each of the agonists. Furthermore, the current responses to 3 μM adenosine were

Table 1 Comparison of pharmacological characteristics of an endogenous *Xenopus* oocyte adenosine receptor and the cloned XA_1 receptor

	Xenopus *oocyte adenosine receptor*				*XA_1 receptor*			
Compound	*EC_{50}*	*Max. efficacy*	*n_H*	(n)	*EC_{50}*	*Max. efficacy*	*n_H*	(n)
Adenosine	917.3±69.4	1	0.88±0.02	(10)	2.91±0.54	1	0.83±0.11	(5)
CPA	96.7±10.1	0.81±0.03	0.91±0.03	(6)	5.72±0.87	1.03±0.01	0.99±0.03	(6)
CHA	86.7±28.3	0.65±0.02	0.89±0.04	(9)	3.60±0.89	1.11±0.04	0.96±0.07	(5)
NECA	398.6±117.9	1.70±0.20*	0.95±0.05	(5)	0.56±0.05	0.96±0.02	0.92±0.04	(5)
CGS21680	N.D.	N.D.	N.D.	(5)	205.6±20.3	0.87±0.03	0.90±0.04	(5)
IB-MECA	N.D.	N.D.	N.D.	(4)	135.2±12.8	0.96±0.04	0.97±0.02	(6)

The EC_{50} values are shown as mean±s.e.mean in nM. The values of maximal efficacy (Max. efficacy) were normalized to magnitude of GIRK currents induced by adenosine at 10 μM in an endogenous *Xenopus* oocyte adenosine receptor and at 1 μM in the XA_1 receptor. The n_H values indicate the mean±s.e.mean of the Hill coefficients. The numbers of oocytes tested are indicated in parentheses. N.D. means that these values were not determined because these compounds induced only small GIRK responses at micromolar concentrations. *The high efficacy raises the possibilities that NECA, a non-selective adenosine receptor agonist, may cause a distinct conformational change of the endogenous adenosine receptor and that there may be additional types of oocyte adenosine receptors which are activated not by A_1 adenosine receptor agonists but by NECA.

almost completely abolished by 1 μM DPCPX, a selective A_1 adenosine receptor antagonist, with an IC_{50} value of 18.0±2.6 nM and a n_H value of 0.96±0.07 ($n=4$, Figure 2a,d). However, a selective A_{2A} adenosine receptor agonist, 2-[*p*-(2-carbonyl-ethyl)-phenylethylamino]-5′-N-ethylcarboxamidoadenosine (CGS21680), and a selective A_3 adenosine receptor agonist, IB-MECA, produced only small current responses as compared with the responses to 10 μM adenosine (0% at 100 nM, 7.9±0.8% at 1 μM and 9.9±5.3% at 30 μM for CGS21680, $n=5$; 0% at 100 nM, 1.7±1.7% at 300 nM and 5.7±2.9% at 1 μM for IB-MECA, $n=4$, respectively). In addition, DMSO, the solvent vehicle, at the highest concentration (0.1%) used had no effect in this study.

As the present results indicated that characteristics of the adenosine receptor found here resembled those of the A_1 adenosine receptor, we next compared the receptor found with the XA_1 receptor identified in *Xenopus laevis* by Nebreda (GenBank/EMBL/DDBJ databases: accession number AJ249842). For functional characterization of the cloned XA_1 receptor, we first carried out molecular cloning of the cDNA from oocytes of *Xenopus laevis* and surrounding tissues by reverse transcription-PCR. The specific PCR product was detected by agarose gel electrophoresis and cloned as described previously (Ikeda *et al*., 1995). The deduced amino acid sequence of the cDNA for the XA_1 receptor was identical with the reported sequence. The XA_1 receptor shares 72% and 72.5% amino acid sequence identity with the rat and human A_1 adenosine receptors, respectively, whereas the overall amino acid sequence identity for the A_1 adenosine receptor between the mammalian species is approximately 95%. To investigate functional coupling of the XA_1 receptor to GIRK channels and pharmacological characteristics of the receptor, we used oocytes co-injected with mRNAs for the XA_1 receptor and GIRK channels, when 3 μM adenosine induced no significant current response in oocytes from the same donor injected with GIRK mRNAs (75.0% of donors). Application of adenosine, CPA or CHA, at low nanomolar concentrations, induced significant inward currents with similarly high potency and similar efficacy (Figure 2b,c and Table 1). However, CGS21680 and IB-MECA induced current responses at relatively high nanomolar concentrations (Table 1). The responses to adenosine were attenuated in the presence of caffeine (IC_{50} value of 327.1±34.4 μM and n_H value of 0.89±0.10, $n=4$) and DPCPX (IC_{50} value of 56.4±5.2 nM and n_H value of 0.85±0.08, $n=4$) (Figure 2d). In oocytes injected with XA_1 receptor mRNA alone, 3 μM adenosine induced no significant current response (3.4±0.7 nA, $n=8$). These results indicate that the XA_1 receptor also couples to GIRK channels and the pharmacological properties of the XA_1 receptor are typical of the A_1 adenosine receptor, although the amino acid sequences for the XA_1 receptor and the mammalian A_1 adenosine receptors exhibit relatively low identity. In addition, the XA_1 receptor was fully activated by low nanomolar concentrations of adenosine, whereas such low concentrations of adenosine induced little or no response in all the oocyte batches injected with GIRK mRNAs (Figure 2c), suggesting that the XA_1 receptor may be located exclusively in follicle cells and outer surrounding tissues or only sparsely in the oocyte membrane. Further studies by *in situ* hybridization analysis and immunohistochemical analysis may identify the location of the XA_1 receptor. As shown in Figure 2c and Table 1, the potency and efficacy of adenosine, NECA and A_1 adenosine receptor agonists for the adenosine receptor found here are distinct from those of the XA_1 receptor. Moreover, the adenosine receptor found here was insensitive to micromolar concentrations of CGS21680 and IB-MECA, whereas the XA_1 receptor was sensitive to moderate nanomolar concentrations of these agonists. While the antagonist effects of caffeine and DPCPX, adenosine receptor antagonists tested, on the adenosine receptor found here and the XA_1 receptor were similar (Figure 2d). In addition, the characteristics of the XA_1 receptor were observed similarly even in the oocytes which showed similar magnitudes of GIRK current responses *via* the XA_1 receptor as compared with magnitudes of the responses *via* the endogenous adenosine receptor found here, suggesting that the differences in pharmacological characteristics between the endogenous adenosine receptor and the XA_1 receptor were not caused by differences in receptor density expressing in oocytes. The present results suggest that the endogenous adenosine receptor found in defolliculated oocytes is different from the XA_1 and A_3 adenosine receptors.

Signal transduction mechanisms of a Xenopus *oocyte adenosine receptor*

Activation of various GPCRs opens GIRK channels *via* direct action of G protein $\beta\gamma$-subunits ($G\beta\gamma$) released from

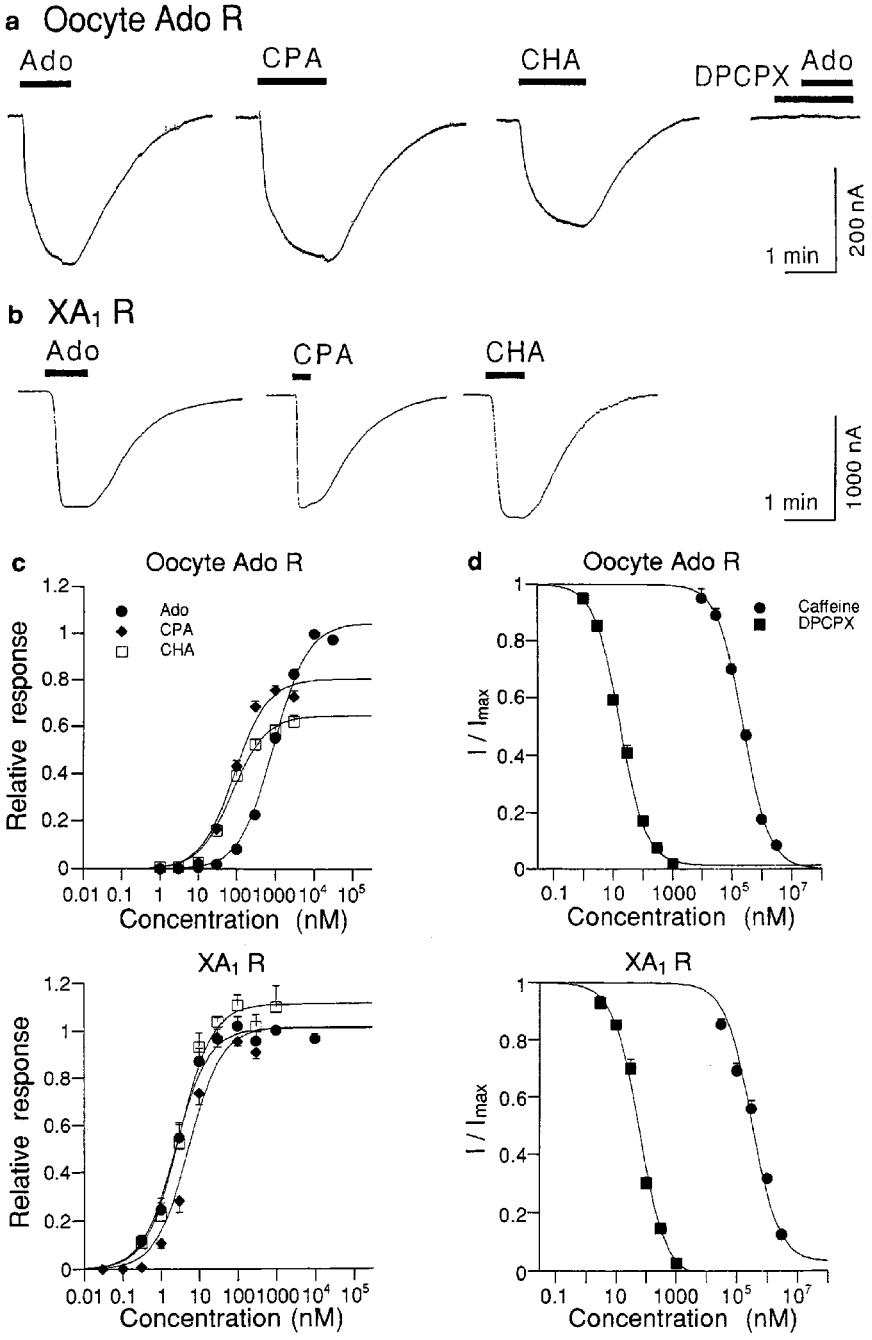

Figure 2 Effects of adenosine receptor ligands on an endogenous *Xenopus* oocyte adenosine receptor (Oocyte Ado R) and the cloned *Xenopus* adenosine A_1 receptor (XA_1 R). (a) In a defolliculated oocyte injected with GIRK1 and GIRK2 mRNAs, current responses to 3 μM Ado, 1 μM CPA, 1 μM CHA and 3 μM Ado in the presence of 1 μM DPCPX are shown. (b) In a defolliculated oocyte injected with mRNAs for the XA_1 receptor and GIRK1/2 channels, current responses to 100 nM Ado, 100 nM CPA and 100 nM CHA are shown. Bars show the duration of application. (c) Concentration-response relationships for adenosine receptor agonists on Oocyte Ado R (top) and XA_1 R (bottom). The magnitudes of GIRK currents induced by adenosine receptor agonists were normalized to the magnitudes of 10 μM Ado-induced currents, which were 355.9 ± 36.7 nA ($n=25$), in oocytes injected with GIRK1 and GIRK2 mRNAs and to the magnitudes of 1 μM Ado-induced currents, which were 574.8 ± 122.2 nA ($n=16$), in oocytes co-injected with mRNAs for the XA_1 receptor and GIRK1/2 channels, respectively. (d) Concentration-dependent inhibition of adenosine-induced currents by adenosine receptor antagonists on Oocyte Ado R (top) and XA_1 R (bottom). I_{max} is the amplitude of GIRK currents induced by Ado at 3 μM in Oocyte Ado R (563.1 ± 91.2 nA, $n=12$) and at 10 nM in XA_1 R (325.7 ± 77.7 nA, $n=8$), and I is the current amplitude in the presence of an adenosine receptor antagonist. Current responses were measured at a membrane potential of -70 mV in a high-potassium solution containing 96 mM K^+. Each point and error bar represents the mean and s.e.mean of the relative responses obtained from 4–10 oocytes. Data points were fitted using a logistic equation.

$G_{i/o}$ proteins, which are sensitive to PTX (Brown & Birnbaumer, 1990; Reuveny *et al*., 1994). To investigate the involvement of G protein-coupled mechanisms in activation of GIRK channels by the endogenous adenosine receptor found here, the responses to adenosine in oocytes expressing GIRK 1/2 channels were compared with and without PTX injection. The current responses to adenosine were almost completely abolished by injection of PTX (Figure 3a). The current responses to adenosine in the PTX-injected oocytes were significantly different from those in the sham-injected oocytes (Figure 3a). These results indicate that the endogenous adenosine receptor opens GIRK channels *via* activation of $G_{i/o}$ proteins and is present on the oocyte membrane.

We next examined whether the receptor regulates intracellular cyclic AMP levels. In the defolliculated oocyte groups which showed the successful current responses to 3 μM adenosine in oocytes expressed with GIRK channels, CPA significantly reduced cyclic AMP accumulation stimulated by forskolin, which produced a modest increase in the cyclic AMP content by 32% (Student's *t*-test, $P<0.01$) in comparison with that of the untreated oocyte group (Figure 3b). Whereas CPA alone had no significant effect on cyclic AMP levels (Figure 3b). In contrast, the defolliculated oocyte groups which showed the unsuccessful current responses to adenosine in oocytes expressed with GIRK channels resulted in no significant change in cyclic AMP levels in any oocyte groups by addition of CPA (data not shown, five donors). The results suggest that the endogenous adenosine receptor inhibits adenylyl cyclase.

Discussion

In the present study we demonstrated that an endogenous *Xenopus* oocyte adenosine receptor on the oocyte membrane can activate GIRK channels expressed in oocytes *via* interaction with PTX-sensitive G proteins. Furthermore, the receptor inhibited forskolin-stimulated cyclic AMP accumulation. These results suggest the existence of a $G_{i/o}$ protein-coupled adenosine receptor in the oocyte.

Among the cloned adenosine receptor subtypes, the A_1 and A_3 adenosine receptors interact with $G_{i/o}$ proteins (Ralevic & Burnstock, 1998). We demonstrated that the adenosine receptor found here was sensitive to two A_1 adenosine receptor agonists with EC_{50} values of moderate nanomolar concentrations, whereas the receptor was insensitive to A_{2A} and A_3 adenosine receptor agonists tested (Figure 2c and Table 1). These results suggested that the receptor resembled an A_1 adenosine receptor. However, when compared with the cloned XA_1 receptor (Figure 2 and Table 1), the pharmacological characteristics of the adenosine receptor found here were distinct from those of the XA_1 receptor. Also, in the *Xenopus* oocyte system co-expressing the cloned rat A_1 adenosine receptor and GIRK channels, the receptor was fully activated by CPA and CGS21680 with EC_{50} values of 7 nM and 2.6 μM, respectively (Pfaff & Karschin, 1997). Therefore, our results suggest that the adenosine receptor found in this study is different from the four subtypes of cloned adenosine receptors reported to date.

In addition to the cloned adenosine receptors, physiological, pharmacological and biochemical studies have suggested the existence of additional adenosine receptors in the *Xenopus* oocyte and in several tissues in mammals. In *Xenopus* follicular oocytes (Lotan *et al*., 1982; 1985; Miledi & Woodward, 1989; Greenfield *et al*., 1990a, b) or defolliculated oocytes (Gelerstein *et al*., 1988), adenosine elevates cyclic AMP levels, and elicits cyclic AMP-mediated outward K^+ currents with an EC_{50} value of approximately 3 μM, indicating that the receptors for adenosine stimulate adenylyl cyclase *via* G_s proteins and resemble A_2 adenosine receptors. In addition, King *et al*. (1996) demonstrated that adenosine and ATP produced similar outward K^+ currents in follicular oocytes with distinct pharmacological characteristics. The characteristics exhibited that adenosine and ATP were equipotent with EC_{50} values of 1.9 and 1.7 μM, respectively, whereas ATP and CGS21680 were 0.5 and 0.7 times as efficient as adenosine, respectively. However, we demonstrated that the adenosine receptor in defolliculated oocytes was coupled to $G_{i/o}$ proteins and inhibited folskolin-stimulated cyclic AMP accumulation and that the receptor was activated by adenosine with an EC_{50} value of 917.3 nM but insensitive to ATP and CGS21680 at micromolar concentrations (Figures 1b, 2c and 3 and Table 1). Finidori *et al*. (1982) showed that adenosine inhibited adenylyl cyclase activity in the defolliculated oocyte membrane fraction, suggesting that the effect may be mediated by interaction with $G_{i/o}$ proteins. However, only high micromolar concentrations of adenosine had such effect. Therefore, our results suggest that the *Xenopus* adenosine receptor found here is different from the *Xenopus* adenosine receptors described previously.

In the rat brain membrane, [^{3}H]-CV 1808 binding study under anomalously low temperature suggested the existence of a novel high affinity component that could only partially be displaced by classical adenosine receptor ligands including CPA and a low affinity component with A_1-like adenosine receptor characteristics (Cornfield *et al*., 1992). Also, [^{3}H]-CV 1808 binding was not affected by 1 μM adenosine or GTP, and the IC_{50} values for NECA and DPCPX were greater than 10 μM. Later, Luthin & Linden (1995) demonstrated that receptors on membranes prepared from both rat brain and COS cells transfected with rat A_{2A} adenosine receptor took on either the unique binding characteristics of [^{3}H]-CV 1808 or the characteristics of A_{2A} adenosine receptor, depending on temperature of the binding assay and the nature of the radioligand. We demonstrated that the receptor found here was sensitive to adenosine, NECA and DPCPX at nanomolar concentrations, and also coupled to $G_{i/o}$ proteins, indicating that the characteristics of the receptor found here are distinct from those of [^{3}H]-CV 1808 binding sites. Like the receptor found here, studies on the inhibitory effect on noradrenaline release in sympathetic nerves of the rat caudal artery (Shinozuka *et al*., 1988) and rat vas deferens (Forsyth *et al*., 1991) and in the rabbit brain cortex (von Kügelgen *et al*., 1992) suggested the existence of putatively novel receptors activated by adenosine and ATP, and antagonized by xanthine derivatives. In the sympathetic nerves, adenosine and ATP were equipotent with EC_{50} values of approximately 10 μM, whereas in the brain, adenosine was more potent than ATP but both drugs were equally effective at 3 μM. In this study, adenosine was more potent than ATP and adenosine at 3 μM was much more efficacious than ATP, suggesting that the receptor found here is distinct from these receptors. In

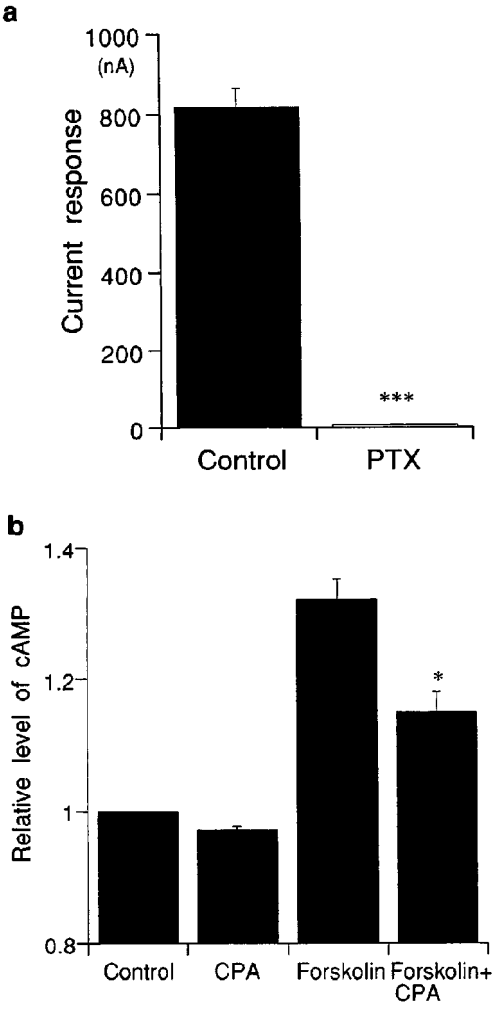

Figure 3 Interaction of an endogenous *Xenopus* oocyte adenosine receptor with $G_{i/o}$ proteins. (a) Effects of pertussis toxin (PTX) on adenosine-induced GIRK currents in oocytes expressing GIRK channels. The relationship of current responses to 3 μM adenosine (Ado) between the PTX-untreated group (817.9$\pm$48.0 nA; $n=14$; black bar) and PTX-treated group (8.46$\pm$2.65 nA; $n=22$; open bar). Current responses were measured at a membrane potential of -70 mV in a high-potassium solution containing 96 mM K^+. (b) Effects of CPA on forskolin-stimulated cyclic AMP accumulation in oocytes. Groups of 25 oocytes were exposed to either 1 μM CPA or 30 μM forskolin, or both drugs for 30 min. The results were obtained from four separate experiments. The basal cyclic AMP level in control group of untreated oocytes was 2389$\pm$54 fmol oocyte^{-1}. The ratios of cyclic AMP levels in the oocyte groups treated with CPA, forskolin or forskolin and CPA to the control group were 0.97$\pm$0.01, 1.32$\pm$0.03 and 1.15$\pm$0.03, respectively. There was significant difference in cyclic AMP contents between the group treated with forskolin and the group treated with forskolin and CPA. Statistical analysis of differences between groups was carried out using Student's *t*-test. Asterisks indicate significant differences between groups (*$P<0.05$; ***$P<0.001$).

addition, a study on relaxation of smooth muscle in the porcine coronary artery suggested the existence of another type of adenosine receptor (Abebe *et al*., 1994). However, the receptor was insensitive to xanthines and the receptor-mediated vasorelaxation was independent of the effects mediated by CPA and CHA. Therefore, we propose the existence of an endogenous *Xenopus* oocyte adenosine receptor with unique pharmacological characteristics distinct from those of known subtypes of adenosine receptors and other proposed adenosine receptors. The receptor may be considered an A_1-like adenosine receptor, although the nomenclature of the newly proposed receptors including the adenosine receptor found in this study remains tentative until identification of the adenosine receptors with the unique pharmacological characteristics.

The coronary vasodilatation mediated by 1 μM adenosine was attenuated by antidiabetic sulfonylureas (Daut *et al*., 1990) and 10 μM adenosine or 100 nM CHA activated the ATP-sensitive K^+ channels, which are blocked by sulfonylureas, *via* $G_i\alpha$ proteins in rat ventricular myocytes (Kirsch *et al*., 1990), but the pharmacological characteristics of adenosine receptor agonists were not reported in these studies. The results suggested that an adenosine receptor sensitive to an A_1 adenosine receptor agonist couples to the ATP-sensitive K^+ channels *via* G_i proteins. Further studies using cDNA clones for ATP-sensitive K^+ channels (Ho *et al*., 1993; Inagaki *et al*., 1995a,b) may clarify whether the adenosine receptor found here can activate the K^+ channels.

Endogenous *Xenopus* oocyte GIRK-related polypeptides (XIR) are also located on the oocyte membrane, although levels of XIR expression are low and variable among oocyte batches from different donors (Duprat *et al*., 1995; Hedin *et al*., 1996). As adenosine induced no significant current response in any of the naive defolliculated oocytes tested, the *Xenopus* adenosine receptor found here may hardly have XIR effect. However, the endogenous receptor was activated by adenosine at nanomolar concentrations, which are estimated to be in the physiological range of extracellular fluid (Fredholm, 1995). Therefore, other intracellular signal pathways *via* $G_{i/o}$ proteins may be involved in some effects in oocyte physiology.

Gelerstein *et al*. (1988) demonstrated in experiments using follicular oocytes and oocytes defolliculated by collagenase treatment and shaking periodically that 10 μM adenosine induced maturation, which was assessed by germinal vesicle breakdown, in stage VI oocytes and accelerated maturation of stage V and VI oocytes induced by approximately 3 μM progesterone. Also, adenosine increased cyclic AMP in defolliculated oocytes to the same extent as in follicular oocytes, suggesting interaction of an adenosine receptor located on the oocyte membrane with G_s proteins. In contrast, cyclic AMP is produced in follicle cells in response to NECA but no cyclic AMP accumulation can be detected in oocytes stripped by manual dissection after hypertonic treatment of collagenase-treated follicles, indicating that the receptor for adenosine appears to be located exclusively on follicle cells (Greenfield *et al*., 1990b). Despite localization of the endogenous G_s protein-coupled adenosine receptor, signal transduction by a $G_{i/o}$ protein-coupled adenosine receptor found here as well as that by the G_s protein-coupled adenosine receptor may have some effects on numerous biochemical events in oocyte maturation. Recently, Lutz *et al*. (2000) demonstrated that *Xenopus* oocyte maturation induced by 50 nM progesterone was inhibited by $G\beta_1\gamma_2$, which can directly activate GIRK channels (Reuveny *et al*., 1994). Activation of GIRK channels by the adenosine receptor found in this study may be mediated by action of $G\beta\gamma$ released from *Xenopus* $G_{i/o}$ proteins. The existence of the receptor may affect the signal transduction pathway induced by progesterone. Identification of the adenosine receptor

found here by molecular biological and genetic approaches may facilitate clarification of the functions and expression mechanisms of the receptor in *Xenopus* oocyte maturation.

In conclusion, we demonstrate that an endogenous *Xenopus* adenosine receptor on the oocyte membrane couples to GIRK channels and adenylyl cyclase *via* interaction with $G_{i/o}$ proteins. Moreover, the unique pharmacological characteristics of the receptor may suggest the existence of a putatively novel adenosine receptor. As the receptor was activated by nanomolar concentrations of adenosine, which is a normal constituent of extracellular fluid, $G_{i/o}$ protein signaling pathways *via* the adenosine receptor may have some effects in ovarian physiology.

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