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Pharmacological characterization of adenosine receptors in PGT-β mouse pineal gland tumour cells

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1 The adenosine receptor in mouse pinealocytes was identified and characterized using pharmacological and physiological approaches.

2 Expression of the two adenosine receptor subtypes A_{2B} and A_3 was detected in mouse pineal glands and PGT-β cells by polymerase chain reaction and nucleotide sequencing.

3 Adenosine and 5′-N-ethylcarboxamidoadenosine (NECA) evoked cyclic AMP generation but the A_{2A}-selective agonist 2-(4-(2-carboxyethyl)phenylethylamino)adenosine-5′-N-ethylcarboxamideadenosine (CGS 21680) and the A_1-specific agonists R-N^6-(2-phenylisopropyl)adenosine (R-PIA) and N^6-cyclopentyladenosine (CPA) had little effect on intracellular cyclic AMP levels. The A_{2B} receptor selective antagonists alloxazine and enprofylline completely blocked NECA-mediated cyclic AMP accumulation.

4 Treatment of cells with the A_3-selective agonist N^6-(3-iodobenzyl)-5′-(N-methylcarbamoyl)adenosine (IB-MECA) inhibited the elevation of the cyclic AMP level induced by NECA or isoproterenol in a concentration-dependent manner with maximal inhibition of 40–50%. These responses were blocked by the specific A_3 adenosine receptor antagonist MRS 1191. Pretreatment of the cells with pertussis toxin attenuated the IB-MECA-induced responses, suggesting that this effect occurred *via* the pertussis toxin-sensitive inhibitory G proteins.

5 IB-MECA also caused a concentration-dependent elevation in $[Ca^{2+}]_i$ and IP_3 content. Both the responses induced by IB-MECA were attenuated by treatment with U73122 or phorbol 12-myristate 13-acetate.

6 These data suggest the presence of both A_{2B} and A_3 adenosine receptors in mouse pineal tumour cells and that the A_{2B} receptor is positively coupled to adenylyl cyclase whereas the A_3 receptor is negatively coupled to adenylyl cyclase and also coupled to phospholipase C.
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Keywords: Mouse pineal gland; A_{2B} Adenosine receptor; A_3 receptor; adenylyl cyclase; phospholipase C; NECA; IB-MECA

Abbreviations: AB-MECA, 4-aminobenzyl-5′-N-methylcarboxamidoadenosine; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; 2-CADO, 2-chloroadenosine; CGS 21680, 2-(4-(2-carboxyethyl)phenylethylamino)adenosine-5′-N-ethylcarboxamideadenosine; CPA, N^6-cyclopentyladenosine; IB-MECA, N^6-(3-iodobenzyl)-5′-(N-methylcarbamoyl)adenosine; IP_3, inositol 1,4,5-trisphosphate; MRS 1191, 3-ethyl-5-benzyl-2-methyl-4-phenylethynyl-6-phenyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate; NAT, serotonin N-acetyltransferase; NECA, N-ethylcarboxamidoadenosine; PKC, protein kinase C; PLC, phospholipase C; R-PIA, R-N^6-(2-phenylisopropyl)adenosine; Ro 20-1724, 4-[(3-butoxy-4-methoxyphenyl)methyl]-2-imidazolidinone

Introduction

Adenosine receptors are found on many neuronal cells of the central and peripheral nervous system. They modulate the general synaptic transmission of neurotransmitters, including norepinephrine, *via* the regulation of synaptic ion channel activity (Haas & Selbach, 2000; Cunha, 2001). The adenosine receptors have been divided into four subtypes, A_1-, A_{2A}-, A_{2B}-, and A_3-receptors on the basis of differences in their affinities for selective ligands, their second-messenger responses, and in the amino acid sequences of the adenosine receptor proteins (Fredholm *et al*., 2000). The A_1- and A_3-adenosine receptors are negatively coupled to adenylyl cyclase and stimulate phospholipase C (PLC) activity, whereas A_{2A}- and A_{2B}-adenosine receptors are positively linked to adenylyl cyclase (Palmer & Stiles, 1995). However, whereas the A_{2A}-adenosine receptor is equally sensitive to the 5′-substituted compounds 2-(4-(2-carboxyethyl)phenylethylamino)adenosine-5′-N-ethylcarboxamide-adenosine (CGS 21680) and 5′-N-ethylcarboxamidoadenosine (NECA), the A_{2B}-adenosine receptor exhibits a much higher sensitivity to NECA than to CGS 21680 (Klotz, 2000). In contrast, the A_1-adenosine receptor exhibits a higher sensitivity to N^6-substituted adenosine analogues, such as R-N^6-(2-phenylisopropyl)adenosine (R-PIA), N^6-cyclopentyladenosine (CPA), and 2-chloro-N^6-cyclopentyladenosine (CCPA) (Klotz *et al*., 1998), whereas the A_3-adenosine receptor preferentially responds to N^6-(3-iodobenzyl)-5′-(N-methylcarbamoyl)adenosine (IB-

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MECA), 2-chloro-N^6-(3-iodobenzyl)-5′-(N-methylcarbamoyl)adenosine (CI-IB-MECA), and 4-aminobenzyl-5′-N-methylcarboxamidoadenosine (AB-MECA) (Jacobson *et al*., 1993; Olah *et al*., 1994; Gallo-Rodriguez *et al*., 1994). Recently, MRE 3008F20 and the isoquinoline analogue VUF5574 were indicated as new potent and selective A_3 adenosine receptor antagonists (Baraldi *et al*., 2000; van muijlwijk-Koezen *et al*., 2000). NECA has also been known to bind to A_3 receptors, but the affinity of NECA for the A_3 receptor is >100 fold lower than that for IB-MECA (Jacobson *et al*., 1995). It has been suggested that the A_1- and A_3-adenosine receptors modulate the spontaneous firing of ion channels and neurotransmitter release *via* a mechanism other than the inhibition of adenylyl cyclase (Ralevic & Burnstock, 1998; Haas & Selbach, 2000).

Several previous studies have addressed that the regulation of pineal functions depends on a variety of first messengers including the nucleoside adenosine (Ebadi & Govitrapong, 1986). Studies of rat pinealocytes have shown that NECA stimulates cyclic AMP accumulation and melatonin synthesis by acting on A_{2B}-adenosine receptors which have been linked to the activation of adenylyl cyclase (Nikodijevic & Klein, 1989; Babey *et al*., 1994). In cultured chicken pinealocytes, nonmetabolizable adenosine analogues exerted an inhibitory action on pineal melatonin production elicited by forskolin probably *via* the A_1 adenosine receptors (Falcon *et al*., 1988a). In addition, pharmacological profiles and molecular studies indicated that A_1- and A_3-adenosine receptors are found most prominently in sheep pineal membranes (Falcon *et al*., 1997; Linden *et al*., 1993). These studies indicate that the distribution of the adenosine receptor subtypes and the effect of the receptors on pineal function are species-specific. At present, the small size and the inefficiency of obtaining pure pinealocytes in primary culture make it difficult to study the mouse pineal gland. Recently, therefore, a transgenic mouse was established by targeted expression of the SV40 T-antigen directed to the pineal gland, and a clonal neuroendocrine pineal cell line, PGT-β, was developed from a pineal tumour (Son *et al*., 1996). Utilizing the pineal tumour cell line, we now attempted to characterize the adenosine receptors of the mouse pineal gland. In the present study, we also provide first evidence that an interaction between A_{2B} and A_3 adenosine receptors does occur during signal transduction.

Methods

Cell culture methods

The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (GIBCO BRL, Gaithersburg, MD, U.S.A.) supplemented with 10% (v v^{-1}) heat-inactivated bovine calf serum (Hyclone, Logan, UT, U.S.A.) and 1% (v v^{-1}) antibiotics containing 5000 units ml^{-1} penicillin G (sodium) and 5000 μg ml^{-1} streptomycin sulphate in 0.85% saline buffer (GIBCO BRL), pH 7.4. Cell cultures were maintained in a humidified atmosphere of 5% CO_2 at 37°C. Cells grown to confluence were removed from the dishes after a 5 min incubation with 0.25% (w v^{-1}) trypsin containing 1 mM EDTA (GIBCO BRL). They were subcultured about twice weekly.

Reverse transcriptase-polymerase chain reaction (RT–PCR)

Total RNA was extracted from PGT-β cells and mouse pineal glands (10 mice, CBA/J) by the acid guanidinium thiocyanate-phenol-chloroform (Chomczynski, 1993). Total RNA concentrations were approximately quantitated by spectrophotometry. One microgram of total RNA was added to 0.5 μg oligo(dT) in DEPC-treated water and incubated at 70°C for 5 min and 4°C for 5 min. A total of 1 mM of all four deoxynucleotides (dNTPs), 5 μl 5X reverse transcriptase (RT) buffer, and 200 units of superscript II reverse transcriptase (GIBCO BRL) were added and the reactions incubated at 42°C for 1 h followed by 10 min at 75°C and stored at 4°C. For PCR amplification, an aliquot of the cDNA synthesis reaction was added to a reaction buffer containing 1 mM dNTPs, 1 mM of oligonucleotide primers, and 2 units of *Taq* DNA polymerase. Forty amplification cycles were conducted as follows: denaturation at 95°C for 1 min, annealing at a temperature specific for each set of primers for 1 min, and extension at 72°C for 1 min in a Minicycler (MJ Research, Watertown, MA, U.S.A.). The resultant amplification products were analysed by gel electrophoresis in a 1.0% agarose gel stained with ethidium bromide.

The following sequence of oligonucleotide primers were used for the amplification of each mouse adenosine receptor: the sense primer 5′-aaatgtactggtgatttggg-3′ (bases 385–404) and the antisense primer 5′-tgatgcagttcaagatgtgt-3′ (bases 1057–1076) for A_1; the sense primer 5′-tattgccatcgacagatata-3′ (bases 521–540) and the antisense primer 5′-aagggaaaactctgaagac-3′ (bases 1442–1461) for A_{2A}; the sense primer 5′-agctccatctttagcctctt-3′ (bases 304–323) and the antisense primer 5′-gtcataagcccagactgaga-3′ (bases 1014–1033) for A_{2B}; the sense primer 5′-ctgtttgcctggggaagtaag-3′ (bases 13–33) and the antisense primer 5′-gagtttgtttcggatgatgt-3′ (bases 923–942) for A_3. Amplified PCR products were sequenced according to the enzymatic method of Sanger *et al*. (1992).

Measurement of cyclic AMP generation

Intracellular cyclic AMP generation was determined by [^{3}H]-cyclic AMP competition assay for binding to cyclic AMP binding protein as previously described (Park *et al*., 1997) with some modification. To determine the cyclic AMP production induced by adenosine or its analogues, the pineal gland tumour cells were detached by trypsin treatment and aliquoted by 5×10^5 cells per Eppendorf tube. The cells were stimulated with agonists for 20 min in the presence or absence of the phosphodiesterase inhibitor Ro 20–1724 (50 μM). The reaction was then quickly terminated by three repeated cycles of freezing and thawing. The samples were centrifuged at $2500\times g$ for 5 min at 4°C. The cyclic AMP assay is based on the competition between [^{3}H]-labelled cyclic AMP and unlabelled cyclic AMP present in the sample for binding to a crude cyclic AMP-binding protein prepared from bovine adrenal cortex following the method of Brown *et al*. (1971). Each sample was incubated with 50 μl [^{3}H]-labelled cyclic AMP (5 μCi) and 100 μl binding protein for 2 h at 4°C. Separation of the protein-bound cyclic AMP from the unbound cyclic AMP was achieved by adsorption of the free

cyclic AMP onto charcoal (100 μl) followed by centrifugation at 12,000 $\times g$ at 4°C. The 200 μl of supernatant was then placed into an Eppendorf tube containing 1.2 ml scintillation cocktail to measure the radioactivity. The cyclic AMP concentration in the sample was determined based on a standard curve and expressed as pmol number of cells^{-1}.

Measurement of IP_3

IP_3 concentration in the cells was determined by [^{3}H]-IP_3 competition assay in binding to IP_3 binding protein (Suh *et al*., 1995). The PGT-β cells were stimulated with agonists and the reaction was terminated by aspirating the medium off the cells followed by addition of 0.3 ml ice-cold 15% (w v^{-1}) trichloroacetic acid containing 10 mM EGTA. The samples were centrifuged at 5000 $\times g$ for 10 min at 4°C. The trichloroacetic acid in the extract was removed by four extractions with diethyl ether. Finally the extract was neutralized with 200 mM Trizma base and its pH adjusted to about 7.4. 20 μl of the cell extract was added to 20 μl of assay buffer (0.1 M tris(hydroxymethyl)aminomethane buffer containing 4 mM EDTA and 4 mg ml^{-1} bovine serum albumin) and 20 μl of [^{3}H]-IP_3 (0.1 μCi ml^{-1}). Then 20 μl of solution containing the binding protein was added and the mixture incubated for 15 min on ice and centrifuged at 2000 $\times g$ for 5 min. The pellet was resuspended in 100 μl of water, and 1 ml of scintillation cocktail was added to measure the radioactivity. IP_3 concentration in the sample was determined based on a standard curve and expressed as pmol μg protein^{-1}. The IP_3 binding protein was prepared from bovine adrenal cortex according to the method of Challiss *et al*. (1990).

Determination of intracellular Ca^{2+} level

The level of intracellular Ca^{2+} was measured using the fluorescent Ca^{2+} indicator fura-2/AM as previously described (Suh *et al*., 1997). Briefly, PGT-β cells were grown to confluency in 75-cm^2 polystyrene dishes and then loaded with fura-2/AM to a final concentration of 3 μM in complete medium at 37°C for 50 min. After the loading, the cells were detached by trypsin treatment and washed twice with Locke's solution (mM: NaCl 154, KCl 5.6, $MgCl_2$ 1.2, $CaCl_2$ 2.2, HEPES 5.0, and glucose 10, pH 7.4) to remove extracellular dye. Sulfinpyrazone which is known to inhibit the dye leakage by blocking the organic-anion transport systems (Di Virgilio *et al*., 1988) was added to all solutions to the final concentration of 250 μM. About 5×10^5 cells of the cell suspension were then transferred to a quartz cuvette and placed in a thermostatically controlled cell holder at 37°C, and the cell suspension was continuously stirred. Changes in fluorescence ratios were measured at the dual excitation wavelengths of 340 and 380 nm and the emission wavelength of 500 nm by an alternative wavelength time scanning method. Calibration of the fluorescence signal in term of $[Ca^{2+}]_i$ was performed according to Grynkiewicz *et al*. (1985).

Analysis of data

All quantitative data are expressed as mean $\pm$ s.e.mean. Comparison between two groups was analysed using Student's unpaired *t*-test. Differences were considered to be significant when the degree of confidence in the significance was 95% or better ($P < 0.05$).

Materials

ATP, ADPβS, forskolin, enprofylline, ethidium bromide, pertussis toxin, phorbol ester, isoproterenol, and EGTA were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). NECA, 2-chloroadenosine, adenosine, AB-MECA, CGS 21680, R-PIA, CPA, IB-MECA, alloxazine, U73122, 3-ethyl-5-benzyl-2-methyl-4-phenylethynyl-6-phenyl-1,4-($\pm$)-dihydropyridine-3,5-dicarboxylate (MRS 1191), and 4-[(3-butoxy-4-methoxyphenyl)methyl]-2-imidazolidinone (Ro 20-1724) were purchased from Research Biochemicals Inc. (Natick, MA, U.S.A.). [^{3}H]-IP_3 and [^{3}H]-adenosine from DuPont NEN Research Products (Boston, MA, U.S.A.). Fura-2 pentaacetoxymethyl ester (fura-2/AM) was obtained from Molecular Probes (Eugene, OR, U.S.A). Taq DNA polymerase and other restriction enzymes were obtained from Promega (Madison, WI, U.S.A.).

Results

Expression of A_{2B} and A_3 adenosine receptors in the mouse pineal gland

In order to investigate which subtype of adenosine receptor was expressed on mouse pinealocytes, we used RT–PCR analysis of total RNA prepared from mouse pineal glands (10 mice, CBA/J) and PGT-β cells. We used primers designed to specifically amplify a fragment of the mouse adenosine receptor cDNAs. As shown in Figure 1, the amplified products were of the expected sizes for A_{2B} (730 bp) and A_3 (930 bp). Also, no cDNA of A_1 and A_{2A} receptors was amplified from the above RNAs (data not shown), while the signal for α-tubulin is commonly detected. RT–PCR analysis of total RNA prepared from PGT-β cells further demonstrated the expression of mRNA for A_{2B} and A_3 (Figure 1). However, cDNA of A_1 and A_{2A} receptors was not detected in the PGT-β cells (data not shown). In contrast, A_{2B} receptors, but not A_1, A_{2A} and A_3, were selectively detected in total RNA prepared from mouse NIH-3T3 fibroblasts, which is consistent with the previous reports (Brackett & Daly, 1994).

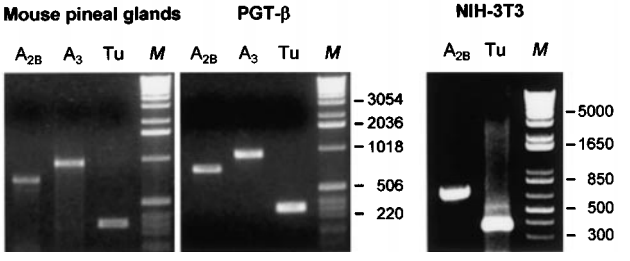

Figure 1 Expression of A_{2B} and A_3 mRNAs in mouse pineal glands and PGT-β cells. Total RNA was extracted for RT–PCR analysis as described under Methods. Both of the transcripts, A_{2B} (730 bp) and A_3 (930 bp), were detected in mouse pineal glands and PGT-β cells, whereas A_{2B} was selectively detected in NIH-3T3 mouse fibroblasts. α-tubulin (Tu) was used as the loading control. Molecular weight markers are in lane *M*. Typical results obtained in more than three separate experiments are shown.

Nucleotide sequence analysis confirmed that the amplified DNA products of pinealocytes were authentic mouse A_{2B} and A_3 adenosine receptors. The results, therefore, suggest that two subtypes of adenosine receptor, A_{2B} and A_3, are specifically co-expressed on mouse pinealocytes and PGT-β cells.

A_{2B} adenosine receptor-mediated adenylyl cyclase activation

Stimulation of the PGT-β cells with adenosine evoked concentration-dependent cyclic AMP generation, the half-maximal effective concentration (EC_{50}) of adenosine being $\sim$87 μM (Figure 2A). Figure 2B shows the time course of cyclic AMP generation induced by 300 μM adenosine, a submaximal effective concentration of the agonist. The peak level of cyclic AMP generation was obtained about 20 min after stimulation, which was then followed by a slow decline to basal levels. In the presence of the phosphodiesterase inhibitor Ro 20-1724 (50 μM), the accumulation of cyclic AMP induced by adenosine was clearly detectable within 1 min and was saturated after about 10 min. Figure 2C shows that treatment of PGT-β cells with the general A_2-adenosine receptor agonist NECA produced a larger maximal cyclic AMP generation, whereas the specific A_{2A}-adenosine receptor agonist CGS 21680 had little effect. The non-hydrolyzable adenosine analogue 2-chloroadenosine (2-CADO) also elevated the cyclic AMP level in a concentration-dependent manner. The EC_{50} for NECA was 3.2 ± 0.5 μM, which was about 10 fold the potency of 2-CADO (29.6 ± 3.1 μM). The selective A_3-adenosine receptor agonists IB-MECA and AB-MECA also continuously increased the cyclic AMP level at concentrations of up to 300 μM, which may be due to their ability to bind to multiple types of adenosine receptors at higher concentrations (Shearman & Weaver, 1997). However, addition of the selective A_1-adenosine receptor agonists R-PIA and CPA had little effect on the cyclic AMP level. Figure 2D shows that addition of the specific A_{2B}-adenosine receptor antagonists alloxazine (Brackett & Daly, 1994) and enprofylline (Auchampach *et al.*, 1997) inhibited the adenosine-induced cyclic AMP generation in a concentration-dependent manner. The half effective inhibitory concentrations (IC_{50}) for alloxazine and enprofylline were 2.9 ± 0.4 and 33.2 ± 4.3 μM, respectively. Treatment of the cells with maximal effective concentrations of alloxazine (30 μM) and enprofylline (300 μM) almost completely inhibited the adenosine-mediated cyclic AMP accumulation (data not shown). These data, therefore, indicate that the effect of adenosine on cyclic AMP generation in these cells occurs primarily through A_{2B}-adenosine receptors.

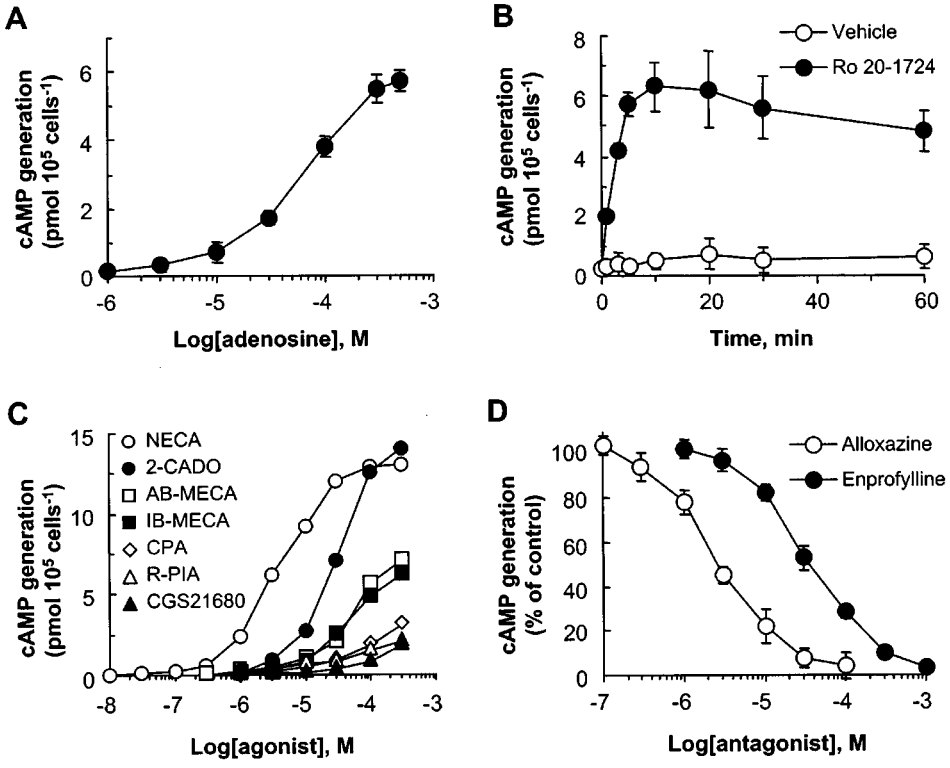

Figure 2 Adenosine-induced cyclic AMP production in PGT-β cells. (A) Cells pretreated with Ro 20-1724 (50 μM) for 15 min were stimulated with various concentrations of adenosine for 20 min. (B) Cells were stimulated with 300 μM adenosine for the indicated lengths of time (0, 1, 3, 5, 10, 20, 30, and 60 min) in the absence or presence of Ro 20-1724. (C) Concentration-dependence curves for cyclic AMP generation evoked by adenosine analogues. Cells were stimulated with various concentrations of the analogues for 20 min in the presence of Ro 20-1724. (D) Interference of adenosine receptor antagonists with adenosine-stimulated cyclic AMP production. Cells were stimulated with 300 μM adenosine in the presence or absence of various concentrations of antagonists for 20 min. Net increase in cyclic AMP production is expressed as percentage of the level obtained upon treatment with adenosine alone. The cyclic AMP levels were measured as described in the Methods, and each point is the mean ($\pm$s.e.mean) of three independent experiments.

To determine the mode of activation of adenylyl cyclase coupled to the A_{2B}-adenosine receptor in comparison to other activating signals, the effects of co-treatments with NECA, isoproterenol, and forskolin were examined. Since we had demonstrated the presence of the β_2-adrenergic receptors on PGT-β cells (Suh *et al*., 1999), we looked for interaction between the signallings of the β_2-receptor and the adenosine receptor. Figure 3A shows that the addition of 5 μM forskolin led to a huge synergistic augmentation of the effect of NECA and isoproterenol on cyclic AMP production, suggesting that the PGT-β cells contained adenylyl cyclase II or IV, which are enzymes that are synergistically activated when they interact with $G_{s\alpha}$ and forskolin (Feinstein *et al*., 1991; Tang & Gilman, 1991). In contrast, co-treatment with the maximal effective concentration of NECA (100 μM) and various concentrations of isoproterenol in the presence of 50 μM MRS 1191, which prevents the involvement of inhibitory A_3 receptor activation, resulted in additively increased cyclic AMP production (Figure 3B). The results thus indicated that the cyclic AMP accumulation stimulated by a $G_{s\alpha}$-coupled receptor is additively stimulated by other G_s protein-mediated signalling.

Inhibition of A_{2B} receptor-mediated cyclic AMP generation by A_3 receptor activation via *pertussis toxin-sensitive G proteins*

Since the A_3-adenosine receptors are negatively coupled to adenylyl cyclase, we investigated the effect of the selective A_3 receptor agonist IB-MECA on the cyclic AMP generation mediated by G_s protein-coupled receptors. Figure 4A shows that treatment of the cells with IB-MECA decreased the NECA-stimulated cyclic AMP production with maximal inhibition of 40–50% obtained at 1 μM IB-MECA. However, this inhibitory effect of IB-MECA was antagonized by addition of the A_3 selective antagonist MRS 1191, indicating involvement of an adenosine A_3 receptor. IB-MECA also

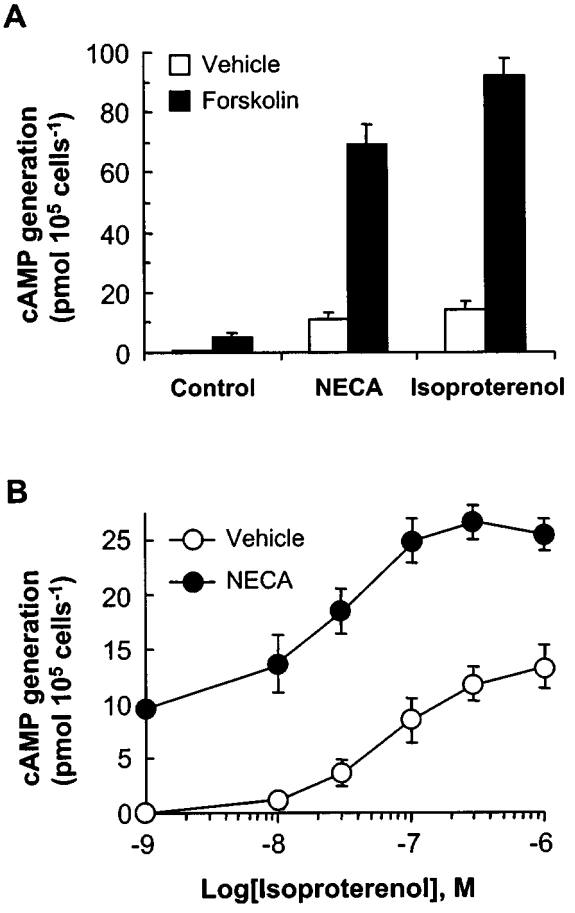

Figure 3 Enhancement of NECA-stimulated cyclic AMP production by forskolin. (A) Effect of forskolin on the NECA- and the isoproterenol-stimulated cyclic AMP production. PGT-β cells were stimulated with NECA (100 μM) or isoproterenol (1 μM) in the presence or absence of forskolin (5 μM) for 20 min after treatment with 50 μM Ro 20-1724, and the cyclic AMP generation was measured as described in the Methods. (B) Additive generation of cyclic AMP by co-treatment of the cells with NECA and isoproterenol. The cells were stimulated with various concentrations of isoproterenol with or without NECA (100 μM) in the presence of 50 μM MRS 1191 for 20 min. The experiments were performed three times and each point is the mean ± s.e.mean.

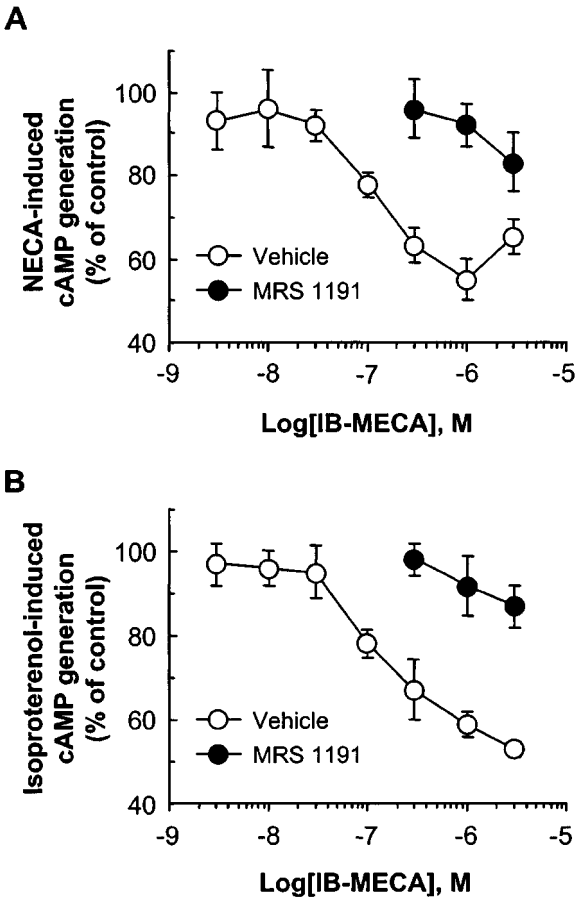

Figure 4 Effect of IB-MECA on NECA- and isoproterenol-stimulated cyclic AMP generation. Cells preincubated with or without 50 μM MRS 1191 for 10 min were stimulated with various concentrations of IB-MECA and 10 μM NECA (A) or 300 nM isoproterenol (B) for 20 min. The cyclic AMP levels were measured as described in the Methods. The net increase in cyclic AMP production is expressed as percentage of the level obtained upon treatment with NECA or isoproterenol alone. Data are the mean ± s.e.mean (bars) of triple experiments.

inhibited isoproterenol-stimulated cyclic AMP generation in a concentration-dependent manner, which could be prevented by addition of MRS 1191 (Figure 4B).

In order to elucidate the role of G protein in the IB-MECA-induced inhibition of cyclic AMP generation, we performed experiments with pertussis toxin which selectively uncouples G_i/G_o proteins from receptors by catalyzing the ADP-ribosylation of the α_i/α_o subunits (Simon *et al*., 1991). As shown in Table 1, the IB-MECA-mediated inhibitory effect was almost completely reversed by a 12-h pretreatment of the cells with pertussis toxin (300 ng ml^{-1}). These data indicate that the A_3 receptors are coupled to adenylyl cyclase through pertussis toxin-sensitive inhibitory G proteins and thus inhibits the G_s-coupled receptor-mediated adenylyl cyclase activation.

A_3 adenosine receptor-mediated PLC activation

The effect of IB-MECA on PLC activation was studied in PGT-β cells. Figure 5A shows that IB-MECA in the presence of 2.2 mM $CaCl_2$ produced a rise in the $[Ca^{2+}]_i$. The $[Ca^{2+}]_i$ peaked within 30 s after stimulation and was followed by a sustained increase above the basal level for more than 3 min. In the absence of extracellular Ca^{2+} (dotted trace), Ca^{2+} mobilization from the intracellular Ca^{2+} stores occurred. The results indicate that the increase in the $[Ca^{2+}]_i$ was caused not only by mobilization of Ca^{2+} from the intracellular stores but also by influx of Ca^{2+} from the extracellular medium. IB-MECA increased the $[Ca^{2+}]_i$ in a concentration-dependent manner (Figure 5B). Although adenosine and NECA also tend to induce an increase in $[Ca^{2+}]_i$ at high concentrations ($\geqslant$100 μM), the intensity of the elevation of the $[Ca^{2+}]_i$ was much below that of IB-MECA (Figure 5A). R-PIA and CPA at concentration of 100 μM had no effect on the $[Ca^{2+}]_i$ rise (data not shown).

To test for phospholipase C activation upon A_3 adenosine receptor stimulation, we treated the cells with IB-MECA and examined phosphoinositide turnover. The time course of the IP_3 formation in the cells in response to IB-MECA is shown in Figure 6A. Addition of IB-MECA to the cells evoked a rapid increase in the level of IP_3 which reached a peak $\sim$30 s after administration of the agonist. The level of IP_3 then slowly declined over time returning to the basal level 10 min after the IB-MECA addition. Figure 6B illustrates the IB-MECA concentration-response curve for IP_3 in terms of peak IP_3 generation time (30 s). In these cells, IB-MECA induced concentration-related (10^{-6} to 10^{-4} M) increases in IP_3 formation. This is consistent with the concentration-dependent Ca^{2+} response. However, NECA treatment had little effect on the IP_3 generation. Preincubation of the cells with the selective A_3 adenosine receptor antagonist MRS 1191 abolished the formation of IP_3 induced by IB-MECA. Figure 6C shows the concentration-dependent inhibition by MRS 1191 of the IB-MECA-induced IP_3 generation, indicating that the IB-MECA-induced responses were mediated through A_3 adenosine receptors.

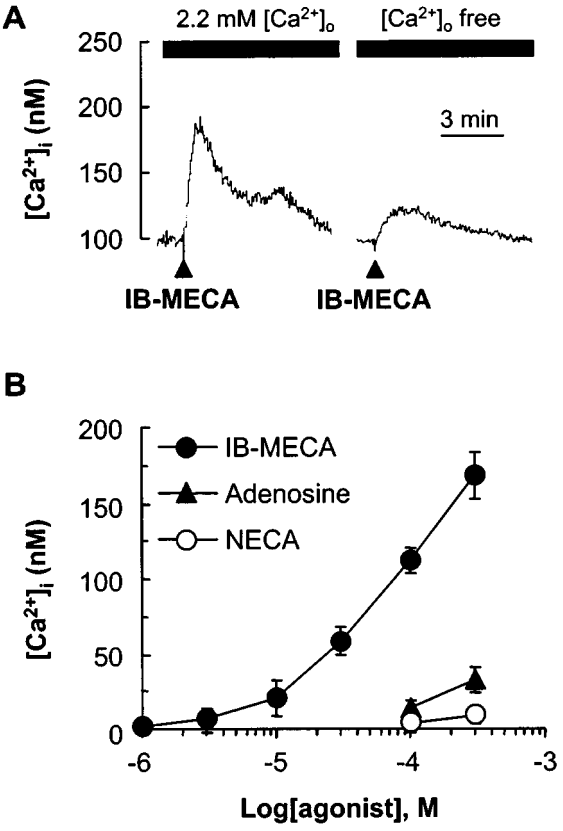

Figure 5 Effects of IB-MECA on the increase in $[Ca^{2+}]_i$ in PGT-β cells. (A) Typical pattern of $[Ca^{2+}]_i$ rise after treatment with IB-MECA (50 μM) in the presence and absence of 2.2 mM extracellular Ca^{2+}. In Ca^{2+}-free experiments, the Locke's solution did not contain Ca^{2+} but rather 200 μM EGTA. Cells were washed with Ca^{2+}-free buffer twice and incubated in Ca^{2+}-free solution for 3 min before stimulation with the agonist. (B) Concentration-dependent effect of adenosine analogues on $[Ca^{2+}]_i$ rise. Fura-2-loaded cells were treated with various concentrations of each nucleotide, and net increases in $[Ca^{2+}]_i$ were measured. Each concentration of IB-MECA was tested four times independently, and the data are mean values$\pm$s.e.mean (bars).

Table 1 Effects of pertussis toxin on IB-MECA-induced inhibition of cyclic AMP production in mouse pineal gland tumour cells

	Cyclic AMP production (pmol 10^5 cells^{-1})			
	Vehicle		*Pertussis toxin-treated*	
Treatment	*Control*	*IB-MECA*	*Control*	*IB-MECA*
Basal	0.12$\pm$0.02	0.13$\pm$0.01	0.16$\pm$0.02	0.18$\pm$0.02
NECA	12.4$\pm$0.07	6.32$\pm$0.05*	13.7$\pm$0.17	12.9$\pm$0.11
Isoproterenol	14.2$\pm$0.13	8.66$\pm$0.08*	15.9$\pm$0.13	13.5$\pm$0.16

Confluent cells were pretreated with 300 ng ml^{-1} pertussis toxin or vehicle for 12 h, and the effect of IB-MECA on cyclic AMP production induced by NECA or isoproterenol were measured as described in the Methods. The cells were stimulated with isoproterenol (300 nM) or NECA (100 μM) in the presence of vehicle (control) or IB-MECA (1 μM) for 20 min. Data are mean$\pm$s.e.mean of three independent experiments. *$P<0.01$, compared to control.

A 5 min pretreatment of the cells with the novel aminosteroid U73122, reportedly a selective inhibitor of phosphatidylinositol-specific PLC, abolished the IB-MECA-mediated $[Ca^{2+}]_i$ rise in a concentration-dependent manner with an IC_{50} of 2.7 ± 0.4 μM (Figure 7A). In addition, the pretreatment with U73122 (5 μM) also decreased the IP_3 formation elicited by IB-MECA by $\sim$60% (Figure 7B). However, U73122 did not have a significant effect on the cyclic AMP production resulting from treatment with IB-MECA or NECA (data not shown). These results indicate that the IB-MECA-mediated $[Ca^{2+}]_i$ rise and IP_3 generation resulted from A_3 receptor-coupled PLC activation.

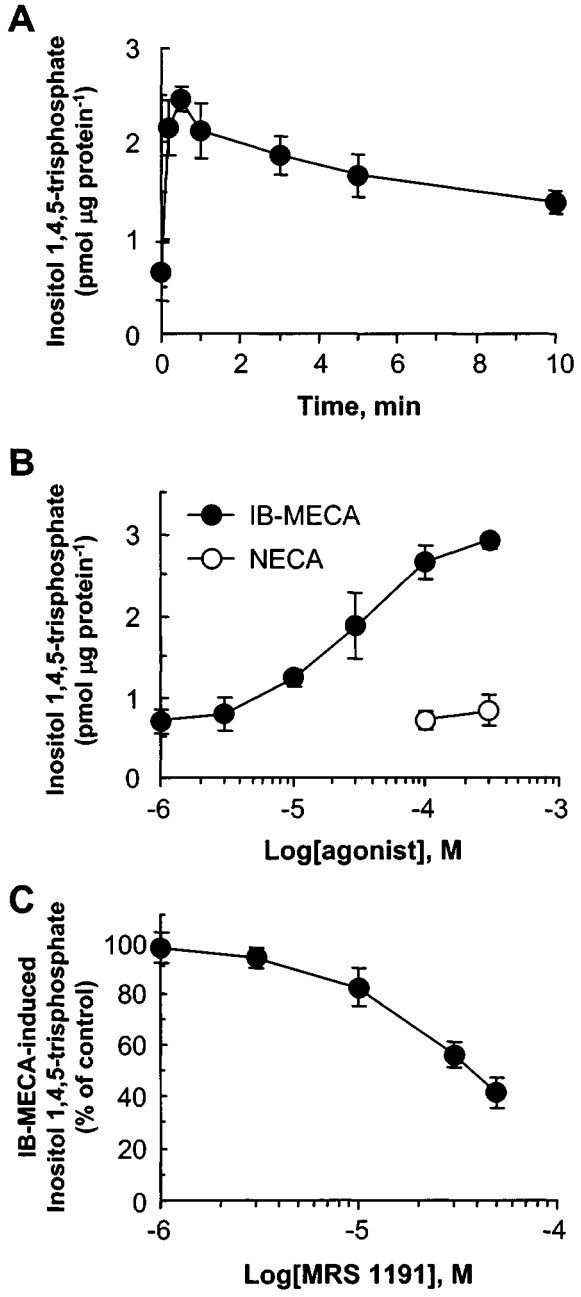

Figure 6 Effect of IB-MECA on IP_3 production. (A) Time course of IP_3 generation stimulated by 50 μM IB-MECA. The cells were treated for the designated time (0, 0.25, 0.5, 1, 3, 5, or 10 min), and the reactions were stopped by addition of 15% (w v^{-1}) TCA containing 10 mM EGTA. (B) Concentration-dependent stimulation of IP_3 formation. The cells were treated with various concentrations of IB-MECA or NECA for 30 s, and the IP_3 production was measured by competition assay as described in the Methods. (C) Concentration-dependent inhibition of IB-MECA-stimulated IP_3 generation by the A_3 selective antagonist MRS 1191. The net increase in IP_3 generation is expressed as percentage of the level obtained after treatment with 50 μM IB-MECA alone. Data are mean$\pm$s.e.mean (bars) values from three experiments.

Pertussis toxin inhibits the IB-MECA-mediated PLC activation

In order to elucidate the mechanism of the IB-MECA-induced PLC activation, we performed experiments with pertussis toxin. Table 2 shows that the IB-MECA-mediated $[Ca^{2+}]_i$ rise was partially inhibited by a 12-h pretreatment of the cells with pertussis toxin (300 ng ml^{-1}). In parallel, pertussis toxin also reduced the IB-MECA-induced IP_3 generation 35–45%. However, the ADPβS-induced $[Ca^{2+}]_i$ rise and IP_3 generation remained unaffected (data not

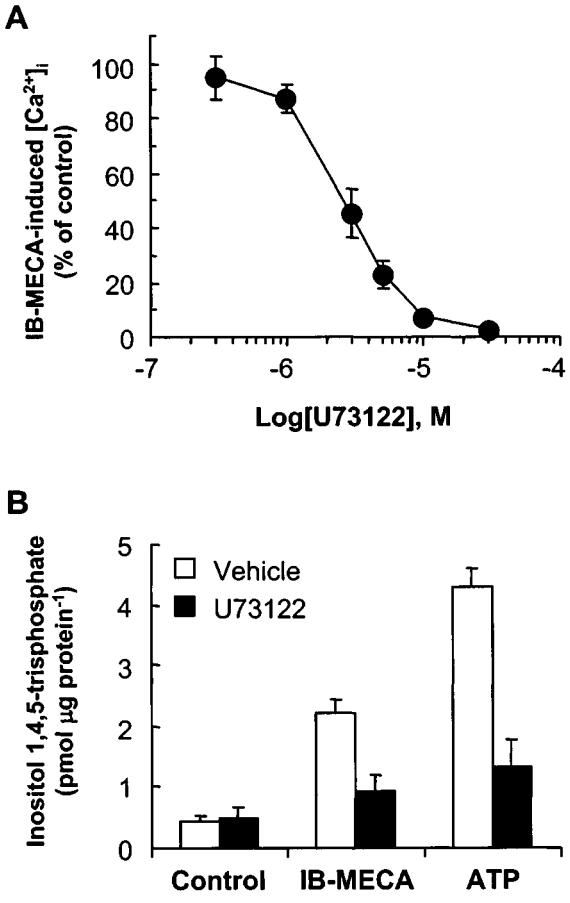

Figure 7 Effect of U73122 on IB-MECA-stimulated $[Ca^{2+}]_i$ rise and IP_3 generation. (A) Cells pretreated with various concentrations of U73122 for 5 min were stimulated with 50 μM IB-MECA. The $[Ca^{2+}]_i$ increase is expressed as percentage of the IB-MECA response in the absence of U73122. (B) The cells pretreated with vehicle or U73122 for 5 min were stimulated with IB-MECA or ATP for 30 s, and the reactions were stopped by addition of 15% (w v^{-1}) TCA containing 10 mM EGTA. The IP_3 generation was measured by competition assay as described in the Methods. Data are mean$\pm$s.e.mean (bars) values from three experiments.

shown), as previously described (Suh *et al*., 1997). The pertussis toxin effect on the IB-MECA-mediated responses indicates that the A_3 receptors are coupled to PLC, at least in part, through pertussis toxin-sensitive G proteins.

Effect of phorbol 12-myristate 13-acetate on IB-MECA-mediated $[Ca^{2+}]_i$ rise and IP_3 generation

The regulation of the IB-MECA-mediated signal transduction by protein kinase C (PKC) was investigated by treating the cells with the phorbol ester PMA. Figure 8A shows that pretreatment of the cells with PMA inhibited the subsequent IB-MECA-elicited $[Ca^{2+}]_i$ elevation. The inhibitory effect of PMA on IB-MECA-induced $[Ca^{2+}]_i$ rise was concentration-dependent and 300 nM PMA inhibited the IB-MECA response maximally by 85–90%, whereas 300 nM PMA inhibited the ATP-induced response by 25–30% (Figure 8B). The results suggest that the signal transduction pathways between the receptors and PLC are negatively regulated by PKC and that the A_3 receptor-mediated signalling is more sensitive to PKC regulation than that of the P2 purinergic receptor. In contrast, PMA treatment slightly increased the forskolin- and NECA-stimulated cyclic AMP accumulation (Figure 8C), indicating that the A_{2B} receptor-mediated signalling is not sensitive to PKC activation.

Discussion

In the present study, we illustrated the presence of both A_{2B} and A_3 adenosine receptors on mouse pineal gland tumour cells and that A_3 receptors have dual coupling to PLC and adenylyl cyclase which is inhibited through pertussis toxin-sensitive G proteins. Our conclusion is based on several lines of evidence. First, RT–PCR and nucleotide sequencing analysis showed that A_{2B} and A_3 receptors were co-expressed on the cells. Second, the general A_2-adenosine receptor agonist NECA significantly raised cyclic AMP production, while the specific A_{2A}-adenosine receptor agonist CGS 21680 had little effect on the cyclic AMP level. In addition, NECA was more effective in stimulating cyclic AMP production than any of the other adenosine analogues, the difference being more than 2-orders of magnitude large. The A_{2B}-selective antagonists alloxazine and enprofylline profoundly inhibited cyclic AMP production induced by adenosine or NECA. The results are consistent with the properties of the A_{2B}-adenosine receptors (Gharib *et al*., 1992), indicating that the A_{2B} receptors are involved in adenosine-mediated cyclic AMP accumulation in the cells. Third, the A_3 selective agonist IB-MECA caused a 40–50% inhibition of isoproterenol- and NECA-stimulated cyclic AMP accumulation. However, pertussis toxin treatment reversed the inhibitory effects elicited by IB-MECA, suggesting that IB-MECA-responsive A_3 receptors are coupled to adenylyl cyclase *via* the pertussis toxin-sensitive G_i/G_o proteins. Fourth, IB-MECA induced Ca^{2+} release from the intracellular Ca^{2+} pools followed by sustained Ca^{2+} influx, which is typical for PLC-coupled receptor-mediated responses. The agonist also induced a concentration-dependent elevation of IP_3 contents in the cells. The IB-MECA-induced IP_3 production and $[Ca^{2+}]_i$ rise was, however, selectively inhibited by the commonly used A_3 receptor antagonist MRS 1191 (Jacobson *et al*., 1997) and the PLC inhibitor U-73122. The result suggests that both of these responses elicited by IB-MECA are mediated *via* PLC-linked A_3 receptors. Overall, the results suggest the involvement of A_3 receptors in the negative regulation of the A_{2B} receptor-mediated adenylyl cyclase activation through G_i proteins in PGT-β cells.

Multiple adenosine receptor coexistence has been identified in many cell types (Ralevic & Burnstock, 1998). These include A_{2A}-, A_{2B}-, and A_3 receptors on mast cells, A_{2A} and A_{2B} receptors on endothelial cells and PC12 cells. The functional significance of this is not entirely clear, but our results in terms of cross-talk between A_{2B} and A_3 receptors suggest an agonistic interplay between two separate signalling pathways. The results of our experiments revealed that stimulation of the adenosine A_3 receptors resulted in a concentration-dependent inhibition of cyclic AMP production induced by NECA and isoproterenol with maximal inhibition of 40–50% at $\sim$1 μM IB-MECA. However, the EC_{50} (>1 μM) and the maximal effective value (>50 μM) of IB-MECA for IP_3 generation and $[Ca^{2+}]_i$ rise were 10 fold higher than those causing inhibition of cyclic AMP production. In addition, pertussis toxin treatment blocked the inhibitory effect of IB-MECA on cyclic AMP production and the stimulatory effect of IB-MECA on PLC activation (Tables 1 and 2). These observations strongly suggest that the A_3 receptor activation resulted in dual effects: a $G_{i\alpha}$-mediated process inhibits adenylyl cyclase and a $G_{\beta\gamma}$-mediated process activates PLC. Indeed, the affinity of $\beta\gamma$ subunits for their target enzyme is much lower (10–100 fold) in comparison to α subunits (Birnbaumer, 1992; Park *et al*., 1993; Sternweiss, 1994). Thus, the coupling of the adenosine A_3 receptor to adenylyl cyclase through G_i protein is more efficient than its coupling to PLCβ and more $\beta\gamma$ subunits are needed to activate PLCβ than α subunits to inhibit adenylyl cyclase. This implies that sufficient $G_{i/o}$ protein must be available to release $\beta\gamma$ subunits to obtain a substantial PLCβ response.

The inhibitory adenosine receptors have been shown to desensitize with different time courses according to a subtype-specific manner. Desensitization of the A_1 adenosine receptor typically occurs over the period of several hours, or even days, and is temporally associated with receptor down-regulation (Ramkumar *et al*., 1991). In contrast, the native A_3 adenosine receptor undergoes a rapid functional desensitization detectable within a few minutes of agonist exposure

Table 2 Effects of pertussis toxin on IB-MECA-stimulated $[Ca^{2+}]_i$ rise and IP_3 production in mouse pineal gland tumour cells

	IB-MECA-mediated responses	
	Net increase in $[Ca^{2+}]_i$	*Net in crease in IP_3*
Pretreatment	(nM)	(pmol μg protein^{-1})
Control	112±14	1.84±0.11
Pertussis toxin	74±9*	1.13±0.08*

PGT-β cells were pretreated with 300 ng ml^{-1} pertussis toxin or vehicle (control) for 12 h, and the $[Ca^{2+}]_i$ rise and IP_3 generation induced by 50 μM IB-MECA were measured. Fura-2-loaded cells were treated with IB-MECA, and the net increase of $[Ca^{2+}]_i$ was measured as described in the Methods. The cells were treated with IB-MECA for 30 s, and the IP_3 production was measured by competition assay. The experiments were performed three to five times and the data are the mean±s.e.mean. $P<0.01$, compared to control.

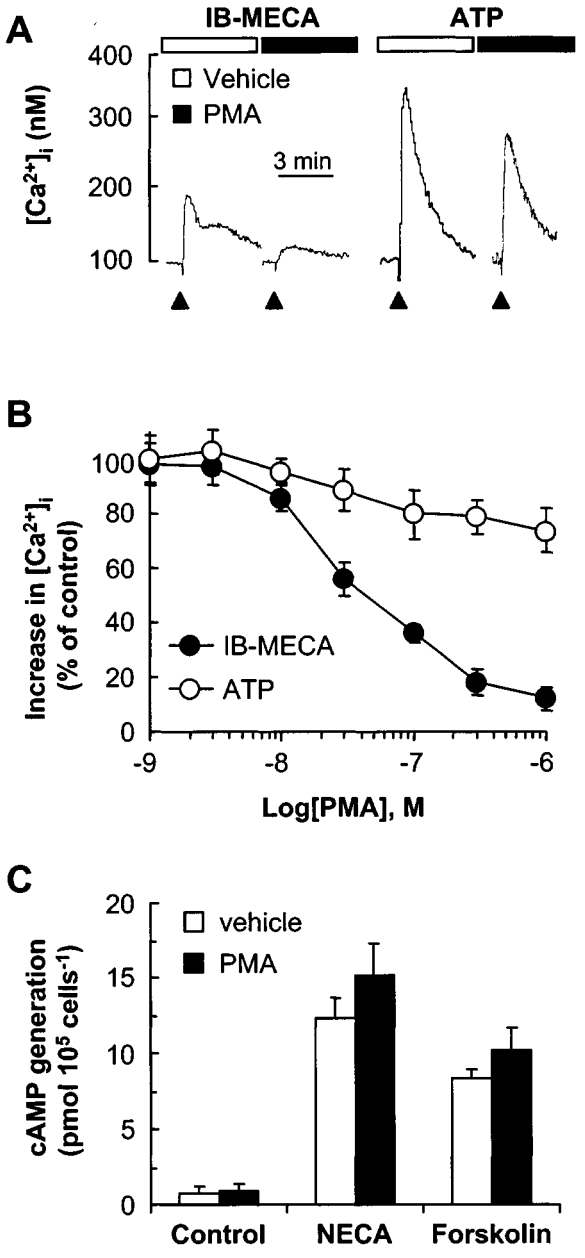

Figure 8 Inhibition of IB-MECA-induced $[Ca^{2+}]_i$ rise by PMA. (A) Cells pretreated for 5 min with vehicle or 300 nM PMA were stimulated with 50 μM IB-MECA or 300 μM ATP. These experiments were repeated more than five times and typical Ca^{2+} transients are presented. (B) Concentration-dependent effects of PMA on IB-MECA-and ATP-induced $[Ca^{2+}]_i$ rise. Cells pretreated for 5 min with vehicle or various concentrations of PMA were stimulated with 50 μM IB-MECA or 300 μM ATP. Net increase in cyclic AMP production is expressed as percentage of the level obtained upon treatment with IB-MECA or ATP alone. (C) Cells were pretreated for 5 min with vehicle or 300 nM PMA then stimulated with 100 μM NECA or 5 μM forskolin for 20 min. The cyclic AMP generation was measured as described in the Methods. Data are mean$\pm$s.e.mean (bars) values from three separate experiments.

(Ramkumar *et al*., 1993; Trincavelli *et al*., 2000). This is associated with the uncoupling of the receptor-G protein complex, as has been indicated by the reduction in the number of high affinity binding sites (Palmer & Stiles, 1995; Palmer *et al*., 1997). It was also reported that the agonist-stimulated phosphorylation of the C-terminal domain of the A_3 adenosine receptor by one or more G protein-coupled receptor kinases is responsible for initiating the events that lead to a rapid desensitization of the A_3 receptor (Palmer & Stiles, 1995). The results of the present study show that A_3 receptor-mediated PLC activation was also highly sensitive to PKC activation, while the A_{2B} receptor-mediated adenylyl cyclase activation was not effected by short-term treatment with PMA in our experimental conditions. In addition, the A_3 receptor-mediated signalling was more sensitive to PMA than was the P2 purinergic receptor. Although the phosphorylation process of A_3 adenosine receptors by second messenger-activated kinase PKC has not yet elucidated, it seems likely that A_3 receptors might be primarily phosphorylated by PKC as evidenced by the involvement of G protein-coupled receptor kinase.

Many studies have suggested that adenosine plays an important role in the trans-synaptic regulation of the pineal function and the modulation of melatonin production through its effect on serotonin N-acetyltransferase (NAT) regulation. Recently, *in vitro* studies with isolated mammalian pineal glands have shown that adenosine elevates NAT gene expression and enzyme activity (Nicholls *et al*., 1997). PGT-β cells express functionally active forms of two characteristic marker enzymes of the pinealocyte, tryptophan hydroxylase and NAT, and the activities of these enzymes can be enhanced by pharmacological stimulation with forskolin and epinephrine through the mediation of β_2-receptors (Son *et al*., 1996; Suh *et al*., 1999). However, NAT activity was only slightly increased (1.2$\sim$1.5 fold) upon stimulation of the cells with NECA (data not shown). This weak activation of NAT may be due to the origin of the cells, since these pineal tumour cells originated from a transgenic mouse that was a hybrid of a NAT-expressing mouse (CBA/J) and a NAT-deficient mouse (C57BL/6J) (Son *et al*., 1996). Goto *et al*. (1989) demonstrates that most laboratory mice do not have pineal melatonin because of a genetic defect in the activity of NAT. For example, C57BL/6J mice do not have NAT activity because of a mutation in an autosomal gene required for normal activity of NAT (Goto *et al*., 1994; Roseboom *et al*., 1998). Recently, experiments with a BALB/c mouse, which did not make pineal melatonin, showed that the night-to-day ratio of NAT transcripts in the retina was much lower than in other mammalian retinae (Sakamoto & Ishida, 1998). However, our finding of the existence of adenosine receptors in pineal gland provides new insights into the biology of adenosine in the pineal gland.

We (Suh *et al*., 1997) and another group (Ferreira *et al*., 1994) have shown that P_2 purinergic receptors are also present in mammalian pineal glands and involved in the modulation of adrenergic receptor-mediated signalling. The results are consistent with evidence that the high concentrations of ATP stored in synaptic vesicles and the release of catecholamine are accompanied by ATP release to rat pineal gland (Mortani Barbosa *et al*., 2000). Previous studies have shown that extracellular adenosine is produced from extracellular ATP, cyclic AMP, or S-adenosylmethionine (Nikodijevic & Klein, 1989; Falcon *et al*., 1988b), presumably by 5′-nucleotidase enzymes on the external surface of pinealocytes. The results suggest that neuronal stimulation

of the pineal gland may involve the activity of nucleotides through P2 purinoceptors and adenosine receptors. Furthermore, the data presented here clearly indicate that the signal flow from the A_3-adenosine receptor negatively regulates the A_{2B}-adenosine and β-adrenergic receptor-mediated signal transduction leading to cyclic AMP production.

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References

AUCHAMPACH, J.A., JIN, X., WAN, T.C., CAUGHEY, G.H. & LINDEN, J. (1997). Canine mast cell adenosine receptors: cloning and expression of the A_3 receptor and evidence that degranulation is mediated by the A_{2B} receptor. *Mol. Pharmacol.,* **52,** 846–860.

BABEY, A.M., PALMOUR, R.M. & YOUNG, S.N. (1994). Caffeine and propranolol block the increase in rat pineal melatonin production produced by stimulation of adenosine receptors. *Neurosci. Lett.,* **176,** 93–96.

BARALDI, P.G., CACCIARI, B., ROMAGNOLI, R., MERIGHI, S., VARANI, K., BOREA, P.A. & SPALLUTO, G. (2000). A_3 adenosine receptor ligands: history and perspectives. *Med. Res. Rev.,* **20,** 103–128.

BIRNBAUMER, L. (1992). Receptor-to-effector signaling through G proteins: roles for $\beta\gamma$ dimers as well as α subunits. *Cell,* **71,** 1069–1072.

BRACKETT, L.E. & DALY, J.W. (1994). Functional characterization of the A_{2B} adenosine receptor in NIH 3T3 fibroblasts. *Biochem. Pharmacol.,* **47,** 801–814.

BROWN, B.L., ALBANO, J.D.M., EKINS, R.P., SGHERZI, A.M. & TAMPION, W. (1971). A simple and sensitive saturation assay method for the measurement of adenosine 3′, 5′-cyclic monophosphate. *Biochem. J.,* **121,** 561–562.

CHALLISS, R.A., CHILVERS, E.R., WILLCOCKS, A.L. & NAHORSKI, S.R. (1990). Heterogeneity of [^{3}H]inositol 1,4,5-trisphosphate binding sites in adrenal-cortical membranes. *Biochem. J.,* **265,** 421–427.

CHOMCZYNSKI, P. (1993). A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. *Biotechniques,* **15,** 532–534.

CUNHA, R.A. (2001). Adenosine as a neuromodulator and as a homeostatic regulator in the nervous system: different roles, different sources and different receptors. *Neurochem. Int.,* **38,** 107–125.

DI VIRGILIO, F., FASOLATO, C. & STEINBERG, T.H. (1988). Inhibitors of membrane transport system for organic anions block fura-2 excretion from PC12 and N2A cells. *Biochem. J.,* **256,** 959–963.

EBADI, M. & GOVITRAPONG, P. (1986). Neural pathways and neurotransmitters affecting melatonin synthesis. *J. Neural Transm. Suppl.,* **21,** 125–155.

FALCON, J., BESSE, C., GUERLOTTE, J. & COLLIN, J.P. (1988b). 5′-Nucleotidase activity in the pineal organ of the pike. An electron-microscopic study. *Cell Tissue Res.,* **251,** 495–502.

FALCON, J., BRUN-MARMILLON, J., CLAUSTRAT, B. & COLLIN, J.P. (1988a). Melatonin production in organ cultured chicken pineal: modulation by adenosine and its analogs. *Pflugers Arch.,* **413,** 93–95.

FALCON, J., PRIVAT, K. & RAVAULT, J.P. (1997). Binding of an adenosine A_1 receptor agonist and adenosine A_1 receptor antagonist to sheep pineal membranes. *Eur. J. Pharmacol.,* **337,** 325–331.

FEINSTEIN, P.G., SCHRADER, K.A., BAKALYAR, H.A., TANG, W.J., KRUPINSKI, J., GILMAN, A.G. & REED, R.R. (1991). Molecular cloning and characterization of a Ca^{2+}/calmodulin-insensitive adenylyl cyclase from rat brain. *Proc. Natl. Acad. Sci. U.S.A.,* **88,** 10173–10177.

FERREIRA, Z.S., CIPOLLA-NETO, J. & MARKUS, R.P. (1994). Presence of P2-purinoceptors in the rat pineal gland. *Br. J. Pharmacol.,* **112,** 107–110.

FREDHOLM, B.B., ARSLAN, G., HALLDNER, L., KULL, B., SCHULTE, G. & WASSERMAN, W. (2000). Structure and function of adenosine receptors and their genes. *Naunyn Schmiedebergs Arch. Pharmacol.,* **362,** 364–374.

GALLO-RODRIGUEZ, C., JI, X.D., MELMAN, N., SIEGMAN, B.D., SANDERS, L.H., ORLINA, J., FISCHER, B., PU, Q., OLAH, M.E., VAN GALEN, P.J.M., STILES, G.L. & JACOBSON, K.A. (1994). Structure-activity relationships of N^6-benzyladenosine-5′-uronamides as A_3-selective adenosine agonists. *J. Med. Chem.,* **37,** 636–646.

GHARIB, A., DELTON, I., LAGARDE, M. & SARDA, N. (1992). Evidence for adenosine A_{2B} receptors in the rat pineal gland. *Eur. J. Pharmacol.,* **225,** 359–360.

GOTO, M., OSHIMA, I., HASEGAWA, M. & EBIHARA, S. (1994). The locus controlling pineal serotonin N-acetyltransferase activity (Nat-2) is located on mouse chromosome 11. *Mol. Brain Res.,* **21,** 349–354.

GOTO, M., OSHIMA, I., TOMITA, T. & EBIHARA, S. (1989). Melatonin content of the pineal gland in different mouse strains. *J. Pineal Res.,* **7,** 195–204.

GRYNKIEWICZ, G., POENIE, M. & TSIEN, R.Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.,* **260,** 3440–3450.

HAAS, H.L. & SELBACH, O. (2000). Functions of neuronal adenosine receptors. *Naunyn Schmiedebergs Arch. Pharmacol.,* **362,** 375–381.

JACOBSON, K.A., NIKODIJEVIC, O., SHI, D., GALLO-RODRIGUEZ, C., OLAH, M.E., STILES, G.L. & DALY, J.W. (1993). A role for central A_3-adenosine receptors. Mediation of behavioral depressant effects. *FEBS Lett.,* **336,** 57–60.

JACOBSON, K.A., PARK, K.S., JIANG, J.L., KIM, Y.C., OLAH, M.E., STILES, G.L. & JI, X.D. (1997). Pharmacological characterization of novel A_3 adenosine receptor-selective antagonists. *Neuropharmacology,* **36,** 1157–1165.

JACOBSON, K.A., SIDDIQI, S.M., OLAH, M.E., JI, X.D., MELMAN, N., BELLAMKONDA, K., MESHULAM, Y., STILES, G.L. & KIM, H.O. (1995). Structure-activity relationships of 9-alkyladenine and ribose-modified adenosine derivatives at rat A_3 adenosine receptors. *J. Med. Chem.,* **38,** 1720–1735.

KLOTZ, K.N. (2000). Adenosine receptors and their ligands. *Naunyn Schmiedebergs Arch. Pharmacol.,* **362,** 382–391.

KLOTZ, K.N., HESSLING, J., HEGLER, J., OWMAN, C., KULL, B., FREDHOLM, B.B. & LOHSE, M.J. (1998). Comparative pharmacology of human adenosine receptor subtypes-characterization of stably transfected receptors in CHO cells. *Naunyn Schmiedebergs Arch. Pharmacol.,* **357,** 1–9.

LINDEN, J., TAYLOR, H.E., ROBEVA, A.S., TUCKER, A.L., STEHLE, J.H., RIVKEES, S.A., FINK, J.S. & REPPERT, S.M. (1993). Molecular cloning and functional expression of a sheep A_3 adenosine receptor with widespread tissue distribution. *Mol. Pharmacol.,* **44,** 524–532.

MORTANI BARBOSA, E.J., FERREIRA, Z.S. & MARKUS, R.P. (2000). Purinergic and noradrenergic cotransmission in the rat pineal gland. *Eur. J. Pharmacol.,* **401,** 59–62.

NICHOLLS, J., SKENE, D.J. & HOURANI, S.M. (1997). Use of a newly developed technique to isolate rat pinealocytes and study the effects of adenosine agonists on melatonin production. *J. Pineal Res.,* **23,** 164–168.

NIKODIJEVIC, O. & KLEIN, D.C. (1989). Adenosine stimulates adenosine 3′,5′-monophosphate and guanosine 3′,5′-monophosphate accumulation in rat pinealocytes: evidence for a role for adenosine in pineal neurotransmission. *Endocrinology,* **125,** 2150–2157.

OLAH, M.E., GALLO-RODRIGUEZ, C., JACOBSON, K.A. & STILES, G.L. (1994). ^{125}I-4-aminobenzyl-5′-*N*-methylcarboxamidoadenosine, a high affinity radioligand for the rat A_3 adenosine receptor. *Mol. Pharmacol.,* **45,** 978–982.

PALMER, T.M. & STILES, G.L. (1995). Adenosine receptors. *Neuropharmacology,* **34,** 683–694.

PALMER, T.M., HARRIS, C.A., COOTE, J. & STILES, G.L. (1997). Induction of multiple effects on adenylyl cyclase regulation by chronic activation of the human A_3 adenosine receptor. *Mol. Pharmacol.,* **52,** 632–640.

PARK, D., JHON, D.Y., LEE, C.W., LEE, K.H. & RHEE, S.G. (1993). Activation of phospholipase C isozymes by G protein $\beta\gamma$ subunits. *J. Biol. Chem.,* **268,** 4573–4576.

PARK, J.Y., KIM, I.J., LEE, M.H., SEO, J.K., SUH, P.G., CHO, B.Y., RYU, S.H. & CHAE, C.B. (1997). Identification of the peptides that inhibit the stimulation of thyrotropin receptor by Graves' immunoglubulin G from peptide libraries. *Endocrinology,* **138,** 617–626.

RALEVIC, V. & BURNSTOCK, G. (1998). Receptors for purines and pyrimidines. *Pharmacol. Rev.,* **50,** 413–492.

RAMKUMAR, V., KWATRA, M., BENOVIC, J.L. & STILES, G.L. (1993). Functional consequences of A_1 adenosine-receptor phosphorylation by the β-adrenergic receptor kinase. *Biochim. Biophys. Acta.,* **1179,** 89–97.

RAMKUMAR, V., OLAH, M.E., JACOBSON, K.A. & STILES, G.L. (1991). Distinct pathways of desensitization of A_1- and A_2-adenosine receptors in DDT1 MF-2 cells. *Mol. Pharmacol.,* **40,** 639–647.

ROSEBOOM, P.H., NAMBOODIRI, M.A., ZIMONJIC, D.B., POPESCU, N.C., RODRIGUEZ, I.R., GASTEL, J.A. & KLEIN, D.C. (1998). Natural melatonin 'knockdown' in C57BL/6J mice: rare mechanism truncates serotonin N-acetyltransferase. *Mol. Brain Res.,* **63,** 189–197.

SAKAMOTO, K. & ISHIDA, N. (1998). Molecular cloning of serotonin N-acetyltransferase gene from the mouse and its daily expression in the retina. *Neurosci. Lett.,* **250,** 181–184.

SANGER, F., NICKLEN, S. & COULSON, A.R. (1992). DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. U.S.A.,* **74,** 5463–5467.

SHEARMAN, L.P. & WEAVER, D.R. (1997). [^{125}I]4-aminobenzyl-5′-N-methylcarboxamidoadenosine (^{125}I-AB-MECA) labels multiple adenosine receptor subtypes in rat brain. *Brain Res.,* **745,** 10–20.

SIMON, M.I., STRATHMANN, M.P. & GAUTAM, N. (1991). Diversity of G proteins in signal transduction. *Science,* **252,** 802–808.

SON, J.H., CHUNG, J.H., HUH, S.O., PARK, D.H., PENG, C., ROSENBLUM, M.G., CHUNG, Y.I. & JOH, T.H. (1996). Immortalization of neuroendocrine pinealocytes from transgenic mice by targeted tumorigenesis using the tryptophan hydroxylase promoter. *Mol. Brain. Res.,* **37,** 32–40.

STERNWEISS, P.C. (1994). The active role of $\beta\gamma$ in signal transduction. *Curr. Opin. Cell. Biol.,* **6,** 198–203.

SUH, B.C., CHAE, H.D., CHUNG, J.H. & KIM, K.T. (1999). Pharmacological characterization of β_2-adrenoceptor in PGT-β mouse pineal gland tumor cells. *Br. J. Pharmacol.,* **126,** 399–406.

SUH, B.C., LEE, C.O. & KIM, K.T. (1995). Signal flows from two phospholipase C-linked receptors are independent in PC12 cells. *J. Neurochem.,* **64,** 1071–1079.

SUH, B.C., SON, J.H., JOH, T.H. & KIM, K.T. (1997). Two distinct P_2-purinergic receptors, P_{2Y} and P_{2U}, are coupled to phospholipase C in mouse pineal gland tumor cells. *J. Neurochem.,* **68,** 1622–1632.

TANG, W.J. & GILMAN, A.G. (1991). Type-specific regulation of adenylyl cyclase by G protein $\beta\gamma$ subunits. *Science,* **254,** 1500–1503.

TRINCAVELLI, M.L., TUSCANO, D., CECCHETTI, P., FALLENI, A., BENZI, L., KLOTZ, K.N., GREMIGNI, V., CATTABENI, F., LUCACCHINI, A. & MARTINI, C. (2000). Agonist-induced internalization and recycling of the human A_3 adenosine receptors: role in receptor desensitization and resensitization. *J. Neurochem.,* **75,** 1493–1501.

VAN MUIJLWIJK-KOEZEN, J.E., TIMMERMAN, H., VAN DER GOOT, H., MENGE, W.M., FRIJTAG VON DRABBE KUNZEL, J., DE GROOTE, M. & IJZERMAN, A.P. (2000). Isoquinoline and quinazoline urea analogues as antagonists for the human adenosine A_3 receptor. *J. Med. Chem.,* **43,** 2227–2238.

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