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Amplification of the Cyclic AMP Response to Forskolin in Pheochromocytoma PC12 Cells through Adenosine A_{2A} Purinoceptors[1]

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ABSTRACT

In this study, we present evidence on the ability of endogenous adenosine to modulate adenylyl cyclase activity in intact PC12 cells. The adenosine receptor antagonists PD 115199, xanthine amine congener, 8-cyclopentyl-1,3-dipropylxanthine, 8-(*p*-sulfophenyl)theophylline, and 3,7-dimethyl-1-propargylxanthine inhibited 10 μM forskolin-induced cyclic AMP (cAMP) accumulation, with IC_{50} values of 2.76 $\pm$ 1.16 nM, 17.4 $\pm$ 1.08 nM, 443 $\pm$ 1.03 nM, 2.00 $\pm$ 1.01 μM, and 2.25 $\pm$ 1.05 μM, respectively. Inhibition by 2.5 nM PD 115199 was only partially reversed by increasing forskolin concentrations up to 100 μM. The addition of PD 115199 with or 60 min after forskolin caused a comparable inhibition of forskolin effect over the next hour. Both exogenous adenosine (0.1 μM) and its precursor, AMP (10 and 100 μM), significantly enhanced forskolin-induced cAMP accumulation, whereas inosine was ineffective. Forskolin activity was also potentiated by the hydrolysis-resistant adenosine receptor agonists 5′-*N*-ethylcarboxamido adenosine and CGS 21680 (8.9- and 12.2-fold increase, respectively). Adenosine deaminase (1 U/ml) and 8-SPT (25 μM), which nearly abolished the response to 1 μM adenosine, also reduced cAMP accumulation caused by AMP (−78 and −54%, respectively). These results demonstrate that in PC12 cells, activation of adenylyl cyclase by forskolin is highly dependent on the occupancy of A_{2A} adenosine receptors and that AMP potentially contributes to the amplification of forskolin response.

Endogenous adenosine modulates the metabolic activity and function of a wide variety of excitable as well as nonexcitable cells. The effects of adenosine are mediated by at least four receptors coupled to adenylyl cyclase through G proteins. Activation of A_1 and A_3 receptors by adenosine leads to inhibition of the enzyme, whereas A_{2A} and A_{2B} receptors are positively linked to it (Fredholm et al., 1994). Among these four subtypes, A_1 and A_{2A} receptors can be activated under basal physiological conditions due to the relatively high affinity for the endogenous ligand (Fredholm, 1995).

PC12 rat pheochromocytoma cells have a number of neuronal characteristics, and they undergo terminal neuronal differentiation in response to cyclic AMP (cAMP)- and cAMP-elevating agents (Roth et al., 1991; Vossler et al., 1997). Therefore, this cell line is often used as an experimental model to study signal transduction mechanisms involved in a variety of processes occurring in neuronal cells. Some of these, such as catecholamine synthesis and neurotransmitter release, are known to undergo physiological control by adenosine through cAMP-dependent pathways (McMahon and Sabban, 1992; Oda et al., 1995; Chae and Kim, 1997; Ono et al., 1998).

PC12 cells posses A_1, A_{2A}, and A_{2B} receptors (Arslan et al., 1997, 1999), but A_1 receptors are not functionally relevant (Noronha-Blob et al., 1986). Some pieces of evidence indicate that in these cells, endogenous adenosine exerts a tonic control on adenylyl cyclase activity. In fact, both inactivation of endogenous adenosine by means of adenosine deaminase (ADA) and blockade of adenosine receptors with theophylline lead to a decrease in cAMP level in unstimulated PC12 cells, whereas an opposite effect has been observed after inhibition of adenosine uptake by dipyridamole (Roskoski and Roskoski, 1989).

The physiological control operated by adenosine can be further stressed on stimulation of adenylyl cyclase with forskolin, a diterpene that acts through a receptor-independent mechanism, by directly interacting with the catalytic site of the enzyme (Dessauer et al., 1997). The cAMP-elevating ef-

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ABBREVIATIONS: cAMP, cyclic AMP; AOPCP, α,β-methyleneadenosine-5′-diphosphate; ADA, adenosine deaminase; CGS 21680, 2-[*p*-(2-carbonylethyl)phenylethylamino]-5′-*N*-ethylcarboxamidoadenosine, NECA, 5′-*N*-ethylcarboxamido adenosine; 8-SPT, 8-(*p*-sulfophenyl)theophylline; DMPX, 3,7-dimethyl-1-propargylxanthine; XAC, xanthine amine congener; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; PD 115199, *N*-[2-(dimethylamino)ethyl]-*N*-methyl-4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1*H*-purin-8-yl)benzenesulfonamide.

fect of forskolin is sensitive to inhibition by ADA (Kim et al., 1993; Rabin et al., 1993; Florio et al., 1999). In accordance with these findings, adenosine receptor desensitization has been shown to reduce the adenylyl cyclase response to subsequent stimulation with adenosine receptor agonists as well as with forskolin in PC12 cell membranes (Chern et al., 1993, 1995). Moreover, in intact cells, an increase in forskolin-induced cAMP accumulation has also been reported after a 4-day exposure to 5′-*N*-ethylcarboxamido adenosine (NECA; Rabin et al., 1993), an hydrolysis-resistant adenosine derivative. These observations prompted us to better define the dependence of the cAMP response to forskolin on the occupancy of purinergic receptors by endogenous adenosine in PC12 cells and to identify the specific receptor or receptors involved.

The direct precursor of adenosine, AMP, is dephosphorylated by 5′-nucleotidase, whose membrane-bound form is an ectoenzyme present in essentially all nervous tissues, including PC12 cells (Zimmermann and Braun, 1996). Because in many cell systems AMP mimics a variety of adenosine receptor-mediated effects, and in PC12 cells it increases cAMP level in an adenosine-like manner (Yakushi et al., 1996), part of the present study was intended to evaluate the dependence of AMP action on 5′-nucleotidase activity and the possible interaction of the mononucleotide with forskolin.

Experimental Procedures

Materials. [2,8-^{3}H]cADP (specific activity, 27 Ci/mmol) was obtained from DuPont-New England Nuclear (Bad Homburg, Germany). Nonlabeled cAMP, adenosine, AMP, α,β-methyleneadenosine 5′-diphosphate (AOPCP), and ADA type VIII (175 U/mg, 25 mg/ml) were obtained from Sigma Chemical Co. (St. Louis, MO). Forskolin, 2-[*p*-(2-carbonylethyl)phenylethylamino]-5′-*N*-ethylcarboxamidoadenosine (CGS 21680), NECA, 8-(*p*-sulfophenyl)theophylline (8-SPT), 3,7-dimethyl-1-propargylxanthine (DMPX), xanthine amine congener (XAC), and 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) were purchased from Research Biochemicals International (Natick, MA). *N*-[2-(Dimethylamino)ethyl]-*N*-methyl-4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1*H*-purin-8-yl)benzenesulfonamide (PD 115199) was obtained from Warner-Lambert/Parke-Davis Pharmaceutical Research Division (Ann Arbor, MI). Forskolin was dissolved in ethanol, kept at −20°C as stock solution, and diluted with culture medium, pH 7.4, immediately before use. Stock solutions (10 mM) of XAC, DPCPX, or PD 115199, dissolved in dimethyl sulfoxide, and of NECA, made in 50% ethanol, were diluted with culture medium on the day of the experiment. Final vehicle concentrations in incubation wells were always less than 0.5% (v/v). All the other reagents were dissolved in distilled water.

Cell Culture. PC12 cells were cultured in RPMI-1640 (GIBCO BRL, Paisley, Scotland) supplemented with 10% heat-inactivated horse serum (Sigma Chemical Co.), 5% fetal calf serum (GIBCO BRL), 100 U/ml penicillin, and 100 μg/ml streptomycin (Sigma Chemical Co.) at 37°C in a humidified 95% air/5% CO_2 atmosphere. The cells were routinely subcultured once weekly. For determination of cAMP production, the cells were seeded at a cell density of 0.3×10^6 cells in 24-well plates 48 h before the experiments.

cAMP Accumulation. Cells were incubated for 30 min at 37°C in 1 ml/well HEPES-buffered culture medium, pH 7.4, containing 1% horse serum-fetal calf serum and, where appropriate, adenosine receptor antagonists. After removal of the preincubation medium, reactions were started by adding 1 ml of the same medium containing test agents or vehicle. After the appropriate time, incubation was terminated by the removal of medium and the addition of 0.25 ml of ice-cold 0.1 N hydrochloric acid to each well. The cells were sonicated, and the supernatants, neutralized by the addition of 0.25 ml of ice-cold 0.1 M Tris, were collected in Eppendorf tubes, centrifuged, and stored at −20°C until assayed.

Measurement of cAMP. cAMP content was determined by displacing [^{3}H]cAMP binding to a bovine adrenal extract as described by Nordstedt and Fredholm (1990), with slight modifications (Florio et al., 1999). Samples or unlabeled cAMP for standard curve were added to a 96-well MultiScreen-FB microtiter plate (Millipore, MA) in a final volume of 250 μl of 100 mM Tris-HCl, pH 7.4, containing 250 mM NaCl, 10 mM EDTA, 0.5 pmol [^{3}H]cAMP, and binding protein. After 150 min at 4°C, the incubation was stopped by vacuum filtration using a MultiScreen vacuum manifold, and the filters were washed twice with 200-μl aliquots of ice-cold Tris-HCl. After the addition of SuperMix liquid scintillation cocktail (25 μl/well), the plate was counted in a MicroBeta Trilux liquid scintillation counter (Wallac, Turku, Finland).

The amount of cAMP in cell supernatants was determined by interpolation of the number of counts per minute of the sample from the linear portion of the standard curve by nonlinear regression.

Calculations and Statistical Analysis. The computer program SigmaPlot (Jandel Scientific, Erkrath, Germany) was used to generate IC_{50} parameters for antagonist concentration-response curves. Data were analyzed using an ANOVA followed by a post hoc multiple-comparison Newman-Keuls test, as indicated in the text. Results from kinetic studies were compared using an ANOVA followed by the post hoc multiple-comparison Bonferroni's test.

Results

Effect of Various Adenosine Receptor Antagonists on Forskolin-Induced Intracellular cAMP Accumulation. A 15-min exposure to 10 μM forskolin caused a large increase in intracellular cAMP levels in PC12 cells (from 1.52 ± 0.13 to 1452 ± 136 pmol/10^6 cells). In the presence of several adenosine receptor antagonists, forskolin-dependent cAMP accumulation was inhibited in a concentration-dependent manner with the following order of potency: PD 115199 > XAC ≫ DPCPX > 8-SPT = DMPX (Fig. 1). The calculated IC_{50} values were 2.76 ± 1.16 nM, 17.4 ± 1.08 nM, 443 ± 1.03 nM, 2.00 ± 1.01 μM, and 2.25 ± 1.05 μM,

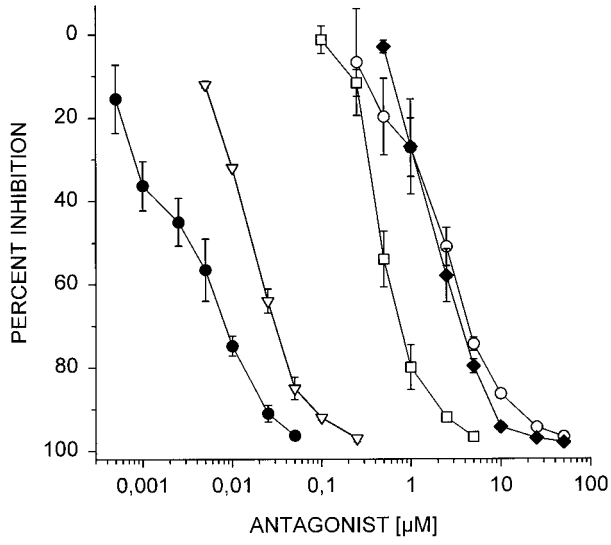

Fig. 1. Effect of increasing concentrations of adenosine antagonists on intracellular cAMP levels in cells exposed to 10 μM forskolin for 15 min. Cells were incubated for 30 min in the presence of the antagonists before the addition of forskolin. Each point is the mean ± S.E.M. of at least two separate experiments, each performed in triplicate. ●, PD 115199; ▽, XAC; □, DPCPX; ♦, 8-SPT; ○, DMPX.

respectively. Maximally effective antagonist concentrations did not fully suppress the response to forskolin, and the residual increase over basal values ranged from 39 ± 10 to 63 ± 5 pmol cAMP/10^6 cells. Moreover, a maximally effective 8-SPT concentration (25 μM) did not alter basal levels of the cyclic nucleotide (1.60 ± 0.15 pmol/10^6 cells).

To evaluate the ability of forskolin to overcome the inhibitory effect of adenosine receptor antagonists, PC12 cells were exposed to increasing concentrations of forskolin in the presence of 2.5 nM PD 115199, which reduced the effect of 10 μM forskolin by 50%. As shown in Fig. 2, in cells treated with 25 μM forskolin plus PD 115199, cAMP levels equaled those measured in the presence of 10 μM forskolin alone. cAMP accumulation in PD 115199-treated cells further increased at 50 and 75 μM forskolin, with no additional increase at 100 μM. However, under these conditions, the response to forskolin was not fully restored, and even at the highest forskolin concentration tested (100 μM), cAMP levels measured in the presence of PD 115199 were significantly lower than those reached in the absence of the antagonist (Fig. 2).

Time Course of Intracellular cAMP Accumulation Induced by Forskolin in Absence and Presence of PD 115199. To further investigate the characteristics of the inhibitory effect of adenosine antagonists on forskolin activity, PC12 cells were incubated with 10 μM forskolin in the absence and the presence of 2.5 nM PD 115199, and intracellular cAMP was measured at different incubation times. The inhibitory effect of the antagonist was already detectable after 5 min of incubation. In the presence of PD 115199, cAMP progressively increased until 30 min of stimulation, with no significant changes occurring thereafter. At the end of the incubation (120 min), when a decline in cAMP levels occurred in cells treated with 10 μM forskolin, the inhibitory effect of the antagonist was no longer significant (Fig. 3).

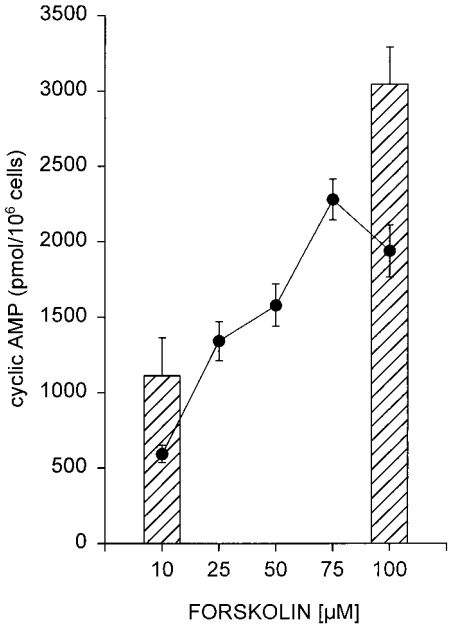

Fig. 2. Effect of increasing concentrations of forskolin on intracellular cAMP levels in the presence (●) or in the absence (bars) of 2.5 nM PD 115199. PC12 cells were incubated for 30 min in the presence of the adenosine receptor antagonist before the addition of forskolin. Stimulation was carried out for 15 min. Values are mean ± S.E.M. of two separate experiments performed in triplicate.

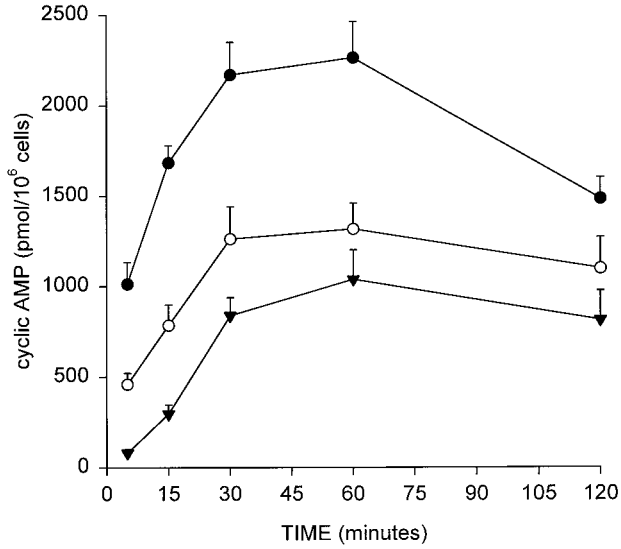

Fig. 3. Time course of intracellular cAMP accumulation in PC12 cells exposed to 10 μM forskolin (●), 1 μM forskolin (▼), or 10 μM forskolin in the continuous presence of 2.5 nM PD 115199 (○). Before the addition of drugs, cells were incubated for 30 min in prewarmed medium. Values are mean ± S.E.M. of three separate experiments performed in triplicate. The curve for 10 μM forskolin was significantly different from the curve for 1 μM forskolin ($P < .01$ for all time points, with the exception of the 120-min time point, where $P < .05$), as well as from the curve for 10 μM forskolin plus 2.5 nM PD 115199 ($P < .01$ for all time points, with the exception of the 120-min time point, not significant). No significant differences were found between the curve for 1 μM forskolin and the curve for 10 μM forskolin plus PD 115199, with the exception of the 15-min time point, where $P < .05$ (ANOVA followed by Bonferroni's test).

The PD 115199-induced changes in time course of the cAMP response to 10 μM forskolin were compared with those obtained by lowering the concentration of the stimulant. As shown in Fig. 3, in the presence of 1 μM forskolin, cAMP accumulation increased up to 30 min, reaching a level that was maintained until the end of the incubation period.

A series of experiments was performed to find an explanation for the loss of PD 115199 inhibitory activity after 120 min of incubation. First, we tested whether inhibition by the antagonist still occurred when PD 115199 was added after 60 min of incubation with forskolin (i.e., when cAMP levels had

TABLE 1
Effect of 2.5 nM PD 115199 added at different times on intracellular cAMP accumulation induced by 1 and 10 μM forskolin over 60 min

Drug Addition		cAMP	
At 0 min	At 60 min	At 60 min	At 120 min
		pmol / 10^6 cells	
Forskolin, 1 μM		660 ± 54	581 ± 54
Forskolin, 1 μM + PD 115199, 2.5 nM		324 ± 30** (−52%)	
Forskolin, 1 μM	PD 115199, 2.5 nM		306 ± 18** (−48%)
Forskolin, 10 μM		2071 ± 201	1319 ± 114
Forskolin, 10 μM + PD 115199, 2.5 nM		1393 ± 118** (−33%)	
Forskolin, 10 μM	PD 115199, 2.5 nM		858 ± 106* (−35%)

Data are the means ± S.E.M. of six to eight replicates. Results were compared using an ANOVA followed by the Newman-Keuls range test. The asterisks (*$P < .05$; **$P < .01$) denote significance of PD 115199 treatment at different times on forskolin activity.

already reached a maximum). As shown in Table 1, the percent reduction of cAMP level caused by a 60-min exposure to the adenosine receptor antagonist was comparable when it was added together with or 60 min after forskolin (1 or 10 μM). Thus, PD 115199 not only prevented but also reversed the effect of the adenylyl cyclase activator. These results also indicate that even after a 120-min treatment with forskolin, cAMP accumulation is still sensitive to inhibition by the adenosine receptor antagonist. Second, we evaluated the possibility that resistance to PD 115199 after 120 min of continuous incubation (Fig. 3) could be related to its time of contact with the cells. Drug-free medium or medium containing 2.5 nM PD 115199 was incubated in wells without cells as well as in cell-plated wells. After 120 min, 1-ml aliquots of the differently treated media were transferred to cell-plated wells, and forskolin was added at this stage in 10 μl of medium (10 μM final concentration). Previous incubation either in the medium alone or in the medium plus cells did not alter the ability of the antagonist to affect forskolin response over 15 min, causing a 48 $\pm$ 6% reduction in cAMP levels, not significantly different from cells incubated in fresh medium (-49 ± 7%; not shown).

Effect of Adenosine Receptor Agonists on Forskolin-Dependent cAMP Accumulation. The above results strongly suggest a role for endogenous adenosine in enhancing the ability of forskolin to stimulate adenylyl cyclase activity. To verify if exogenously applied adenosine could accordingly facilitate forskolin action, the effect of increasing concentrations of forskolin was investigated in the absence and in the presence of adenosine at 0.1 μM, a concentration below its EC_{50} value of 513 $\pm$ 61 nM (Florio et al., 1999). In this set of experiments, 0.1 μM adenosine-induced intracellular cAMP accumulation was equal to 114 $\pm$ 20 pmol/10^6 cells. As shown in Fig. 4, adenosine markedly potentiated the effect of forskolin during 30 min of incubation, causing a parallel shift of the concentration-response curve for forskolin to the left. Adenosine potentiated the response to 1 and 3 μM forskolin to a comparable extent, enhancing intracellular cAMP levels by 5.3 $\pm$ 0.3- and 5.4 $\pm$ 0.8-fold, respectively, whereas 30 μM forskolin-induced cAMP levels were augmented by 1.8 $\pm$ 0.2-fold. At 100 μM forskolin, a concentration that was shown previously to be maximally effective (Florio et al., 1999) the potentiation (1.7 $\pm$ 0.1-fold increase) was still significant.

The adenosine receptor agonists NECA and CGS 21680 concentration-dependently stimulated cAMP accumulation to a similar extent (Fig. 5). A 30-min exposure of the cells to the lowest agonist concentration tested (3 nM) markedly potentiated forskolin activity. Intracellular cAMP accumulation evoked by 1 μM forskolin was increased 8.9 $\pm$ 0.5- and 12.2 $\pm$ 0.6-fold by NECA and CGS 21680, respectively. The latter agonist was significantly more potent than NECA in enhancing forskolin activity. Results are summarized in Table 2.

Effect of Exogenous AMP and Inosine on Forskolin-Dependent cAMP Accumulation. The possible interaction between forskolin and the adenosine precursor AMP was also evaluated. AMP was less potent than adenosine in promoting cAMP accumulation, with a calculated EC_{50} value equal to 49 $\pm$ 13 μM (not shown). AMP, at concentrations both below (10 μM) and above (100 μM) its EC_{50} value, mimicked the ability of adenosine to potentiate the effect of forskolin (Fig. 6). The response to 1 μM forskolin was increased by 4.8 $\pm$ 0.5- and 6.7 $\pm$ 0.7-fold in the presence of 10 and 100 μM AMP, respectively, whereas the same concentrations of the nucleotide augmented the effect of 10 μM forskolin by 1.1 $\pm$ 0.1- and 1.2 $\pm$ 0.2-fold, respectively.

In contrast, inosine, the deamination product of adenosine, up to 100 μM did not affect basal cAMP level (5.2 $\pm$ 1.9 and 4.4 $\pm$ 0.8 pmol cAMP/10^6 cells for control and 100 μM ino-

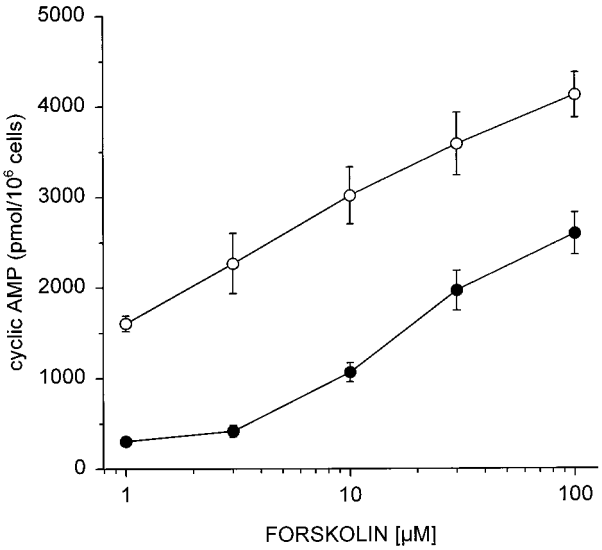

Fig. 4. Effect of the presence of 0.1 μM adenosine on intracellular cAMP in PC12 exposed to increasing concentrations of forskolin. Cells were exposed to forskolin alone (●) or to forskolin plus adenosine (○) for 30 min. Adenosine (0.1 μM)-dependent cAMP synthesis was equal to 114 $\pm$ 20 pmol/10^6 cells. Data are mean $\pm$ S.E.M. of four separate experiments performed in triplicate. Results were compared using ANOVA followed by the Newman-Keuls range test; the curve for forskolin plus adenosine was significantly different from the forskolin curve ($P < .01$ for all forskolin concentrations, with the exception of the lowest concentration, where $P < .05$).

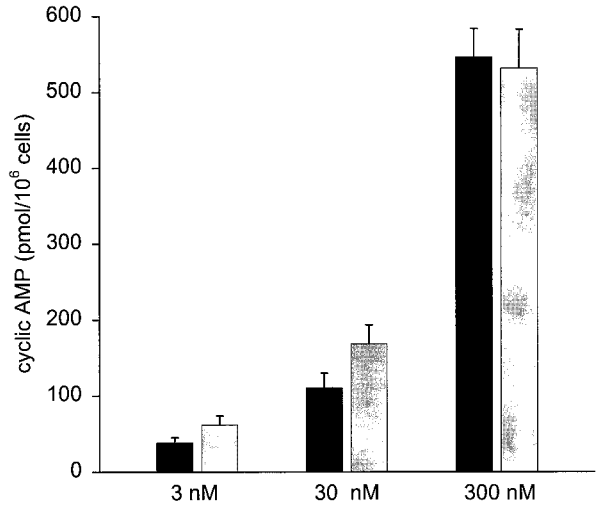

Fig. 5. Effect of increasing concentrations of the adenosine receptor agonists NECA (black columns) and CGS 21680 (gray columns) on intracellular cAMP levels. Each point is the mean $\pm$ S.E.M. of two separate experiments performed in duplicate.

TABLE 2
Effect of 3 nM NECA and CGS 21680 on 1 μM forskolin-induced cAMP accumulation

	cAMP	
	No Forskolin	1 μM Forskolin
	pmol / 10^6 cells	
None	3.5 ± 0.9	210 ± 16
NECA 3 nM	38 ± 7	1864 ± 99**
CGS 21680 3 nM	62 ± 12	2562 ± 134**

Cells were incubated for 30 min with vehicle, NECA, or CGS 21680 in the absence (left column) or presence (right column) of 1 μM forskolin. Data are means ± S.E.M. of four separate experiments carried out in triplicate. Results were compared using an ANOVA followed by the Newman-Keuls range test and significance at a $P < .01$ level (**) was found among the treatment agonists plus forskolin with respect to forskolin alone and between the associations of NECA plus forskolin and CGS 21680 plus forskolin.

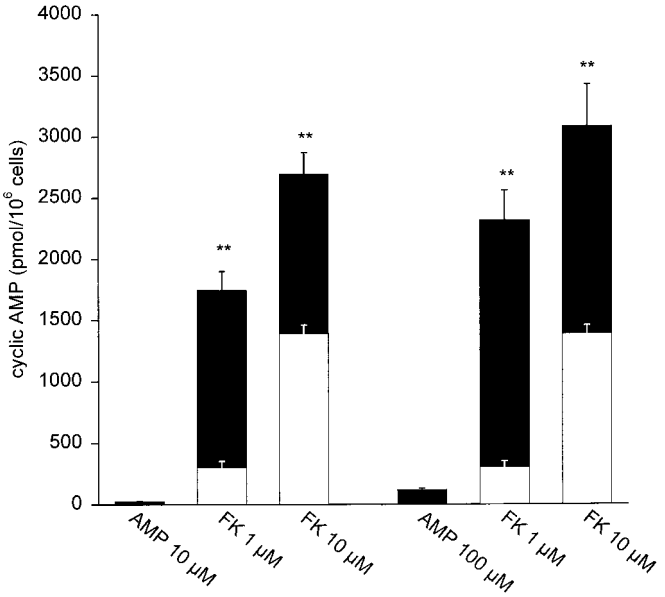

Fig. 6. Effect of 10 or 100 μM AMP on intracellular cAMP in PC12 cells exposed to 1 and 10 μM forskolin. Cells were exposed to forskolin alone (open columns) or to forskolin plus AMP (open plus filled columns) for 30 min. AMP (10 and 100 μM)-dependent intracellular cAMP synthesis was equal to 24 ± 5 and 120 ± 11 pmol/10^6 cells, respectively. Data are mean ± S.E.M. of 6 to 12 separate determinations assayed in duplicate. Results were compared using ANOVA followed by the Newman-Keuls range test, and the results for 1 and 10 μM forskolin plus 10 and 100 μM AMP were found to be significantly different from those obtained with forskolin alone (**$P < .01$).

sine, respectively) and did not cause significant changes in 1 or 10 μM forskolin-induced cAMP accumulation (Fig. 7).

Effect of ADA and of 8-SPT on AMP- and Adenosine-Dependent Intracellular Accumulation of cAMP. ADA, the enzyme that promotes the deamination of adenosine into inosine; 8-SPT; and AOPCP, an inhibitor of ecto-5′-nucleotidase activity, were used to evaluate whether the ability of exogenous AMP to increase intracellular cAMP was due to interaction of the nucleotide with adenosine receptors per se or after conversion to adenosine or to some other unrelated mechanism. cAMP accumulation caused by 100 μM AMP was reduced after a 30-min incubation together with 1 U/ml ADA (−78%) or with 25 μM 8-SPT (−54%) (Fig. 8). At these concentrations, ADA nearly suppressed the response to 1 and 10 μM exogenous adenosine, whereas 8-SPT decreased the response to 1 and 10 μM exogenous adenosine by 96 and 48%,

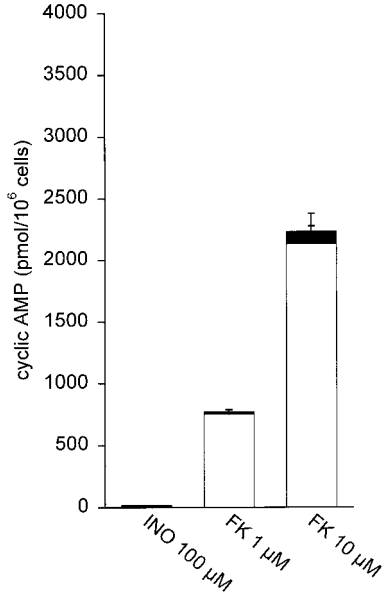

Fig. 7. Effect of 10 μM inosine on intracellular cAMP in PC12 cells exposed to 1 and 10 μM forskolin. Cells were exposed to forskolin alone (open columns) or to forskolin plus inosine (INO, open plus filled columns) for 30 min. Data are mean ± S.E.M. of three to nine separate determinations assayed in duplicate. Results were compared using ANOVA followed by the Newman-Keuls range test. No significant difference was found between 1 and 10 μM forskolin plus 100 μM inosine with respect to forskolin alone.

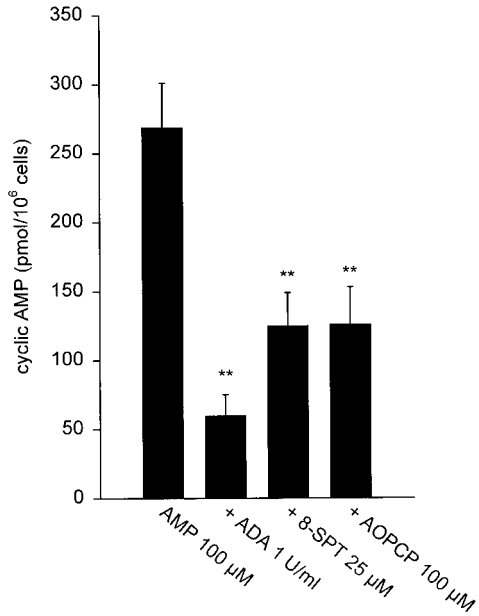

Fig. 8. Effect of 1 U/ml ADA, 25 μM 8-SPT, and 100 μM AOPCP on intracellular cAMP accumulation in PC12 cells exposed to 100 μM AMP. Cells were exposed to AMP alone or plus test agents for 30 min. Basal values (3.34 ± 0.53 pmol/10^6 cells) were not significantly affected by the presence of ADA (2.80 ± 0.58 pmol/10^6 cells) or 8-SPT (3.52 ± 0.37). Data are mean ± S.E.M. of 5 to 12 separate determinations assayed in duplicate. Results were compared using ANOVA followed by the Newman-Keuls range test, and the results for the three different treatments (AMP plus ADA, 8-SPT, or AOPCP) were found to be significantly different from those with AMP alone (**$P < .01$).

respectively (Fig. 9). The effect of 100 μM AMP was also sensitive to inhibition by AOPCP (100 μM), which reduced cAMP accumulation by 54% (Fig. 8).

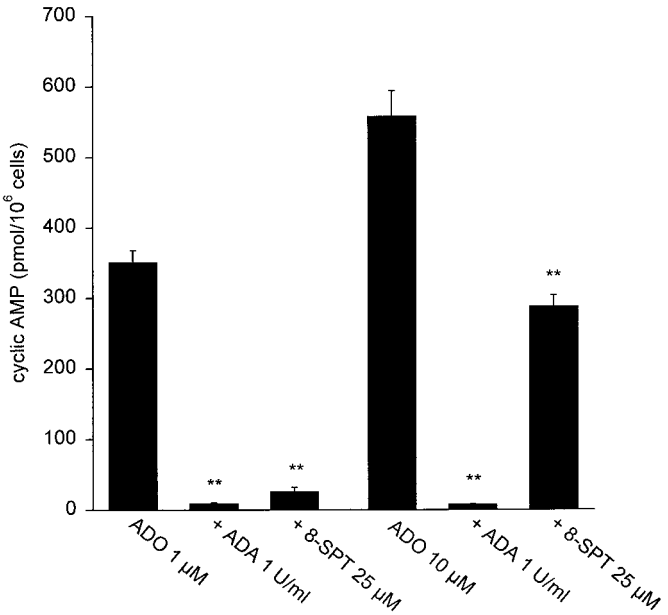

Fig. 9. Effect of 1 U/ml ADA and 25 μM 8-SPT on intracellular cAMP accumulation in PC12 cells exposed to 1 or 10 μM adenosine (ADO). Cells were exposed to adenosine alone or to adenosine plus test agents for 30 min. Data are mean ± S.E.M. of six separate determinations assayed in duplicate. Results were compared using ANOVA followed by the Newman-Keuls range test, and the results for 1 and 10 μM adenosine plus ADA or 8-SPT were found significantly different from those with adenosine alone (**$P < .01$).

Discussion

The present results demonstrate that the ability of forskolin to stimulate cAMP production in PC12 cells is strictly modulated by the occupancy of adenosine receptors by endogenous adenosine. In fact, several adenosine antagonists dramatically impaired activation of adenylyl cyclase by 10 μM forskolin. The inhibition was concentration-dependent with the following order of potency: PD 115199 > XAC ≫ DPCPX > 8-SPT = DMPX. The antagonist potencies are in good agreement with the IC_{50} values reported in several studies for antagonism at adenosine A_2 receptors (Daly et al., 1986; Bruns et al., 1987; Jarvis et al., 1989; Hide et al., 1992; Kirk and Richardson, 1995).

PC12 cells predominantly express adenosine A_{2A} receptors but also possess a small proportion of A_{2B} receptors (Van der Ploeg et al., 1996), and both binding sites are positively coupled to adenylyl cyclase. On the basis of the potency of two antagonists that have high affinity at the A_{2A} receptor, PD 115199 and XAC (Bruns et al., 1987; Jacobson et al., 1987), it appears that the receptor subtype predominantly involved in modulating forskolin activity is the A_{2A} subtype. This would be in keeping with the relatively high affinity of this receptor for the endogenous ligand, allowing its activation under basal physiological conditions (Fredholm, 1995).

Because it is highly improbable that forskolin and adenosine antagonists may share a common binding site, the remaining alternative is that adenosine antagonists impair forskolin activity by acting in a noncompetitive manner (i.e., by blocking the chain of events that leads to the production of cAMP in response to the diterpene). To better clarify the type of antagonism involved, the effect of the reversible and relatively selective A_{2A} receptor antagonist PD 115199 (Bruns et al., 1987) was investigated in the presence of increasing concentrations of forskolin. The results show that the inhibition of the response to 10 μM forskolin caused by PD 115199 could be surmounted by increasing the concentration of the stimulant but that the reversibility of PD 115199 antagonism was only partial because a maximally effective forskolin concentration did not fully restore the cAMP response. These results mirror previous findings showing that the nonselective P_1 receptor antagonist 8-SPT at a concentration that was maximally effective in inhibiting 10 μM forskolin-dependent cAMP accumulation caused a downward shift in the concentration-response curve for forskolin (Florio et al., 1999).

Inclusion of the antagonist PD 115199 in the incubation medium reduced the early burst of cAMP accumulation evoked by 10 μM forskolin, thus allowing it to maintain its levels unchanged during the second hour of incubation. A similar profile was found in time course experiments performed using a 10-fold lower concentration of forskolin. Thus, the presence of the antagonist mimics a condition in which a low concentration (1 μM) of forskolin is used, possibly indicating that the recruitment of A_{2A} receptors for the amplification of forskolin response is directly proportional to forskolin concentration.

Time course studies also demonstrated that the ability of forskolin to stimulate adenylyl cyclase activity was not only prevented but also reversed by PD 115199. Thus, a prolonged increase in intracellular cAMP levels caused by forskolin does not seem to impair the function of the purinergic receptor system, in accordance with previous reports indicating that adenosine A_{2A} receptor desensitization, which occurs rapidly in PC12 cells (Mundell and Kelly, 1998), is a homologous process (Palmer et al., 1994). However, when PD 115199 was added together with forskolin at the beginning of the incubation, after 120 min its inhibitory effect was no longer significant. A 2-h preincubation of this compound with the culture medium alone or with culture medium preincubated in cell-plated wells did not reduce its ability to inhibit the response to a subsequent challenge of untreated cells with forskolin, excluding a significant chemical or metabolic instability of PD 115199. Thus, PC12 cells appear to undergo time-dependent "adaptation" to the antagonist. The cellular mechanisms involved in this phenomenon remain to be identified.

In recent years, much experimental work has been done to better understand the molecular processes that lead to activation of adenylyl cyclase by forskolin (McHugh Sutkowski et al., 1994; Juska and de Foresta, 1995; Dessauer et al., 1997; Tesmer et al., 1997; Yan et al., 1997). Several lines of evidence now indicate the presence of at least one binding site for the drug that resides on the catalyst, whose affinity for forskolin is enhanced by almost 500-fold in the presence of the G protein-derived $G_{\alpha s}$ subunit (Dessauer et al., 1997). The recently demonstrated atypical interaction between adenosine receptors and G proteins may explain the dramatic effect of adenosine antagonists on forskolin-dependent cAMP accumulation observed in PC12 cells. Compared with other G protein-coupled receptors, adenosine A_1 and A_2 receptors are tightly coupled to G proteins, with the ternary complex stabilized in a high-affinity conformation (Freissmuth et al., 1991; Nanoff et al., 1991, 1995; Nanoff and Stiles, 1993; Nanoff and Freissmuth, 1997). Displacement of endog-

enous adenosine from the receptor by A_2 receptor antagonists is likely to unhinge the equilibrium between endogenous adenosine and its binding site, thus facilitating the uncoupling of the ternary complex. This would finally result in a reduction of forskolin affinity toward its binding site, the complex $G_{\alpha s}$ subunit/catalyst. Interestingly, it has been reported that adenylyl cyclase type VI, which in PC12 cells mediates adenosine-dependent cAMP synthesis (Chern et al., 1995), is not detectable by photolabeling with iodinated forskolin derivatives in PC12 cell membranes unless $G_{\alpha s}$ subunits are present (McHugh Sutkowski et al., 1994).

The presence of adenosine antagonists would not affect the binding of forskolin on the catalyst, which is independent from the presence of $G_{\alpha s}$. In agreement with this hypothesis is the finding that the ability of forskolin to stimulate adenylyl cyclase was not totally dependent on adenosine receptor stimulation. In fact, even at maximally effective antagonist concentrations, a residual stimulatory response to forskolin was always found, resulting in a 25-fold increase of cAMP over basal levels.

The prominent role of adenosine in facilitating adenylyl cyclase activation by forskolin was further stressed by the finding that exogenous adenosine strongly potentiated cAMP synthesis and that the relative enhancing effect was more evident at low forskolin concentrations, which are apparently more largely dependent on occupancy of adenosine receptors. In fact, the response to 1 μM forskolin was increased 5-fold by 0.1 μM adenosine, whereas that of 10 μM forskolin was only doubled. The parallel leftward shift of the concentration-response curve for forskolin caused by adenosine indicates an increased affinity of the diterpene for its binding site on adenylyl cyclase.

As reported previously (Chern et al., 1993), the nonselective adenosine receptor agonist NECA was equieffective to the selective adenosine receptor agonist CGS 21680 in increasing cAMP levels. The effect of forskolin was potentiated by NECA and, to an even larger extent, by CGS 21680. These results straighten the conclusion that the enhancing effect is predominantly mediated by adenosine receptor of the A_{2A} subtype.

Another possibility worthy of investigation was that, besides adenosine, other endogenous purines might contribute in facilitating forskolin response. The present data indicate that, like adenosine, its precursor, AMP, causes intracellular accumulation of the cyclic nucleotide and significantly enhances forskolin activity when added exogenously. In contrast, inosine, the deamination product of adenosine, neither alters basal cAMP nor affects the response evoked by forskolin. The ability of 100 μM AMP to stimulate intracellular cAMP accumulation in the absence of forskolin was reduced by 50% by the inhibitor of ecto-5′-nucleotidase, AOPCP, and by the adenosine receptor antagonist 8-SPT, suggesting that conversion of AMP to adenosine by ecto-5′-nucleotidase and the interaction of the nucleoside with its receptors are at least in part responsible for the enhancing effect of AMP. In this respect, Roskoski and Roskoski (1989) reported that conversion of 100 μM [^{14}C]AMP into [^{14}C]adenosine by PC12 is relatively slow, with approximately 35% of the nucleotide being lost within 20 min of incubation. This may explain the lower potency of AMP in stimulating cAMP accumulation compared with adenosine or NECA. The stimulatory effect of 100 μM AMP was markedly inhibited by 1 U/ml ADA, and 5 U/ml concentration of the enzyme was previously shown to completely abolish 100 μM AMP-induced accumulation of cAMP in these cells (Yakushi et al., 1996). Thus, adenosine fully or at least in part accounts for the observed effects of AMP. It should be stressed that the cAMP response to forskolin in PC12 cells is inhibited by ADA but is not significantly affected by AOPCP (Florio et al., 1999). Thus, even though these cells are capable of releasing AMP (Braumann et al., 1986), which can undergo extracellular degradation to adenosine via ecto-nucleotidase, the latter pathway would not contribute significantly to the adenosine pool involved in the amplification of forskolin response.

In conclusion, the present study demonstrates that occupancy of adenosine A_{2A} receptors by adenosine receptor ligands strictly modulates the ability of forskolin to stimulate adenylyl cyclase and that endogenous adenosine has a determinant role in facilitating forskolin-dependent cAMP accumulation in PC12 cells.

Although no putative endogenous ligand for the forskolin-binding site or sites has been so far identified, the dependence of forskolin action on the neuroprotective metabolite adenosine opens new perspectives in the pharmacological control of neuronal function.

References

Arslan G, Kontny E and Fredholm BB (1997) Down regulation of adenosine A_{2A} receptors upon NGF-induced differentiation of PC12 cells. *Neuropharmacology* **36:**1319–1326.

Arslan G, Kull B and Fredholm BB (1999) Signaling via A_{2A} adenosine receptor in four PC12 cell clones. *Naunyn-Schmiedeberg's Arch Pharmacol* **359:**28–32.

Braumann T, Jastorff B and Richter-Landsberg C (1986) Fate of cyclic nucleotides in PC12 cell cultures: Uptake, metabolism, and effects of metabolites on nerve growth factor-induced neurite outgrowth. *J Neurochem* **47:**912–919.

Bruns RF, Fergus JH, Badger EW, Bristol JA, Santay LA and Hays SJ (1987) PD 115,199: An antagonist ligand for adenosine A_2 receptors. *Naunyn-Schmiedeberg's Arch Pharmacol* **335:**64–69.

Chae HD and Kim KT (1997) Stimulation of the A2A adenosine receptor increases expression of the tyrosine hydroxylase gene. *Mol Brain Res* **44:**31–38.

Chern Y, Chiou JY, Lai HL and Tsai MH (1995) Regulation of adenylyl cyclase type VI activity during desensitization of the A2a adenosine receptor-mediated cyclic AMP response: Role for protein phosphatase 2A. *Mol Pharmacol* **48:**1–8.

Chern Y, Lai HL, Fong JC and Liang Y (1993) Multiple mechanisms for desensitization of A2a adenosine receptor-mediated cAMP elevation in rat pheochromocytoma PC12 cells. *Mol Pharmacol* **44:**950–958.

Daly JW, Padgett WL and Shamim MT (1986) Analogues of caffeine and theophylline: Effect of structural alterations on affinity at adenosine receptors. *J Med Chem* **29:**1305–1308.

Dessauer CW, Scully TT and Gilman AG (1997) Interactions of forskolin and ATP with the cytosolic domains of mammalian adenylyl cyclase. *J Biol Chem* **272:** 22272–22277.

Florio C, Frausin F, Vertua R and Gaion RM (1999) Involvement of P_1 receptors in the effect of forskolin on cyclic AMP accumulation and export in PC12 cells. *Biochem Pharmacol* **57:**355–364.

Fredholm BB (1995) Purinoceptors in the nervous system. *Pharmacol Toxicol* **76:** 228–239.

Fredholm BB, Abbracchio MP, Burnstock G, Daly JW, Harden TK, Jacobson KA, Leff P and Williams M (1994) Nomenclature and classification of purinoceptors. *Pharmacol Rev* **46:**143–156.

Freissmuth M, Selzer E and Schütz W (1991) Interaction of purified bovine brain A_1-adenosine receptors with G-proteins. *Biochem J* **275:**651–656.

Hide I, Padgett WL, Jacobson KA and Daly JW (1992) A_{2A} adenosine receptors from rat striatum and rat pheochromocytoma PC12 cells: Characterization with radioligand binding and by activation of adenylate cyclase. *Mol Pharmacol* **41:**352–359.

Jacobson KA, Ukena D, Padgett WL, Daly JW and Kirk KL (1987) Xanthine functionalized congeners as potent ligands at A_2-adenosine receptors. *J Med Chem* **30:**211–214.

Jarvis MF, Schulz R, Hutchison AJ, Do UH, Sills MA and Williams M (1989) [^{3}H]CGS 21680, a selective A_2 adenosine receptor agonist directly labels A_2 receptors in rat brain. *J Pharmacol Exp Ther* **251:**888–893.

Juska A and de Foresta B (1995) Analysis of effects of corticotropin, forskolin and fluoride on activity of adenylate cyclase of bovine adrenal cortex. *Biochim Biophys Acta* **1236:**289–298.

Kim WK, Hawthorn MH and Rabin RA (1993) Chronic ethanol exposure potentiates muscarinic receptor and α_2-adrenoceptor-mediated inhibition of cAMP accumulation in PC12 cells. *Eur J Pharmacol* **247:**89–92.

Kirk IP and Richardson PJ (1995) Further characterization of [^{3}H]-CGS 21680 binding sites in the rat striatum and cortex. *Br J Pharmacol* **114:**537–543.

McHugh Sutkowski E, Tang WJ, Broome CW, Robbins JD and Seamon KB (1994)

Regulation of forskolin interactions with type I, II, V, and VI adenylyl cyclases by $G_{s\alpha}$. *Biochemistry* **33:**12852–12859.
McMahon A and Sabban EL (1992) Regulation of expression of dopamine β-hydroxylase in PC12 cells by glucocorticoids and cyclic AMP analogues. *J Neurochem* **59:**2040–2047.
Mundell SJ and Kelly E (1998) Evidence for co-expression and desensitization of A_{2a} and A_{2b} adenosine receptors in NG108–15 cells. *Biochem Pharmacol* **55:**595–603.
Nanoff C and Freissmuth M (1997) Coupling cofactor: A novel regulatory component in G protein-mediated signalling? *Physiol Res* **46:**79–87.
Nanoff C, Jacobson KA and Stiles GL (1991) The A_2 adenosine receptor: Guanine nucleotide modulation of agonist binding is enhanced by proteolysis. *Mol Pharmacol* **39:**130–135.
Nanoff C, Mitterauer T, Roka F, Hohenegger M and Freissmuth M (1995) Species differences in A_1 adenosine receptor/G protein coupling: Identification of a membrane protein that stabilizes the association of the receptor/G protein complex. *Mol Pharmacol* **48:**806–817.
Nanoff C and Stiles GL (1993) Solubilization and characterization of the A_2-adenosine receptor. *J Recept Res* **13:**961–973.
Nordstedt C and Fredholm BB (1990) A modification of a protein-binding method for rapid quantification of cAMP in cell-culture supernatants and body fluid. *Anal Biochem* **189:**231–234.
Noronha-Blob L, Marshall RP, Kinnier WJ and U'Prichard DC (1986) Pharmacological profile of adenosine A_2 receptor in PC12 cells. *Life Sci* **39:**1059–1067.
Oda H, Murayama T and Nomura Y (1995) Effect of protein kinase C and A activation on ATP-stimulated release of [^{3}H]noradrenaline from PC12 cells. *J Biochem* **118:**325–331.
Ono T, Matsuoka I, Ohkubo S, Kimura J and Nakanishi H (1998) Effects of YT-146 [2-(1-octynil) adenosine], an adenosine A_{2A} receptor agonist, on cAMP production and noradrenaline release in PC12. *Jpn J Pharmacol* **78:**269–277.
Palmer TM, Gettys TW, Jacobson KA and Stiles GL (1994) Desensitization of the canine A2a adenosine receptor: Delineation of multiple processes. *Mol Pharmacol* **45:**1082–1094.
Rabin RA, Fiorella D and Van Wylen DGL (1993) Role of extracellular adenosine in ethanol-induced desensitization of cyclic AMP production. *J Neurochem* **60:**1012–1017.
Roskoski R and Roskoski LM (1989) Adenosine receptor activation and the regulation of tyrosine hydroxylase activity in PC12 and PC18 cells. *J Neurochem* **53:**1934–1940.
Roth JA, Marcucci K, Lin WH, Napoli JL, Wagner JA and Rabin R (1991) Increase in beta-1,4-galactosyltransferase activity during PC12 cell differentiation induced by forskolin and 2-chloroadenosine. *J Neurochem* **57:**708–713.
Tesmer JJ, Sunahara RK, Gilman AG and Sprang SR (1997) Crystal structure of the catalytic domains of adenylyl cyclase in a complex with $G_{s\alpha}$ GTPγS. *Science (Wash DC)* **278:**1907–1916.
Van der Ploeg I, Ahlberg S, Parkinson FE and Fredholm BB (1996) Functional characterization of adenosine A_2 receptors in Jurkat cells and PC12 cells using adenosine receptor agonists. *Naunyn-Schmiedeberg's Arch Pharmacol* **353:**250–260.
Vossler MR, Yao H, York RD, Pan MG, Rim CS and Stork PJ (1997) cAMP activates MAP kinase and Elk-1 through a B-Raf- and Rap1-dependent pathway. *Cell* **89:**73–82.
Yakushi Y, Watanabe A, Murayama T and Nomura Y (1996) P_2 purinoceptor-mediated stimulation of adenylyl cyclase in PC12 cells. *Eur J Pharmacol* **314:**243–248.
Yan SZ, Huang ZH, Rao VD, Hurley JH and Tang WJ (1997) Three discrete regions of mammalian adenylyl cyclase form a site for $G_{s\alpha}$ activation. *J Biol Chem* **272:**18849–18854.
Zimmermann H and Braun N (1996) Extracellular metabolism of nucleotides in the nervous system. *J Auton Pharmacol* **16:**397–400.

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