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EFFECTS OF ADENOSINE ANALOGUES ON BASAL, PROSTAGLANDIN E_1- AND FORSKOLIN-STIMULATED CYCLIC AMP FORMATION IN INTACT NEUROBLASTOMA CELLS

MARY G. MURPHY* and ZENOBIA BYCZKO
Department of Pharmacology, Dalhousie University, Halifax, Nova Scotia, Canada B3H 4H7

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Abstract—We have examined the effects of *R*-phenylisopropyladenosine (*R*-PIA) and other adenosine analogues on basal, prostaglandin E_1 (PGE_1)- and forskolin-stimulated cyclic AMP (cAMP) formation in intact N1E-115 neuroblastoma cells, to determine whether the cells contain A_1 adenosine receptors that are negatively coupled with adenylate cyclase. Basal levels of cAMP (68 ± 7 pmol/mg protein; mean ± SE, N = 15) were not altered by low concentrations of *R*-PIA. The apparent lack of inhibition was not due to increases in cAMP due to activation of a stimulatory A_2 receptor by endogenously-synthesized adenosine. By comparison, low levels of *R*-PIA did reduce significantly ($P < 0.05$) PGE_1-dependent increases in cAMP formation (maximum response to PGE_1, 972 ± 77 pmol cAMP/mg protein; EC_{50} for PGE_1, 0.2 μM). Inhibition was dose dependent, and resulted in a 30–50% maximum reduction in production stimulated by PGE_1. Nanomolar concentrations of *R*-PIA elicited half-maximal inhibition; the inhibitory response was blocked by 8-phenyltheophylline (8-PT). The order of potencies of several adenosine analogues in eliciting this response suggested that inhibition was mediated by an A_1 adenosine receptor. Examination of the effects of *R*-PIA on forskolin-stimulated cAMP formation yielded several interesting findings. First, stimulation by the diterpene by itself was blocked by both adenosine deaminase (ADA) and 8-PT (40 and 25% inhibition respectively). Low concentrations of *R*-PIA ($<10^{-6}$ M) had no effect on forskolin-stimulated cAMP production. At higher levels ($\geq 10^{-6}$ M) the analogues acted synergistically with the diterpene, to yield cAMP levels that were up to 3-fold higher than the additive effect of the two agents. Potentiation was stereospecific, Ca^{2+} dependent, and was blocked by 8-PT. The results of this study suggest that, in N1E-115 neuroblastoma cells, inhibitory A_1 receptors are not stimulated in response to non-specific elevations in cAMP, but are associated with specific stimulatory receptors such as those activated by PGE_1.

Adenosine is widely recognized to be an important modulator of central nervous system function [1]; however, the molecular mechanisms whereby it exerts its effects are largely unknown. One possible mechanism involves modulation of adenylate cyclase activity [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1], and, as such, intracellular levels of the second messenger, cyclic AMP (cAMP†). At high (micromolar) concentrations, adenosine activates an extracellular receptor, designated A_2, which stimulates adenylate cyclase activity in a GTP-dependent manner. This stimulatory receptor has been demonstrated in a variety of organs and cell types, including transformed lines of neuroblastoma [2–7] and neuroblastoma × glioma hybrid cells [8, 9], and in primary cultures of neural cells [10, 11]. The physiological function(s) of stimulatory receptors in the CNS has not been defined.

A second set of pharmacologically-distinct adenosine receptors, designated A_1, appears to be the major modulators of electrophysiological activity in the CNS, and at least some of the modulatory effects are related to inhibition of adenylate cyclase activity [12, 13]. Van Calker *et al.* [10, 14] found that activation of A_1 receptors at nanomolar concentrations of adenosine results in inhibition of isoproterenol-stimulated cAMP formation in primary cultures of rat astrocytes. A human astrocytoma cell line, clone 1321-N1, has also been shown to contain inhibitory A_1 adenosine receptors [15]. However, data from recent studies have suggested that the neuroendocrine pheochromocytoma cell line, PC_{12}, has only stimulatory A_2 receptors [16, 17]. To date, no one has ascertained whether neuroblastoma cell lines contain adenosine receptors that are negatively linked with adenylate cyclase.

In this laboratory, one major focus concerns the molecular mechanisms of receptor function in cultured neuroblastoma cells, with particular emphasis on receptor regulation by membrane polyunsaturated fatty acids (PUFA) [18]. Prior to examining the effects of PUFA on A_1 receptor function, however, it was first necessary to demonstrate that N1E-115 neuroblastoma cells contain these inhibitory receptors.

MATERIALS AND METHODS

Materials. *R*(*S*)-N^6-(2-Phenylisopropyl)adenosine

* Correspondence: Dr M. G. Murphy, Department of Pharmacology, Sir Charles Tupper Medical School, Dalhousie University, Halifax, Nova Scotia, Canada B3H 4H7.

† Abbreviations: cAMP, cyclic AMP; *R*(*S*)-PIA, *R*(*S*)-N^6-(phenylisopropyl)adenosine; NECA, 5′-*N*-ethylcarboxyamide adenosine; CPA, N^6-cyclopentyladenosine; PGE_1, prostaglandin E_1; 8-PT, 8-phenyltheophylline; ADA, adenosine deaminase; Ro 20-1724, 4-(3-butoxy-4-methoxybenzyl)imiazolidin-2-one; DMEM, Dulbecco's modified Eagle's medium; and HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

(*R*(*S*)-PIA), 5′-*N*-ethylcarboxyamide adenosine (NECA), N^6-cyclopentyladenosine (CPA), and 8-phenyltheophylline (8-PT) were purchased from Research Biochemicals Inc. (Natick, MA). Forskolin (7β - acetoxy-8,13-epoxy-1α,6β,9α-trihydroxy-labd-14-ene-11-one) was obtained from Calbiochem Behring (LaJolla, CA). Ro 20-1724 [4-(3-butoxy-4-methoxybenzyl)imiazolidin-2-one] was provided by Hoffmann–LaRoche Ltd. (Etobicoke, Canada). Other chemicals were purchased from the Sigma Chemical Co. (St Louis, MO).

Cell culture. Murine neuroblastoma cells, clone N1E-115, were a gift from Dr E Richelson. The cells were seeded at a density of 5–7 × 10^4 cells/ml into 35 mm Primaria culture dishes (Becton Dickinson, Mississauga, Canada) in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal bovine serum (Flow Laboratories, Mississauga, Canada). Cultures were maintained in a humidified atmosphere of 5% CO_2/95% air at 37°, and subculture was carried out every 5–7 days. Only cells between passages 18 and 35 were used for this study.

Cyclic AMP assays. Three days following subculture, the medium was aspirated and the cells were washed with DMEM buffered at pH 7.6 with 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid). Incubation medium (serum-free HEPES-buffered DMEM containing 0.7 mM Ro 20-1724) was added to each dish, and the incubation was carried out at 30° for a total of 40 min. Where indicated, the adenosine agonists were added in 100 μl HEPES/DMEM 20 min after the incubation was initiated. When prostaglandin E_1 (PGE_1) or forskolin were included in the assays, each was added in 10 μl ethanol 10 min prior to termination of the assay. When the antagonist 8-PT was included, it was added in 0.01 N NaOH at the beginning of the assay. Cyclic AMP formation was not affected by addition of either ethanol or 0.01 N NaOH alone. Adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4; 1.5 units/ml) was added to the culture dishes a minimum of 1 hr before assay and was included throughout the 40-min incubation period.

Reactions were terminated by aspirating the medium, and the cells were washed with ice-cold HEPES-buffered DMEM. Ethanol (250 μl) was added to the dishes, and the cells were dislodged and disrupted by brief sonication. The dish contents were transferred to disposable tubes, and membranes and supernatant fractions were separated by centrifugation (1000 *g*, 15 min). The membrane pellets were digested overnight in 1 N NaOH for protein analysis according to Lowry *et al.* [19], using bovine serum albumin (BSA) as the standard. Supernatant fractions were dried under N_2, and the residues were resuspended in 50 mM sodium acetate buffer (pH 4.5) containing 4 mM EDTA; duplicate aliquots from each dish were analyzed for cyclic AMP according to Gilman [20]. Each assay was carried out in triplicate dishes.

Statistical analysis. Data are the means (± SE) of values obtained from triplicate dishes in two or more independent experiments. Data were analyzed using Student's *t*-test for unpaired data. Differences were considered significant at a level of $P < 0.05$.

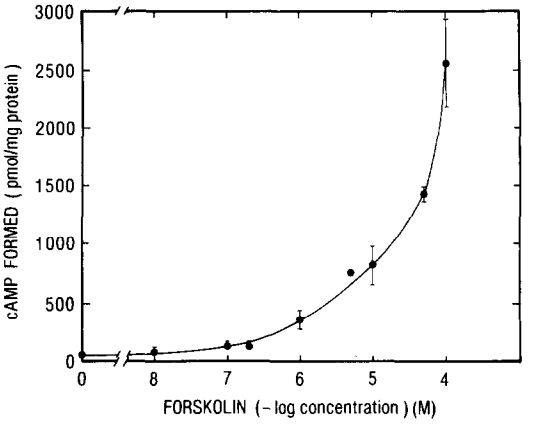

Fig. 1. Effect of forskolin on cyclic AMP formation in N1E-115 neuroblastoma cells. Three days following subculture, the cells were washed and assayed *in situ* in HEPES-buffered DMEM containing 0.7 mM Ro 20-1724. Incubations were carried out at 30° for a total of 40 min. Forskolin was added at the concentrations indicated for the final 10 min of the assay. The data are means (± SE) of values obtained in three separate experiments, each of which was carried out in triplicate dishes.

RESULTS

Basal and stimulated cyclic AMP formation. Incubation of intact N1E-115 neuroblastoma cells for 40 min in the presence of the phosphodiesterase inhibitor, Ro 20-1724, resulted in an intracellular accumulation of 40-100 pmol cAMP/mg protein. Addition of PGE_1 for the final 10 min of the assay produced rapid, dose-dependent increases in cyclic AMP formation [18]. Eadie–Hofstee analysis of four separate saturation experiments indicated 15- to 20-fold increases in cAMP formation (mean maximum response, 970 ± 80 pmol cAMP/mg protein) with an EC_{50} value for PGE_1 of approximately 0.2 μM. Addition of the plant diterpene, forskolin, for the final 10 min of the assay also increased cyclic nucleotide formation significantly (Fig. 1). In three separate saturation experiments, synthesis of cAMP increased exponentially as concentrations of forskolin were increased above 10^{-6} M. Maximum values (pmol cAMP/mg protein) obtained with 10^{-4} M forskolin ranged from 1960 to 3240 in the three experiments. Since there was no apparent saturation of the response even at the highest concentration of forskolin tested, it was not possible to calculate a value for EC_{50}. However, inspection of each of the concentration–response curves suggested values in excess of 10 μM. Values in this range are consistent with those reported by Seamon and Daly [21] and by Stenstrom *et al* [22].

Effects of adenosine deaminase on basal and stimulated cAMP formation. Cultured cells of neural origin have been shown to release adenosine into the culture medium at concentrations that could partially occupy adenosine receptors and alter the effects of exogenously-added agents [23]. Accordingly, we examined the effects on cAMP production of treating the cells with adenosine deaminase (ADA) for 1–3 hr before, and during, the 40-min incubation. This

Table 1. Effects of adenosine deaminase and 8-phenyltheophylline on *R*-PIA-dependent modulation of basal, PGE_1- and forskolin-stimulated cAMP formation

Assay additions	cAMP formation (pmol/mg protein)		
	−ADA, −8PT	+ADA (1.5 units/ml)	+8PT (10^{-4} M)
None (basal)	68 ± 7	66 ± 2	67 ± 3
R-PIA (10^{-6} M)	117 ± 9	129 ± 4	80 ± 10
PGE_1 (10^{-6} M)	1112 ± 140	1038 ± 38	1177 ± 29
PGE_1 (10^{-6} M) + *R*-PIA (10^{-6} M)	788 ± 147	710 ± 25	1143 ± 95
Forskolin (10^{-5} M)	1057 ± 201	640 ± 66	799 ± 65
Forskolin (10^{-5} M) + *R*-PIA (10^{-6} M)	2240 ± 298	2329, 2460	1264 ± 232

† Data are the means (± SE) of values obtained from three to six separate experiments, each of which was run in triplicate dishes. Where two values are shown, they represent means of data obtained from individual experiments. The cells were subcultured and seeded as described in Materials and Methods. After 3 days in culture, the cells were washed and incubated in HEPES-buffered serum-free medium containing Ro 20-1724. Where indicated, *R*-PIA was added after 20 min of incubation; PGE_1 or forskolin was added 10 min later. For the experiments with adenosine deaminase, the cells were exposed to the enzyme (1.5 units/ml) for at least 1 hr before and throughout the 40-min assay. Where indicated, 8-PT was added at the beginning of the incubation.

treatment completely blocked the stimulatory effect of exogenously-added adenosine (10^{-4} M), reducing levels of cAMP formed from 295 ± 16 (−ADA) to 69 ± 2 (+ADA) pmol/mg protein (not shown). However, as shown in Table 1, exposure of the cells to the enzyme did not alter basal cAMP production appreciably, nor did it change cyclic nucleotide formation stimulated by *R*-PIA or PGE_1. Surprisingly, exposure to ADA did have a marked effect on forskolin-stimulated cAMP formation, reducing levels to between 40 and 70% of those that accumulated in its absence. Inclusion of 8-PT in the incubations with forskolin also reduced markedly the levels of cAMP formed (Table 1).

Effect of R-*PIA on basal cAMP formation.* Addition of *R*-PIA (10^{-10} to 10^{-7} M) to the incubation dishes for 20 min had essentially no effect on the amount of cAMP formed under basal assay conditions (data not shown). This did not reflect a time-dependent reduction in responsiveness, as no effect was seen even when the time of exposure of the cells to *R*-PIA was reduced to 3 min (data not shown). When the concentration of *R*-PIA was increased above 10^{-7} M, nucleotide accumulation was elevated to maximum responses of 264 and 306 pmol cAMP/mg protein in two separate saturation experiments, with a mean value for EC_{50} of 5 μM. The results of studies of the effects of other adenosine analogues on the stimulatory response indicated the rank order of their potencies to be NECA > *R*-PIA > CPA, which suggested activation of an A_2 adenosine receptor (Fig. 2) [24]. PIA-dependent enhancement was stereospecific (maximum response to *S*-PIA, 140 pmol cAMP/mg protein), and was blocked by inclusion of 8-phenyltheophylline in the incubation medium (Table 1).

Effects of adenosine analogues on PGE_1*-stimulated cAMP formation.* When *R*-PIA was added to the assay dishes 10 min before PGE_1, the stimulatory effect of PGE_1 was reduced in a dose-dependent manner (Fig. 3A). Maximum inhibition of PGE_1-dependent accumulation ranged from 30–50%; half-maximal inhibition required concentrations of agonist in the low (1–10) nanomolar range. Values obtained at and above 10^{-9} M *R*-PIA were significantly lower than those determined in the presence of PGE_1 alone. The inhibitory effect of *R*-PIA was not altered appreciably by treatment with ADA and was blocked completely when 8-PT was included in the incubation mixture (Table 1). Inhibition was stereospecific, since *S*-PIA was only half as effective as *R*-PIA in blocking PGE_1-stimulated enzyme activity (not shown). To determine whether the effects observed were consistent with activation of an A_1 adenosine receptor, we examined the abilities

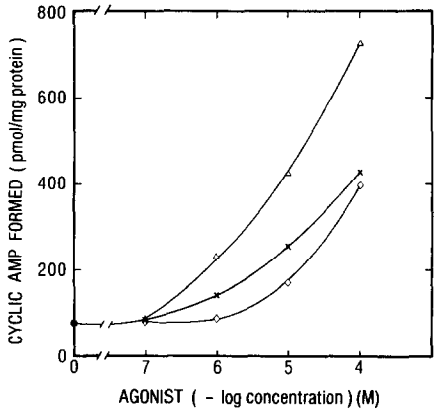

Fig. 2. Stimulatory effects of adenosine analogues on cAMP formation in intact N1E-115 neuroblastoma cells. The analogues, NECA (△), *R*-PIA (×) or CPA (◇), were added at the concentrations indicated after a 20 min preincubation of the cells with Ro 20-1724 (0.7 mM). Incubation was continued for 20 min, and the assays were terminated and processed as described in the text. The data are valucs obtained in one of three separate experiments, each of which was run in triplicate. In each of these experiments the cells had been preincubated for 1–3 hr with adenosine deaminase (1.5 units/ml), and ADA was included throughout the 40-min incubation. Individual values varied from the means by less than 10%.

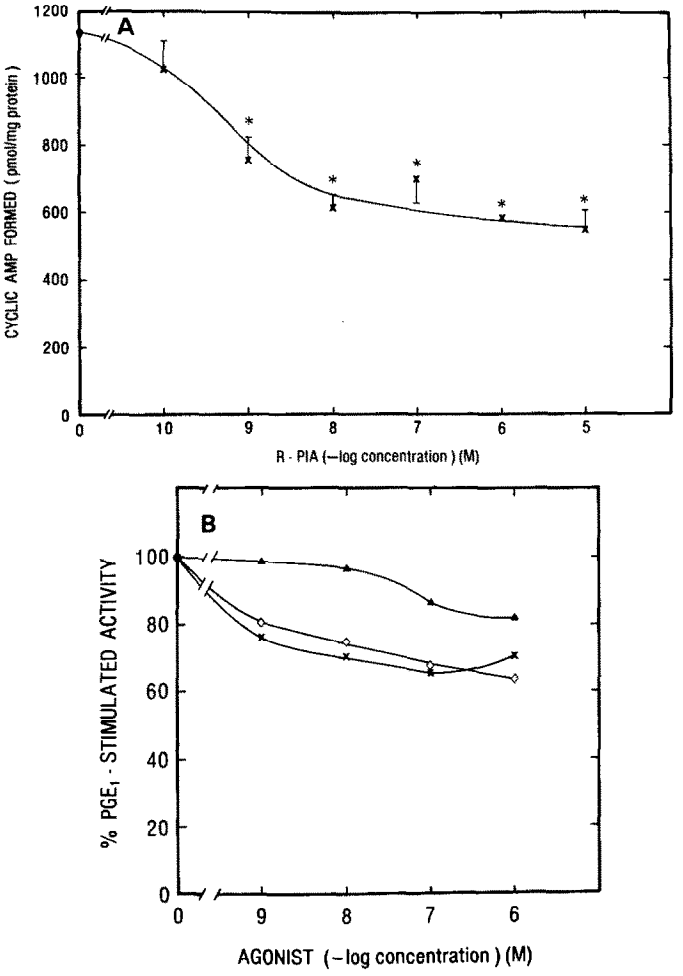

Fig. 3. (A) Inhibition of prostaglandin E_1-stimulated cyclic AMP formation by *R*-PIA. Assays were carried out *in situ* as described in the legend to Fig. 1. PGE_1 was added 10 min before the termination of the assay, and *R*-PIA was added at the concentrations indicated, 10 min prior to PGE_1. The data are means (± SE) of values obtained in one of four separate experiments. Asterisks denote values that differ significantly ($P < 0.05$) from those obtained with PGE_1 alone. (B) Inhibition of PGE_1-stimulated cAMP formation by adenosine analogues. The analogues [NECA (▲), *R*-PIA (×) and CPA (◇)] were added as described for panel A, and PGE_1 (10^{-6} M) was added 10 min later. In the absence of the analogues, 1370 pmol cAMP were formed per mg protein during the 10-min exposure to PGE_1. The data are values obtained in one of three separate experiments, each of which was run in triplicate. In each of these experiments the cells had been preincubated for 1–3 hr with adenosine deaminase (1.5 units/ml), and ADA was included throughout the 40-min incubation. Individual values varied from the means by less than 10%.

of two other adenosine analogues to block PGE_1-stimulated cAMP formation. As shown in Fig. 3B, *N*-ethylcarboxyamide adenosine (NECA), an analogue that acts at both receptors but has a relatively low potency at the A_1 site [24], was least effective of the three in blocking PGE_1-mediated cAMP accumulation. Cyclopentyladenosine (CPA), a selective and potent A_1 agonist, was equivalent to *R*-PIA in eliciting the inhibitory response.

Effects of R-PIA on forskolin-stimulated cAMP formation. To determine whether the inhibitory adenosine receptors functioned only in association with receptors that activated adenylate cyclase, or whether they could be activated nonselectively in response to elevated cyclic nucleotide levels, we examined the effects of *R*-PIA on forskolin-stimulated cAMP formation. As shown in Fig. 4, addition of *R*-PIA at concentrations up to 10^{-7} M did not alter appreciably levels formed in the presence of the diterpene alone. However, in both the presence and absence of adenosine deaminase, further increases in the adenosine analogue had a synergistic effect with forskolin, resulting in levels of cAMP significantly in excess (> 3-fold) of those that would have resulted if the effects of the two agents had been additive. The forskolin-mediated potentiation also increased the potency of *R*-PIA in eliciting the stimulatory response, as evidenced by a reduction in EC_{50} values from 5 to 1 μM. Potentiation was stereospecific (Fig. 4), and Ca^{2+} dependent, as it was

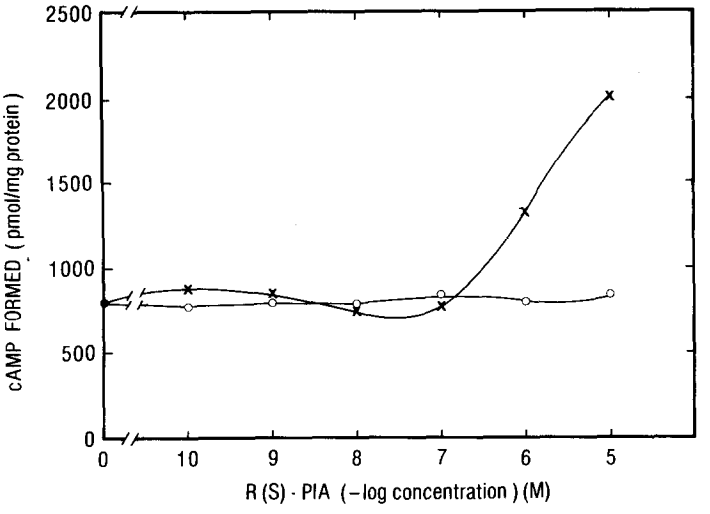

Fig. 4. Forskolin-dependent potentiation of *R*-PIA-stimulated cAMP formation in N1E-115 neuroblastoma cells. *R*-PIA (×) or *S*-PIA (○) and forskolin (10^{-5} M) were added at 20 and 30 min, respectively, of the 40 min incubation. Assay termination and processing are described in the text. Data are means of values obtained in one of four separate experiments. Individual values differed from the means by less than 10%.

blocked by inclusion of ethyleneglycolbis(aminoethylether)tetra-acetate (EGTA) (2 mM) in the incubation medium (data not shown). This concentration of the calcium chelator did not alter responses to *R*-PIA or forskolin alone (not shown). Forskolin-dependent potentiation of the *R*-PIA response was blocked almost completely (>90%) by inclusion of ADA in the incubation medium (Table 1).

DISCUSSION

The purpose of the present study was to determine whether there were inhibitory adenosine receptors on N1E-115 neuroblastoma cells. Data from initial studies clearly showed that the A_1-selective adenosine agonist, *R*-PIA, did not alter basal formation of cAMP at the low concentrations of agonist known to activate this receptor. Higher concentrations of *R*-PIA activated adenylate cyclase appreciably, and we (Fig. 2) and others [2] have provided evidence that this involves the stimulatory A_2 receptor. The lack of obvious inhibition at low concentrations of *R*-PIA could have at least two explanations in addition to the possibility that there are no A_1 receptors on these cells. The first would be if the cells had become desensitized to *R*-PIA over the 20-min course of exposure, and that during this time basal adenylate cyclase had increased to a level where it compensated for the short-term loss in cAMP synthesis. This seems unlikely, since we did not see any effects of low amounts of *R*-PIA on basal cyclic nucleotide formation even when the incubation time was reduced to 3 min (not shown). A second explanation would have been that the cells were releasing endogenously-synthesized adenosine into the culture medium in amounts that were sufficient to activate A_2 stimulatory receptors to the extent that *R*-PIA-dependent losses in cAMP were countered by activation of the A_2 site by the native agonist. This phenomenon has been evident in studies with both astrocytoma [15] and some neuroblastoma [23] cell lines. However, this proved an unlikely possibility in the case of N1E-115, since cAMP synthesis was not altered by *R*-PIA when the cells were exposed to adenosine deaminase under conditions (1.5 units/ml, ≥ 1 hr plus 40 min) that were sufficient to abrogate the effects of 10^{-4} M exogenously-added adenosine (not shown). The notion that N1E-115 cells do not release sufficient adenosine into the incubation mixture to alter adenylate cyclase activity appreciably was further supported by the finding that the potent adenosine antagonist, 8-phenyltheophylline, also did not affect basal cAMP synthesis. Thus, it would appear that N1E-115 cells do not release adenosine under our assay conditions.

The second approach that we used to ascertain whether these cells contained inhibitory adenosine receptors was to determine whether the analogues could block cAMP formation stimulated by other agents. This is an approach frequently used by investigators, since it serves to magnify inhibitory responses that may not be evident if basal adenylate cyclase activities are low. The data from this study clearly demonstrated that *R*-PIA significantly blocked PGE_1-stimulated synthesis of cAMP in N1E-115 neuroblastoma cells. From the few studies that have examined the effects of interactions between these two agonists, Penit *et al.* [4] and Matsuzawa and Nirenberg [8] reported that high concentrations of adenosine have only weak inhibitory effects on PGE_1-dependent adenylate cyclase activity, and the attempt by Penit *et al.* to demonstrate theophylline blockade was unsuccessful. We have also found theophylline to be a weak antagonist in the intact cell assay. Data from other studies have suggested that some cell lines, including the NS20 line examined by Blume and Foster [5], do not contain inhibitory adenosine receptors. Noronha-Blob and coworkers

[16] concluded that PC_{12} cells also lack functional A_1 receptors, based on the failure of *R*-PIA to inhibit basal or forskolin-stimulated adenylate cyclase activity. Interestingly, both the PC_{12} studies and those of Blume and Foster were carried out using cell homogenates, and it is conceivable that expression of the inhibitory response requires cellular integrity.

In carrying out these studies, it would have been interesting to know whether the A_1 receptor interacted with other stimulatory receptors, such as those for catecholamines, or whether adenosine-mediated inhibition was associated only with specific stimulatory sites. However, this question could not be addressed with the N1E-115 cells, since there are no stimulatory adrenergic or dopaminergic receptors on N1E-115 neuroblastoma cells (unpublished observations). The only two stimulatory receptors that we have identified in this cell line are those for PGE_1 and for adenosine (Fig. 2). As an alternative approach, we examined the effects of *R*-PIA on forskolin-stimulated cAMP formation, to determine whether the analogue would block synthesis due to direct activation of the catalytic unit of adenylate cyclase. However, the data in Fig. 4 indicated that this was not the case. The fact that low concentrations of *R*-PIA failed to inhibit forskolin-stimulated cAMP formation was surprising, since one would predict that occupation of receptors that were negatively coupled with the catalytic unit would block its activity. At least one explanation for the lack of inhibition is that forskolin enhances the release of endogenously-synthesized adenosine in amounts that are sufficient to produce compensatory increases in cAMP by occupying and activating the A_2 receptors. This phenomenon is discussed in greater detail below. At concentrations of either *R*-PIA or adenosine (not shown) above 10^{-7} M, there was significant, dose-dependent potentiation of the stimulatory effect of forskolin. This was not surprising, since, in addition to its ability to activate adenylate cyclase direcly, forskolin is known to potentiate stimulatory responses to other agonists, including catecholamines [25–27], serotonin [28], histamine [29] and adenosine [25, 30]. Using C6-2B glioma cells, Barovsky *et al.* found that the synergism between isoproterenol and forskolin is characterized by increases in both potency and efficacy for the hormone [26], which is consistent with our findings. The precise site(s) and mechanism(s) of action of forskolin in the potentiation phenomenon are not well understood at present, although it appears to involve an enhanced interaction between the regulatory protein, N_s, and the catalytic unit of adenylate cyclase [27, 31, 32]. Observations that synergism is more pronounced in intact cells than in membrane preparations suggest that forskolin may act by stabilizing the membrane [33]. If this is the case, it could influence adenosine receptor conformation in such a manner that agonist binding is more effective, or it could stabilize one or more post-receptor events [31]. One finding that was particularly interesting in this study was that inclusion of ADA or 8-PT in the incubation mixture dramatically reduced forskolin-stimulated cAMP formation (40 and 25% reductions respectively). By contrast, Seamon *et al.* [25] had reported earlier that forskolin-dependent stimulation of cAMP formation in rat cerebral cortical slices is not blocked by a variety of neurotransmitter antagonists, including 8-PT. Whether this reflects a distinction between the transformed cell lines and neural slices, or a limited accessibility of the antagonist in the latter, is not known at present. The most likely explanation for our findings with the neuroblastoma line is that forskolin stimulates release of endogenous adenosine (and/or nucleotides that are catabolized extracellularly to adenosine) in quantities sufficiently high to stimulate the A_2 receptors. However, the fact that neither basal nor PGE_1-stimulated cAMP formation was reduced in the presence of ADA or 8-PT suggests that, under these conditions at least, there is not a large amount of endogenous substrate acting at the A_2 receptors.

In summary, the data that we have obtained from the present study provides strong evidence that neuroblastoma cells contain adenosine receptors that are negatively linked with adenylate cyclase. Since low concentrations of the selective A_1 analogue, *R*-PIA, did not inhibit basal or forskolin-stimulated cAMP production, we perceive the A_1 receptor as being one that operates in close association with cyclase-stimulating receptors. In the case of N1E-115 cells, the stimulatory receptor is that which is activated by PGE_1. The levels of *R*-PIA required for half-maximal inhibition were in the low nanomolar range (Fig. 3), which is consistent with the known potency of agonists at the A_1 receptor [14]. *R*-PIA and CPA were considerably more potent in eliciting the inhibitory response than was NECA, which is also consistent with activation of the A_1 site [24]. It is interesting to speculate that one of the mechanisms of adenosine neuroregulation may involve its ability to reduce (or prevent) increases in cyclic nucleotides resulting from enhanced synthesis and release of prostaglandins, for example, during cerebral stress.

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