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Involvement of P_1 Receptors in the Effect of Forskolin on Cyclic AMP Accumulation and Export in PC12 Cells

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ABSTRACT. In PC12 cells, forskolin as well as the adenosine receptor agonist 5′-*N*-ethylcarboxamidoadenosine (NECA) increased intracellular adenosine-3′,5′-cyclic monophosphate (cyclic AMP) levels, which peaked at 45–60 minutes and declined thereafter. Maximum levels were 3000 and 1700 pmol/10^6 cells during treatment with 10 μM forskolin or 0.1 μM NECA, respectively. Extracellular cyclic AMP rose with time, at mean rates of 24.7 (forskolin) and 11.3 (NECA) pmol/min/10^6 cells. With either drug, a linear correlation was obtained between the calculated time integral of intracellular cyclic AMP and the measured extracellular cyclic AMP levels, indicating that the outflow of cyclic AMP was sustained by a nonsaturated transport system. The ability of forskolin to increase intracellular and extracellular cyclic AMP levels was hindered in a concentration-dependent manner by 8-(*p*-sulfophenyl)theophylline (8-SPT). A similar inhibition was exerted by other two adenosine receptor antagonists, 8-cyclopentyl-1,3-dipropylxanthine and 3,7-dimethyl-1-propargylxanthine. The concentration-response curve to adenosine was shifted to the right by 25 μM 8-SPT, whereas that of forskolin was shifted downwards. Adenosine deaminase (ADA, EC 3.5.44, 1 U/mL) reduced the intracellular cyclic AMP response to forskolin by 68%, whereas the adenosine transport inhibitor, dipyridamole (10 μM), significantly increased 1 and 10 μM forskolin-dependent cyclic AMP accumulation. Erythro-9-(2-hydroxy-3-nonyl)adenine (10 μM), an inhibitor of ADA, and α,β-methyleneadenosine 5′-diphosphate (100 μM), an inhibitor of ecto-5′-nucleotidase, did not alter forskolin activity. These results demonstrate that a cyclic AMP extrusion system operates in PC12 cells during adenylyl cyclase stimulation by forskolin and that this stimulation involves a synergistic interaction with endogenous adenosine. However, extruded cyclic AMP does not appear to significantly contribute to the formation of the endogenous adenosine pool. BIOCHEM PHARMACOL **57**;4: 355–364, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. adenosine deaminase; cyclic AMP efflux; intracellular cyclic AMP; forskolin; PC12 cells; 8-(*p*-sulfophenyl)theophylline

Cyclic AMP† is an ubiquitous intracellular second messenger that mediates short- and long-term effects of several hormones and neurotransmitters in living cells. Most of our knowledge on the physiopathological role of cyclic AMP is based on studies dealing with the intracellular changes in this cyclic nucleotide. Much less attention has been paid to the functional significance of extracellular levels of cyclic AMP, even though several cells release cyclic AMP into the extracellular environment by means of an energy-dependent transporter [1, 2]. Like intracellular cyclic AMP, extracellular cyclic AMP influences cellular functions by acting on membrane-bound protein kinases [3, 4] and on cation channels [5]. Furthermore, the cyclic nucleotide may act as a signal molecule after metabolic degradation in the extracellular space [6, 7].

Intracellular and extracellular variations of cyclic AMP levels can be monitored *in vitro* and *in vivo* following activation of adenylyl cyclase by either receptor-dependent or -independent mechanisms [8, 9]. Forskolin, a natural diterpene extracted from the plant *Coleus forskohlii*, stimulates adenylyl cyclase activity by a direct interaction with the catalytic subunit of the enzyme [10]. Because of this unique ability, forskolin is used extensively to investigate the functional consequences of increased cyclic AMP. In rat brain slices, however, forskolin-induced cyclic AMP accumulation is reduced by the adenosine antagonists caffeine and 8-sulfophenyltheophylline [11, 12], and inhibition of forskolin activity is also observed following treatment with adenosine deaminase, the enzyme that hydrolyzes adenosine to the inactive compound, inosine [13, 14]. It thus appears that in some tissues the ability of forskolin

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† *Abbreviations:* AOPCP, α,β-methyleneadenosine-5′-diphosphate; ADA, adenosine deaminase; cyclic AMP, adenosine-3′,5′-cyclic monophosphate; DMPX, 3,7-dimethyl-1-propargylxanthine; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; EHNA, erythro-9-(2-hydroxy-3-nonyl)adenine; NECA, 5′-*N*-ethylcarboxamidoadenosine; and 8-SPT, 8-(*p*-sulfophenyl)theophylline.

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to stimulate cyclic AMP production is partly dependent upon stimulation of P_1 purinergic receptors by endogenous adenosine.

In the rat pheochromocytoma PC12 cell line, both forskolin and adenosine cause large accumulation of cyclic AMP [15, 16]. In these cells, adenosine activity is mediated only by the adenosine A_2 receptors, positively linked to adenylyl cyclase, while functional A_1 receptors, negatively coupled to the enzyme, are lacking [17]. These cells are hence a good model for studying the intracellular and extracellular changes in cyclic AMP levels that follow the challenge with forskolin and with adenosine and for investigating the role of P_1 purinergic receptors in forskolin activity.

The aim of this study was first to characterize the process of cyclic AMP synthesis and possible outflow during activation of adenylyl cyclase by a receptor-independent, as well as by a receptor-dependent mechanism. For this purpose, PC12 cells were exposed to forskolin and to the hydrolysis-resistant adenosine agonist, NECA, respectively. Second, considering that adenosine is known to facilitate the ability of several neurotransmitters to stimulate adenylyl cyclase activity, the possible involvement of adenosine receptors in modulating the intracellular and extracellular levels of cyclic AMP in response to forskolin was evaluated. Finally, an attempt was made to evaluate the contribution of extracellular cyclic AMP to the formation of the extracellular pool of adenosine.

MATERIALS AND METHODS

Materials

[2,8-^{3}H] Adenosine 3′,5′-cyclic phosphate (specific activity 27 Ci/mmol) was obtained from Du Pont New England Nuclear. Nonlabeled cyclic AMP, adenosine, ADA type VIII (175 U/mg, 25 mg/mL), AOPCP, dipyridamole, and probenecid were obtained from Sigma Chemical Co. Forskolin, 8-SPT, DPCPX, DMPX, and NECA were from Research Biochemicals International. EHNA was from Burroughs Wellcome Co.

Cell Culture

PC12 cells were cultured in RPMI-1640 (Gibco BRL) supplemented with 10% heat-inactivated horse serum (Sigma), 5% fetal bovine serum (Gibco BRL), 100 U/mL of penicillin, and 100 μg/mL of streptomycin (Sigma) at 37° in a humidified 95% air/5% CO_2 atmosphere. The cells were routinely subcultured once weekly. For determination of cyclic AMP production, the cells were seeded at a cell density of 0.3×10^6 cells in 24-well Primaria surface plates (Falcon, Becton Dickinson) 48 hr before the experiments.

Production of Cyclic AMP

Cells were preincubated for 30 min at 37° in 1 mL/well of HEPES-buffered culture medium, pH 7.4, containing 1% horse serum-fetal bovine serum. Reactions were started by removing the preincubation medium by aspiration, followed by the immediate addition of 1 mL of the same medium containing test agents or vehicle. The reactions were terminated after the appropriate time by removal of medium and addition of 0.25 mL of ice-cold 0.084 N hydrochloric acid to each well. The cells were sonicated and the supernatants neutralized by addition of 0.25 mL of ice-cold 0.1 M tris[hydroxymethyl]amino-methane. Supernatants were collected in Eppendorf tubes, centrifuged in an Eppendorf microfuge for 1 min, and stored at −20°. When indicated, the incubation media from each well were collected individually in Eppendorf tubes and stored at −20° for analysis of cyclic AMP content. At the end of each experiment, the cells present in one well not employed for the determination of cyclic AMP synthesis were detached by vigorous pipetting and counted. The cell number ranged from 350,000 to 500,000 cells/well.

Cyclic AMP Assay

Aliquots of cell supernatants or of cell media (50 μL) were assayed according to the modified protein-binding method described by Nordstedt and Fredholm [18], with slight modifications. Samples or unlabeled cyclic AMP for standard curves (0–8 pmol), binding protein diluted in 100 mM Tris–HCl, pH 7.4, containing 250 mM NaCl and 10 mM EDTA, and [^{3}H] cyclic AMP (0.5 pmol, final concentration) were added to a 96-well MultiScreen-FB microtiter plate (Millipore) in a final volume of 250 μL. After 150 min at 4°, the plate was placed on a MultiScreen vacuum manifold (Millipore) and vacuum was applied. Wells were washed twice with 200 μL of ice-cold Tris–HCl, 50 mM, pH 7.4. After washing, the flexible plate underdrain was removed and the plate placed inside the Wallac cassette for Millipore MultiScreen Filtration Plates. SuperMix liquid scintillation cocktail (25 μL/well) was added and the plate counted in a MicroBeta Trilux liquid scintillation counter (Wallac).

The amount of cyclic AMP in cell supernatants and in cell medium was determined by interpolation of the number of cpm of the sample from the linear portion of the standard curve by nonlinear regression [19]. The specificity of the protein-binding assay was assessed using increasing concentrations of AMP and adenosine, at a constant volume of 50 μL. No significant displacement of [^{3}H] cyclic AMP was observed at concentrations of AMP and adenosine up to 10,000 pmoles/well.

Calculations and Statistical Analysis

The computer program SigmaPlot (Jandel Scientific) was used to generate EC_{50} parameters for concentration-response curves. Statistical analysis of differences between means was performed by using the Student's unpaired *t*-test.

Fractional release rates were calculated by plotting the cumulative extracellular cyclic AMP as a function of the

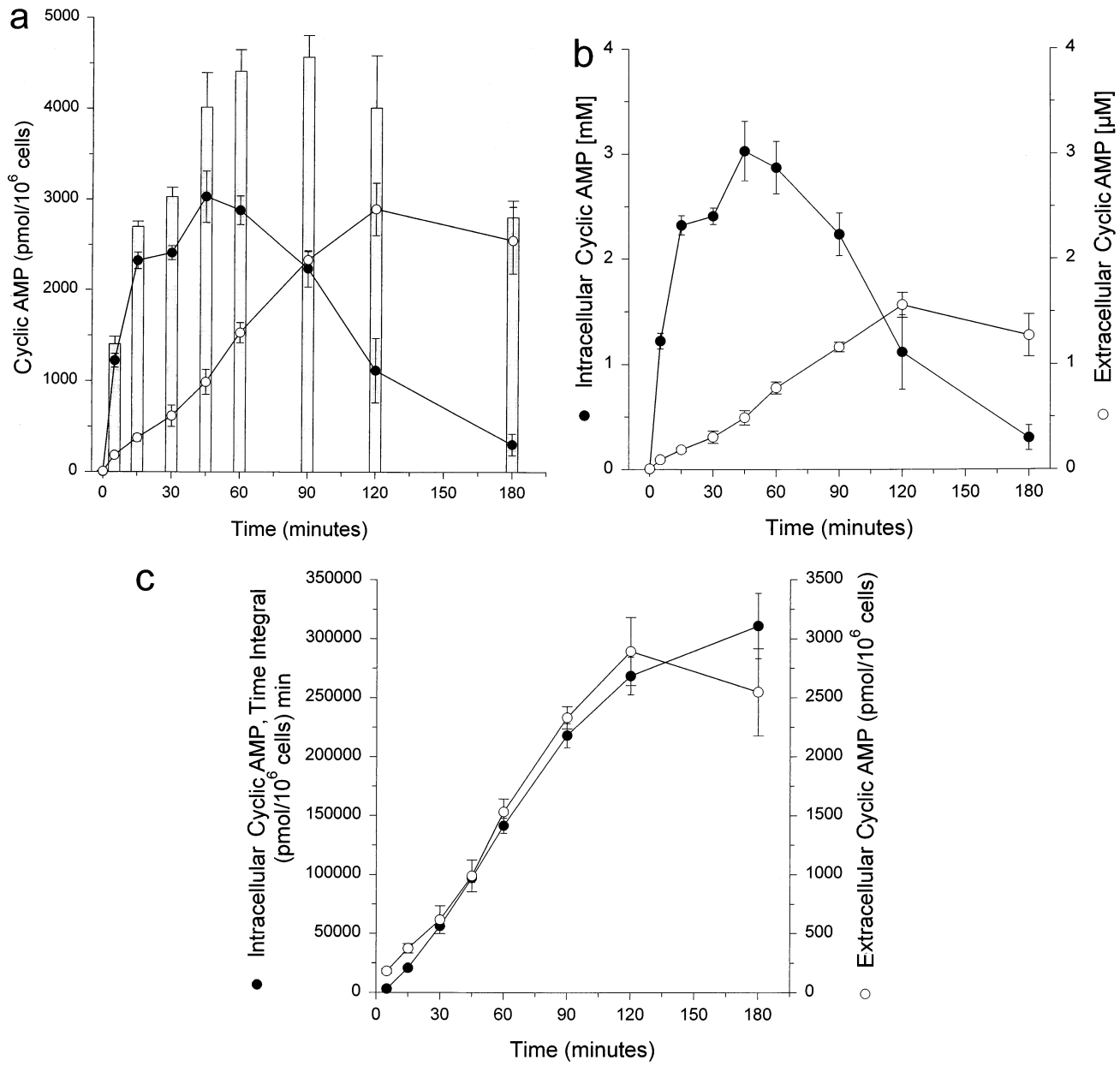

FIG. 1. (a) Time-course of intracellular (●), extracellular (○), and total (intracellular plus extracellular, closed bars) cyclic AMP during exposure of PC12 cells to 10 µM forskolin. Values are means ± SEM of 3 separate determinations, assayed in duplicate. (b) Time-dependent changes in intracellular (●) and extracellular (○) cyclic AMP concentrations. Data plotted are from Fig. 1a. (c) Time-dependent changes in extracellular cyclic AMP (○) and in time integral of intracellular cyclic AMP (●). Data plotted are from Fig. 1a.

time integral of the intracellular cyclic AMP, as described by Fehr *et al.* [20]. The linear regression was thus calculated and the slope gives the fractional release rate.

RESULTS

Time-dependent Changes in Intracellular and Extracellular Cyclic AMP in response to Forskolin

The time-course of forskolin-induced changes in intracellular and extracellular cyclic AMP was examined, using a concentration (10 µM) that is close to its EC_{50} (see Fig. 5b). Exposure of PC12 cells to forskolin caused a progressive increase in intracellular cyclic AMP levels, which reached a maximum of about 3000 pmol/10^6 cells within 45 to 60 min and fell thereafter (Fig. 1a). Accumulation of extracellular cyclic AMP started soon after the addition of forskolin and proceeded almost linearly at the mean rate of 24.7 pmol/min/10^6 cells until 120 min, when the amount of cyclic AMP released equaled the peak level reached in the cells. At the end of the 180-min incubation, extracellular cyclic AMP did not increase any further and its level, expressed as pmol/10^6 cells, was 8-fold higher than the intracellular level (Fig. 1a). As shown in Fig. 1a, total cyclic AMP (intracellular plus extracellular), which represents the balance between formation and elimination of the

TABLE 1. Effect of 50 μM probenecid on intracellular and extracellular cyclic AMP in PC12 cells after 60 min exposure to 10 μM forskolin

	Intracellular cyclic AMP	Extracellular cyclic AMP	Total [intracellular plus extracellular]	Intracellular to extracellular ratio
Basal	1.52 ± 0.13	4.26 ± 0.50	5.78 ± 0.52	0.38 ± 0.05
Forskolin	1526 ± 43	1011 ± 13	2536 ± 55	1.51 ± 0.03
Forskolin + probenecid	1171 ± 136*	589 ± 88†	1760 ± 222‡	2.01 ± 0.09

Values are given as pmol/10^6 cells and are the means ± SEM of 12 separate determinations assayed in duplicate.
Significance of the difference from forskolin: *, $P < 0.05$; †, $P < 0.001$ and ‡, $P < 0.005$.

cyclic nucleotide in the whole system, reached a plateau level at 45 min that was maintained up to 2 h, decreasing during the next hour.

Based on the estimate of 1 mL of intracellular fluid/10^9 cells [21], and on the mean number of cells present in each well (see Methods), the intracellular and extracellular concentrations of cyclic AMP were calculated. As shown in Fig. 1b, the intracellular peak was close to 3 mM. The extracellular concentration of cyclic AMP between 120 and 180 min was in the order of 1 μM, i.e. 300 to 1000 times lower than the intracellular concentration measured during the same time interval.

In the absence of forskolin intracellular levels of cyclic AMP were very low, ranging from a maximum of 2.83 ± 1.27 pmol/10^6 cells at 15 min to a minimum of 1.04 ± 0.23 pmol/10^6 cells at 120 min, corresponding to an intracellular concentration of 3 and 1 μM, respectively. The intracellular to extracellular ratio did not change during the incubation and was approximately equal to 0.4.

Intracellular accumulation of cyclic AMP in response to forskolin was evaluated by calculating the time integral of measured intracellular amounts, as suggested by Fehr *et al.* [20]. The time-dependent profiles of intracellular accumulation and extracellular levels of cyclic AMP were very similar (Fig. 1c), and a highly significant ($P < 0.001$) linear correlation was obtained between the two parameters. Thus, the measured efflux fits well with the model of a nonsaturated transport process with a first order rate constant (fractional release rate) of 0.0093 min^{-1}.

Preincubation of PC12 cells with 50 μM probenecid before addition of 10 μM forskolin reduced the extracellular accumulation of cyclic AMP by 38% over 60 min (Table 1). Under these conditions, a marginal decrease in intracellular cyclic AMP was detected that was at the limit of statistical significance. As a result of these changes, the total amount of the cyclic nucleotide was reduced upon treatment of the cells with probenecid (Table 1).

Time-dependent Changes in Intracellular and Extracellular Cyclic AMP in response to NECA

The metabolically stable adenosine derivative, NECA (0.1 μM), was used at a concentration near its EC_{50} [15] in order to study the time-related changes in cyclic AMP levels following stimulation of P_1 receptors. NECA increased cyclic AMP content in the cells as well as its accumulation in the extracellular medium with a time-course similar to that obtained with forskolin (Fig. 2). Peak intracellular levels of 1700 pmol/10^6 cells were reached at 45–60 min and declined at longer incubation times, while extracellular cyclic AMP increased throughout the incubation at a mean rate of 11.4 pmol/min/10^6 cells. Total cyclic AMP increased within the first hour, reaching a level that was maintained until the end of the experiments. Extracellular cyclic AMP levels were linearly correlated with the time integral of the intracellular levels ($P < 0.001$) and the fractional release rate was 0.0093 min^{-1}.

Effect of the Adenosine Receptor Antagonist 8-SPT on Forskolin-induced Cyclic AMP

Before addition of 10 μM forskolin, PC12 cells were exposed for 30 min to the P_1 receptor antagonist, 8-SPT

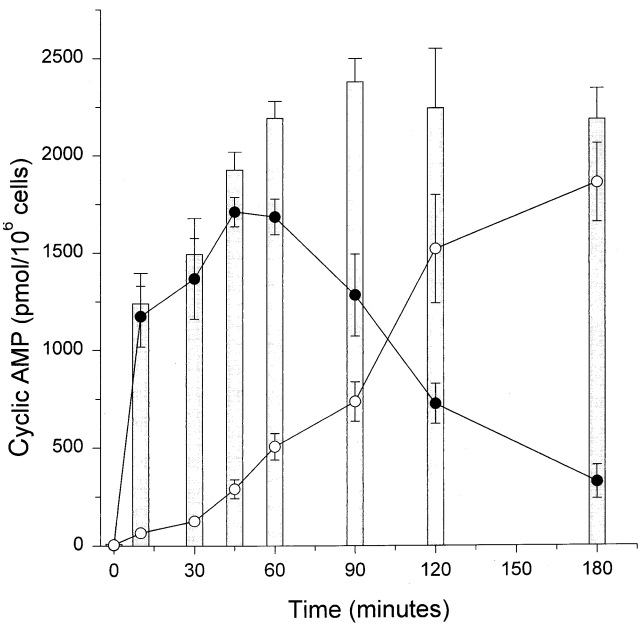

FIG. 2. Time-course of intracellular (●), extracellular (○), and total (intracellular plus extracellular, closed bars) cyclic AMP accumulation in the presence of 100 nM NECA in PC12 cells over 3 hr. Values are means ± SEM of 3 separate determinations, assayed in duplicate.

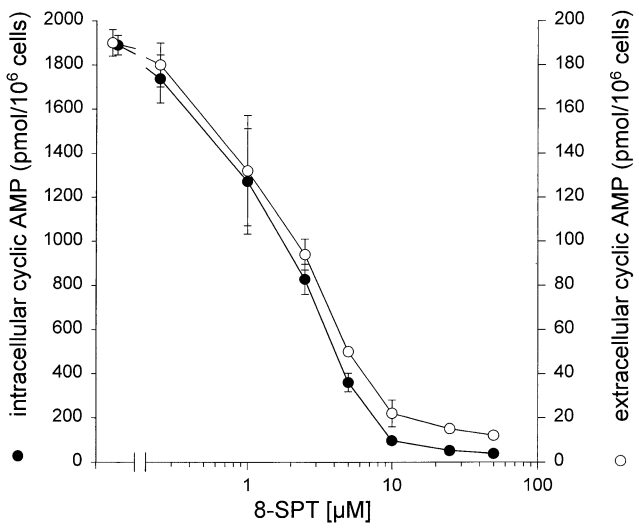

FIG. 3. Effect of increasing concentrations of 8-SPT on intracellular (●) and extracellular (○) cyclic AMP levels in PC12 cells exposed to 10 μM forskolin for 15 min. Cells were preincubated for 30 min in the presence of the antagonist before the addition of forskolin. Values are means ± SEM of 3 separate determination, assayed in duplicate.

(0.25–50 μM). A 15-min stimulation with forskolin was used, because preliminary experiments had shown that this time was as suitable as longer periods for detecting the effect of antagonists as well as making possible the comparison with adenosine, the physiological agonist at these receptors that undergoes time-dependent degradation by adenosine deaminase.

The ability of forskolin to increase intracellular cyclic AMP was progressively reduced by increasing 8-SPT concentrations, with an IC_{50} of 1.97 ± 0.28 μM (Fig. 3). With the same potency (IC_{50} = 1.97 ± 0.35 μM), 8-SPT also decreased the accumulation of cyclic AMP in the extracellular medium, and the relative changes in intracellular and extracellular cyclic AMP were superimposable at any 8-SPT concentration tested (Fig. 3), indicating that cyclic AMP efflux is strictly dependent on its intracellular level.

In the presence of 50 μM 8-SPT, forskolin appeared to be almost ineffective (Fig. 3). However, in time-course studies it was found that, notwithstanding the presence of 25 μM of 8-SPT, 10 μM forskolin was still able to evoke a small but detectable rise in intracellular cyclic AMP levels, which peaked after 30 min (122 ± 16 pmol/106 cells) and declined thereafter (Fig. 4). In the same experiments, 25 μM 8-SPT did not cause any significant change in basal intracellular or extracellular cyclic AMP levels (data not shown).

The presence of 8-SPT differentially affected the concentration-effect curve for adenosine and forskolin. Upon preincubation of the cells with 25 μM 8-SPT, the concentration-effect curve for exogenous adenosine (EC_{50}: 0.513 ± 0.061 μM) on intracellular cyclic AMP was parallelly shifted to the right (Fig. 5a), while the concentration-response curve for forskolin (EC_{50}: 11.0 ± 3.9 μM) was shifted downwards (Fig. 5b) and the diterpene was much more effective than adenosine in elevating cyclic AMP.

A marked inhibitory effect on forskolin activity was also observed in the presence of other adenosine receptor antagonists. In fact, reduced intracellular cyclic AMP levels were found after exposure of the cells to the receptor blockers DPCPX and DMPX. The results are summarized in Table 2.

Involvement of Adenosine in Forskolin-induced Cyclic AMP Accumulation

ADA mimicked the inhibitory effect of 8-SPT on the response of PC12 cells to forskolin. A 30-min preincubation with 1 U/mL of the enzyme caused a 68% inhibition of intracellular cyclic AMP accumulation induced by 10 μM forskolin (Fig. 6). Conversely, in the presence of exogenously applied adenosine (0.1 μM) the ability of 1 μM forskolin to stimulate adenylyl cyclase activity was greatly enhanced. As shown in Fig. 7, intracellular cyclic AMP accumulation was increased by more than 3-fold when cells were simultaneously exposed to both drugs (1698 ± 125 pmol/10^6 cells) with respect to forskolin alone (437 ± 54 pmol/10^6 cells), indicating marked cooperativity in the cyclic AMP response.

Finally, the possibility that newly synthesized cyclic AMP extruded into the extracellular medium during 30 min of adenylyl cyclase stimulation may contribute to the

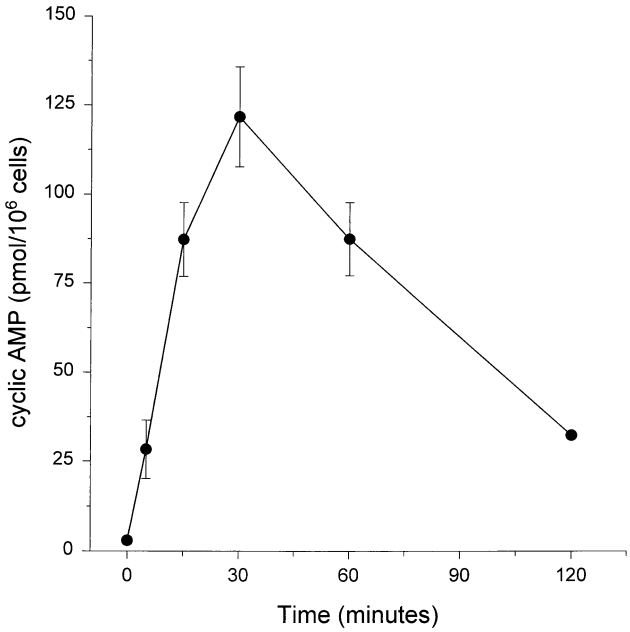

FIG. 4. Time-course of intracellular cyclic AMP levels during exposure of PC12 cells to 10 μM forskolin in the presence of 25 μM 8-SPT. Cells were incubated for 30 min in the presence of the adenosine receptor antagonist before the addition of forskolin. Values are means ± SEM of 6 separate determinations, assayed in duplicate.

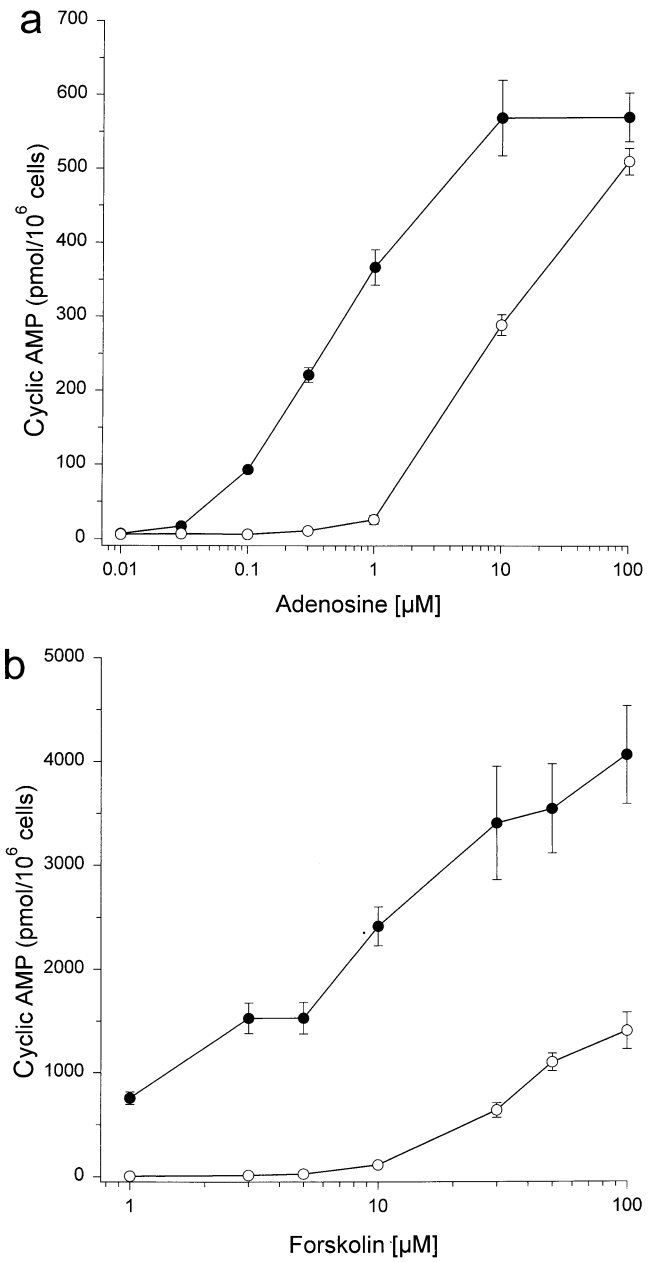

FIG. 5. Concentration-dependent changes in intracellular cyclic AMP in PC12 cells exposed to adenosine (a) or forskolin (b) in the absence (●) or in the presence (○) of 25 μM 8-SPT. Values are means ± SEM of 5 (adenosine) or 6 (forskolin) separate determinations, assayed in duplicate.

formation of the extracellular pool of endogenous adenosine, and hence to the enhancing effect on forskolin activity, was investigated. PC12 cells were exposed for 30 min to 1 or 10 μM forskolin, in the absence or presence of 100 μM AOPCP, a competitive inhibitor of ecto 5′-nucleotidase activity, 10 μM dipyridamole, a nucleotide transport inhibitor, or 10 μM EHNA, an inhibitor of ADA. Dipyridamole significantly enhanced the effect of 1 and 10 μM forskolin, increasing intracellular cyclic AMP levels by 85% and 57%, respectively ($P < 0.001$). Conversely, AOPCP and EHNA did not significantly affect forskolin response. The results are summarized in Table 3.

TABLE 2. Inhibition of 10 μM of forskolin-dependent intracellular cyclic AMP accumulation in PC12 cells exposed to the adenosine receptor antagonists DPCPX, DMPX, and 8-SPT

		Cyclic AMP pmol/10^6 cells	% inhibition
FK 10 μM		1521 ± 95	
FK 10 μM + DPCPX	1 μM	111 ± 16†	−93%
	10 μM	47 ± 18†	−97%
FK 10 μM + DMPX	1 μM	1258 ± 139*	
	10 μM	208 ± 10†	−86%
FK10 μM + 8-SPT	1 μM	820 ± 73†	−46%
	10 μM	96 ± 15†	−94%

Values are means ± SEM of 3–6 separate determinations assayed in duplicate.
*, not significant, †, $P < 0.001$ with respect to forskolin alone.

DISCUSSION

In a wide variety of cells, extrusion of cyclic AMP into the extracellular space acts synergistically with phosphodiesterase to limit intracellular accumulation of the cyclic nucleotide in response to adenylyl cyclase stimulation [1, 2]. In the present study, evidence has been obtained that the cyclic AMP transport system also operates in PC12 cells, where both forskolin and adenosine receptor agonists are known to be very effective in stimulating cyclic AMP formation [15, 17, 22].

Intracellular cyclic AMP levels increased soon after the addition of forskolin and peaked at 45–60 min, declining thereafter. The maximum concentration reached within the cells can be estimated as being close to 3 mM.

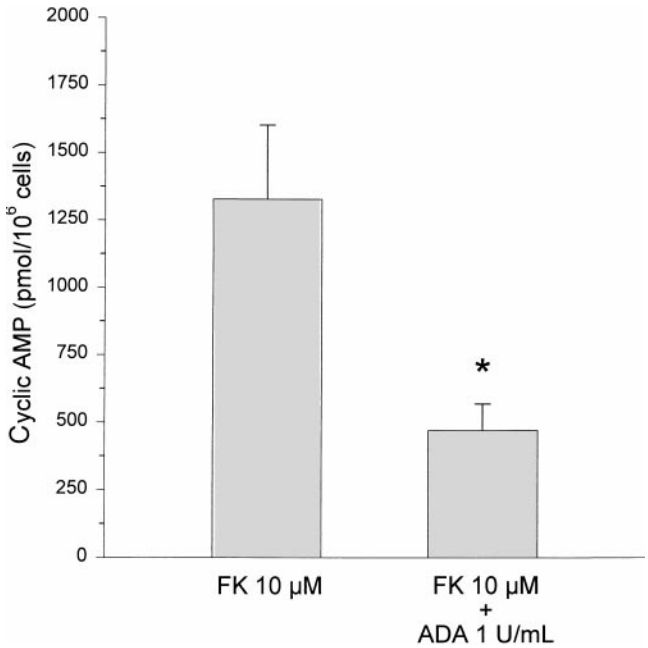

FIG. 6. Effect of 1 U/mL ADA on intracellular cyclic AMP accumulation in PC12 cells exposed to 10 μM forskolin (FK) over 15 min. Cells were incubated for 30 min in the presence of ADA before the addition of forskolin. Values are means ± SEM of 9 separate determinations, assayed in duplicate. *, $P < 0.005$.

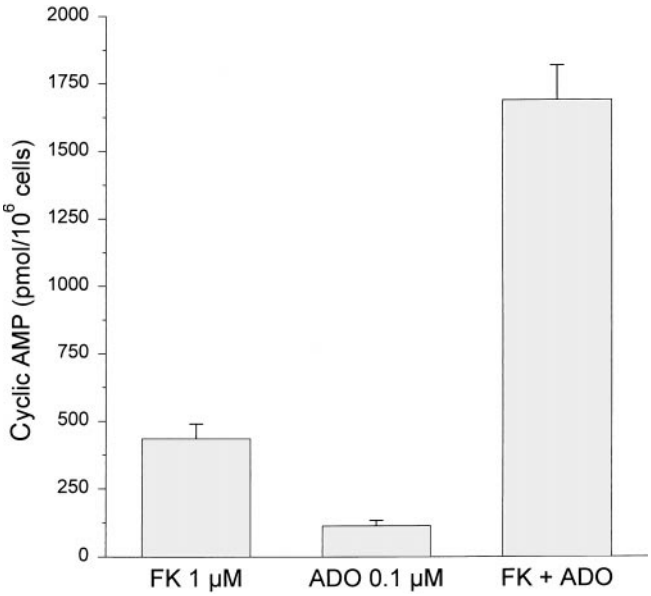

FIG. 7. Effect of 0.1 μM adenosine (ADO) on intracellular cyclic AMP accumulation in PC12 cells exposed to 1 μM forskolin (FK) over 15 min. Values are means ± SEM of 6 separate determinations, assayed in duplicate.

Accumulation of the cyclic nucleotide in the extracellular medium started immediately and proceeded almost linearly up to 120 min, when the concentration reached was about 1 μM. At 180 min of incubation, when intracellular cyclic AMP was reduced to 10% of the peak level, no further change occurred in the medium, notwithstanding the large concentration gradient that still should drive the cyclic nucleotide out of the cells if this were a passive diffusion process. A tight dependence of efflux process on the intracellular availability of the cyclic nucleotide is indicated by the superimposable time-dependent changes in cyclic AMP accumulation into the cells, evaluated as the time integral of intracellular cyclic AMP levels [20], and in the medium. Again, the increase in extracellular cyclic AMP stops after 120 min, when intracellular accumulation is approaching a plateau level.

TABLE 3. Effect of the ecto 5′-nucleotidase inhibitor AOPCP, of the adenosine transport inhibitor dipyridamole, and of the adenosine deaminase inhibitor EHNA on intracellular cyclic AMP in PC12 cells after 30 min exposure to 1 or 10 μM forskolin

	Cyclic AMP pmol/10^6 cells
FK 1 μM	423 ± 47
FK 1 μM + AOPCP 100 μM	363 ± 34
FK 1 μM + dipyridamole 10 μM	784 ± 66*
FK 1 μM + EHNA 10 μM	562 ± 89
FK 10 μM	1259 ± 82
FK 10 μM + AOPCP 100 μM	1124 ± 87
FK 10 μM + dipyridamole 10 μM	1972 ± 93*
FK 10 μM + EHNA 10 μM	1414 ± 143

Values are means ± SEM of 6–8 separate determinations assayed in duplicate.
*, $P < 0.001$ with respect to forskolin alone.

The proportionality between accumulation (time integral) and extracellular levels of cyclic AMP is indicative of a nonsaturated transport process that drives the cyclic nucleotide out of the cells [20]. The fractional release rate is 0.0093 min^{-1}, which means that an amount of cyclic AMP equivalent to that within the cells is released every 2 hr.

The total level of cyclic AMP remained constant from 45 to 120 minutes, indicating that in this time interval the amount formed equals the amount that is hydrolyzed. However, between 120 and 180 minutes a decrease in the rate of synthesis and/or an increase in the rate of degradation occurs, leading to a reduced total level. In PC12 cells incubated with 1 μM forskolin, Chern *et al.* [23] did not detect significant changes in phosphodiesterase activity after 30 minutes of treatment, but did observe a 100% increase after 16 hr. While the concentration-dependence of this effect has not been described, a significant increase in cyclic AMP hydrolysis may well occur even within 3 hours when forskolin is used at a concentration of 10 μM. Alternatively or in addition, a possible decrease in cyclic AMP synthesis should be considered. In this respect, it is tempting to speculate a role for extracellular cyclic AMP in turning on some negative control mechanism that decreases the sensitivity of adenylyl cyclase to forskolin stimulation, thus limiting ATP expenditure for the synthesis and export of the cyclic nucleotide. Alternatively, desensitization of the adenylyl cyclase system may occur, through a cyclic AMP-independent mechanism. Independently from the mechanisms that account for the lowering of total cyclic AMP, after 180 minutes of incubation the amount still present in the cells was very low, less than 10% of the total, and under these conditions the lack of any further accumulation in the medium is a likely consequence of decreased intracellular synthesis and/or faster degradation. Probenecid significantly reduced extracellular accumulation of cyclic AMP during forskolin stimulation, suggesting that the extrusion process could be mediated by a probenecid-sensitive anion transporter, as previously demonstrated [24].

The adenosine receptor agonist, NECA (100 nM), stimulated cyclic AMP formation and efflux, causing time-dependent changes in its cellular and medium levels that were qualitatively similar to those evoked by forskolin. Since both drugs were used at a concentration close to their EC_{50}s, from a quantitative point of view NECA appears to be less effective than forskolin, but again the amount of the cyclic nucleotide found in the medium at the end of the incubation with NECA was close to the maximum intracellular level measured after 45 min of exposure to the agonist. In NECA-stimulated PC12 cells, the fractional release rate, which in aortic smooth muscle was shown to be a function of NECA and of adenosine concentration [20, 25], is 0.0093 min^{-1}, rather low as compared to other types of cells, in which a lower accumulation of cyclic AMP

occurs [2, 20, 25]. However, this rate is comparable to that obtained in our experiments with forskolin and thus appears to be independent of the type of agonist. On the other hand, the mean rate of cyclic AMP appearance in the medium during stimulation with NECA (11.3 pmol/min/10^6 cells) as well as peak intracellular levels of the cyclic nucleotide were about half of those associated with forskolin treatment. These results stress that extrusion follows first order kinetics, i.e. its rate is proportional to the amount of cyclic AMP in the intracellular compartment and the possibility arises that stimulation of adenosine receptors might be involved in the effect of forskolin. This point was worthy of being investigated, because some relevant interactions between forskolin and adenosine have been stressed in PC12 cells. Not only does forskolin cause a marked leftward shift of the concentration-effect curve for NECA on cyclic AMP accumulation [15], but exposure of the cells to NECA, which causes adenosine receptor desensitization, reduces the adenylyl cyclase response to subsequent stimulation with adenosine receptor agonists as well as with forskolin [23, 26]. Moreover, treatment of PC12 cells with forskolin causes changes in $A_{2A}R$ mRNA that strictly resemble those that occur during incubation with NECA [27]. Very recently, forskolin has also been shown to mimic the ability of A_{2a} receptor agonists to increase phosphodiesterase IV activity [28] and their inhibitory effect on Ca^{2+} influx through P_2 receptor-operated cation channels [29].

The hypothesis of a direct involvement of P_1 purinoceptors in the response of PC12 cells to forskolin was tested by evaluating the possible influence of 8-SPT, a P_1 purinoceptor antagonist, on forskolin-induced changes in cyclic AMP accumulation and extrusion. Indeed, in the concentration range normally used to block P_1 receptors [15, 30–32], this compound caused a concentration-dependent decrease in cyclic AMP levels, without altering the intracellular to extracellular ratio. Similarly, 8-SPT did not alter this ratio when the P_1 receptor agonist NECA was used to stimulate adenylyl cyclase activity (data not shown). These results indicate that 8-SPT interferes with the ability of forskolin to stimulate cyclic AMP formation and does not affect the transport process. Because this xanthine derivative does not easily penetrate the cell membrane, it is conceivable that it interferes with forskolin by acting at an extracellular site that facilitates the effect of this direct activator of adenylyl cyclase. Since the effect of 8-SPT was shared by other two, more selective adenosine receptor antagonists DPCPX and DMPX, it is possible that the extracellular sites involved are the same receptors through which adenosine and its analog NECA stimulate adenylyl cyclase activity. Furthermore, the kinetic profile of forskolin-induced cyclic AMP accumulation in the presence of 25 μM 8-SPT indicates that direct adenylyl cyclase activation and amplification of the response via P_1 receptors are concomitant events.

Concentration-response curves for adenosine and for forskolin show that the former is more potent but less effective than forskolin. Moreover, the type of antagonism by 8-SPT is clearly different, because the curve for forskolin was shifted downwards. Thus, a direct activation of P_1 purinoceptors by forskolin can be excluded. This is also supported by the previously reported lack of 8-SPT influence on forskolin-stimulated adenylyl cyclase activity in isolated membranes prepared from PC12 cells [26]. Still, P_1 receptors are likely to be the membrane structure involved in the facilitation of forskolin-induced cyclic AMP accumulation in intact cells, because degradation of endogenous adenosine by ADA results in a marked inhibition of this response. Moreover, addition of exogenous adenosine, at a concentration that with respect to forskolin poorly stimulated cyclic AMP formation, greatly enhanced the ability of 1 μM forskolin to increase intracellular cyclic AMP levels. A similar type of synergistic interaction between forskolin and endogenous adenosine at the level of cyclic AMP accumulation has been previously observed in human neuroblastoma SH-SY5Y cells [33], in rat hippocampal slices [13], and in rat brain cortex slices [11, 12]. Taken together, these results strongly suggest that occupancy of adenosine receptors by endogenous adenosine enhances the ability of forskolin to directly stimulate adenylyl cyclase activity.

From a quantitative point of view, the inhibition by ADA was lower than that caused by maximally effective 8-SPT concentrations. This difference may suggest the existence of some additional component (e.g. phosphodiesterase inhibition) of 8-SPT action when the drug is used at concentrations of 10 μM and higher. However, some pieces of evidence argue against this possibility. Phosphodiesterase inhibitors have been shown to increase basal cyclic AMP levels in PC12 cells [34] while, under our conditions, 25 μM 8-SPT did not affect basal intracellular or extracellular cyclic AMP levels and 10 μM 8-SPT was also found to have a negligible effect on soluble phosphodiesterase from guinea pig ventricle [35]. Finally, 8-SPT caused a parallel rightward shift of the concentration-response curve for adenosine, with no change in the slope. On the other hand, the existence of an adenosine pool in tissues that is not available for metabolic degradation has been known for a long time [36, 37]. More recently, the resistance of endogenous adenosine to exogenous ADA has often been demonstrated [11, 12], and this may well account for the quantitative difference between the inhibitory effects of 8-SPT and ADA found in the present study.

Experiments performed using 1 μM forskolin in combination with 0.1 μM adenosine show that the effect of the exogenous nucleoside is much more than additive. Thus, very small amounts of endogenous adenosine are likely sufficient to amplify the effect of forskolin. Similar interactions between the diterpene and other receptor agonists have been observed [38, 39].

Adenosine can be formed inside the cell and then released through the plasma membrane, but its formation from precursors released from the cell can also occur in the extracellular compartment. According to the present results, incubation of PC12 cells with forskolin results in a time-dependent increase in cyclic AMP in the medium that

reaches a concentration in the micromolar range after 2 hr; at 15 min, a time sufficient for detecting the inhibitory effect of 8-SPT on forskolin stimulation, extracellular cyclic AMP is 10 times lower. Because at present very little is known about the role of cyclic AMP extrusion, the possible involvement of extracellular cyclic AMP in the observed decay of the response to forskolin following prolonged exposure should be evaluated. Similarly, the possibility that extracellular cyclic AMP can be a precursor of adenosine in these cells is worthy of being considered. Conversion of secreted cyclic AMP into adenosine has been documented in rat cerebral cultures, where stimulation of receptors positively coupled to adenylyl cyclase [40–42] as well as direct activation of the enzyme by forskolin [43], besides promoting intracellular cyclic AMP accumulation, also increase the accumulation of extracellular adenosine. In this respect, the marginal reduction in the intracellular cyclic AMP level caused by probenecid after 60-min contact would be in keeping with this hypothesis. However, the finding that the ecto 5′-nucleotidase inhibitor AOPCP does not significantly reduce forskolin activity seems to argue against a role of newly synthesized cyclic AMP as an extracellular source of endogenous adenosine. It has recently been shown that, in contrast to the potent activity of superfused cyclic AMP on field excitatory postsynaptic potentials in hippocampal slices, locally applied cyclic AMP failed to produce a significant activation of adenosine receptors. These results suggested that either ecto-phosphodiesterase activity is the rate-limiting step in the conversion of extracellular cyclic AMP to adenosine, or a very large increase in extracellular cyclic AMP is required [44]. Clearly, this issue would best be addressed in a direct assay on ecto-phosphodiesterase activity. On the other hand, the enhancing effect of dipyridamole, an inhibitor of adenosine transport, on forskolin activity further stresses the prominent role of endogenous adenosine in facilitating forskolin-induced adenylyl cyclase activation, whereas the lack of effect of the ADA inhibitor, EHNA, is consistent with a marginal role of ADA, with respect to the nucleoside uptake system, as an inactivating mechanism of adenosine action [45, 46].

In summary, the presented data show that in PC12 cells synergistic interaction between forskolin and endogenous adenosine accounts for the large accumulation of cyclic AMP caused by forskolin and that extrusion of the cyclic nucleotide through the unidirectional anion transport system contributes to cyclic AMP elimination from the intracellular space. Since extruded cyclic AMP does not appear to significantly contribute to the formation of the endogenous adenosine pool, some additional role for cyclic AMP extrusion may exist, one that is now under investigation.

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