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Is Adenosine Involved in Inhibition of Forskolin-Stimulated Cyclic AMP Accumulation by Caffeine in Rat Brain?

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SUMMARY

Caffeine potently inhibited forskolin-stimulated cyclic AMP accumulation in slices of rat cerebral cortex, with an IC_{50} of 21 ± 3 μM. Because caffeine competitively blocks adenosine receptors, we examined whether the action of forskolin involved endogenous adenosine or whether caffeine was acting through some novel mechanism. Inhibition by caffeine was observed at all forskolin concentrations examined, although the degree of inhibition decreased at higher concentrations of forskolin. The effect of caffeine was not blocked by the presence of a phosphodiesterase inhibitor but was mimicked by several other methylxanthines. The most potent of these was 8-(p-sulfophenyl)-theophylline, which does not readily cross cell membranes, arguing for an extracellular site of action. Addition of either adenosine or the adenosine uptake blocker dipyridamole potentiated the forskolin response, suggesting that forskolin and adenosine act synergistically in increasing cyclic AMP accumulation. The nonxanthine adenosine receptor antagonist CGS 15943 potently blocked cyclic AMP responses to forskolin, adenosine, and combinations. 3-Isobutyl-1-methylxanthine potently blocked the response to adenosine but caused little or no inhibition of the response to forskolin. Adenosine deaminase (ADA) was added to eliminate contributions of endogenous adenosine. ADA inhibited the response to both adenosine and forskolin; however, 200 times as much enzyme was necessary to inhibit the forskolin response. Inhibition of added ADA with 2′deoxycoformycin dramatically increased the concentration of ADA required to inhibit the adenosine response, without altering the concentration required to inhibit the forskolin response. These results suggest that forskolin-stimulated cyclic AMP accumulation may be partially dependent on endogenous adenosine but that the inhibition observed with caffeine is not solely due to blockade of adenosine receptors.

Forskolin is a plant alkaloid that is commonly used to stimulate cyclic AMP accumulation in a variety of tissues (1). Forskolin can directly activate the catalytic site of adenylate cyclase, leading to accumulation of cyclic AMP (2). However, forskolin also has many other actions in mammalian cells, and increases in cyclic AMP accumulation in response to this compound may be a result of a composite of these actions (2, 3).

Sattin and Rall (4) originally demonstrated that adenosine stimulated cyclic AMP formation in brain slices and that this response was blocked by methylxanthines. Subsequent investigations have shown that there are two main classes of cell surface adenosine receptors, the A_1 receptor, which inhibits adenylate cyclase through G_i, and the A_2 receptor, which activates adenylate cyclase through G_s (5). Both adenosine receptor subtypes are competitively antagonized by caffeine and other methylxanthines (6), and the behavioral stimulant actions of these compounds are now thought to be due to blockade of adenosine receptors (7). Although the relative potencies of most methylxanthines in stimulating locomotor activity parallel their affinities for blocking adenosine receptors (7, 8), there are some exceptions. For example, IBMX is a potent adenosine receptor antagonist that does not stimulate locomotor activity but produces behavioral depression (6–8).

We have recently reported that caffeine (1,3,5-trimethylxanthine) potently inhibits forskolin-stimulated cyclic AMP accumulation in slices of rat cerebral cortex (9). The potency and pharmacological specificity of this action suggest that it may have some relevance to the behavioral stimulant effects of the methylxanthines. Because caffeine is an adenosine receptor antagonist, we have examined the possibility that the forskolin response is dependent on endogenous adenosine and that the inhibition by caffeine may be due to adenosine receptor blockade.

Experimental Procedures

Materials. Caffeine sodium benzoate, theophylline, dipyridamole, forskolin, isoproterenol HCl, adenosine, IBMX, and adenosine deaminase (Type II) were obtained from Sigma Chemical Co. (St. Louis, MO), 8-PST and 7-BCT from Research Biochemicals Inc. (Natick,

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ABBREVIATIONS: IBMX, 3-isobutyl-1-methylxanthine; ADA, adenosine deaminase; 2′dCF, 2′-deoxycoformycin; 8-PST, 8-(*p*-sulfophenyl)-theophylline; 7-BCT, 7-(β-chloroethyl)-theophylline; KRB, Krebs-Ringer bicarbonate buffer; IC_{50}, concentration necessary to inhibit 50% of a response.

MA), CGS 15943 [9-chloro-2-(2-furanyl)-5,6-dihydro-1,2,4-triazolo[1,5-c]quinazolin-5-imine] from Dr. R. A. Lovell at Ciba-Geigy Corp. (Summit, NJ), and 2′dCF from Dr. R. Glazer (National Cancer Institute). [2,8-^{3}H]adenine (10–25 Ci/mmol) was obtained from DuPont NEN (Wilmington, DE).

Tissue preparation. Male Sprague-Dawley rats were sacrificed by cervical dislocation and decapitated, and the brains were removed. Cerebral cortices were dissected and transferred to a beaker of cold KRB (120 mM NaCl, 5.5 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM NaH_2PO_4, 1.2 mM $MgCl_2$, 20 mM $NaHCO_3$, 11 mM glucose, 0.019 mM Ca-Na_2EDTA), which had been equilibrated with 95% O_2/5% CO_2.

Cyclic AMP accumulation. Cyclic AMP accumulation in slices of rat cerebral cortex was measured by the [^{3}H]adenine prelabeling technique described by Shimizu *et al.* (10), as previously described (11). Cerebral cortices were chopped into 350- × 350-μm trapezoids on a McIlwain tissue chopper, dispersed in KRB, and incubated at 37° under 95% O_2/5% CO_2 for 15 min. The medium was decanted and slices from a pair of hemicortices were added to 30 ml of warm KRB containing 60 μCi of [^{3}H]adenine and 3 μM unlabeled adenine. The mixture was preincubated at 37° with shaking for 40 min under O_2/CO_2 and slices were collected on nylon mesh, washed with warm KRB, and resuspended in the same buffer. An aliquot of 50 μl of gravity-packed slices was added to each incubation tube. Incubations were carried out in a final volume of 1 ml of KRB containing appropriate drugs, at 37° under O_2/CO_2, in a shaking water bath for 15 min. Incubations were terminated by addition of 100 μl of 77% trichloroacetic acid, after which 50 μl of 10 mM unlabeled cyclic AMP were added as a carrier. Each sample was homogenized briefly with a Polytron and centrifuged at 19,000 × *g* for 15 min. An aliquot of 50 μl of supernatant was removed from each sample for determination of the total radioactivity incorporated into the tissue. [^{3}H]cAMP was isolated by sequential Dowex and alumina chromatography, as previously described (11). Recovery was determined on parallel columns with [^{3}H]cAMP and ranged from 50 to 95%.

Results

Inhibition of forskolin-stimulated cyclic AMP accumulation by methylxanthines. Forskolin (0.5–4 μM) caused a concentration-dependent 50-fold increase in cyclic AMP accumulation in slices from rat cerebral cortex (Fig. 1). Caffeine (200 μM) inhibited this response at all forskolin concentrations examined, causing an 84, 78, 70, and 54% inhibition at 0.5, 1, 2, and 4 μM forskolin, respectively.

The potencies of several methylxanthines in inhibiting cyclic AMP accumulation stimulated by 0.5 μM forskolin in slices of rat cerebral cortex are shown in Fig. 2. Caffeine, theophylline, 7-BCT, and 8-PST all inhibited the response to forskolin (0.5 μM) by 80–100%, with an order of potency of 8-PST > 7-BCT > theophylline > caffeine. The mean IC_{50} values for these compounds are listed in Table 1. When similar experiments were performed using a higher concentration of forskolin (10 μM), 8-PST, theophylline, and caffeine were all able to almost completely inhibit the forskolin response (not shown), although with 5–10-fold lower potency (Table 1).

Inhibition of 3′,5′-cyclic nucleotide phosphodiesterase. The effect of the specific phosphodiesterase inhibitor Ro20 1724 (200 μM) on the interaction between forskolin and caffeine was examined (Table 2). The presence of Ro20 1724 caused a doubling of both basal and forskolin-stimulated cyclic AMP

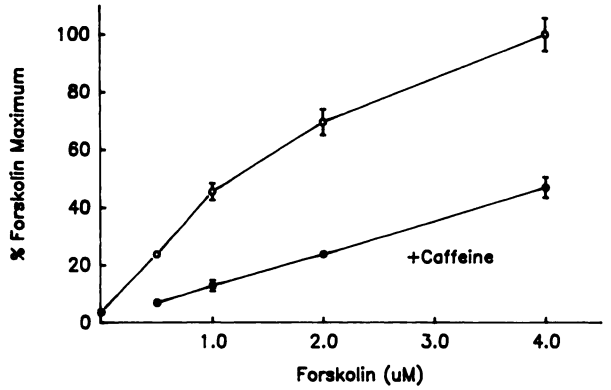

Fig. 1. Inhibition of forskolin-stimulated cyclic AMP accumulation by caffeine in slices of rat cerebral cortex. Tissue was incubated with increasing concentrations of forskolin (0.5–4 μM) in the absence (○) or presence (●) of 200 μM caffeine, as described in Experimental Procedures. Ordinate, percentage of the maximum cyclic AMP levels in the presence of the highest forskolin concentration (3.6% conversion). Each *point* is the mean ± standard error of triplicate determinations from two experiments.

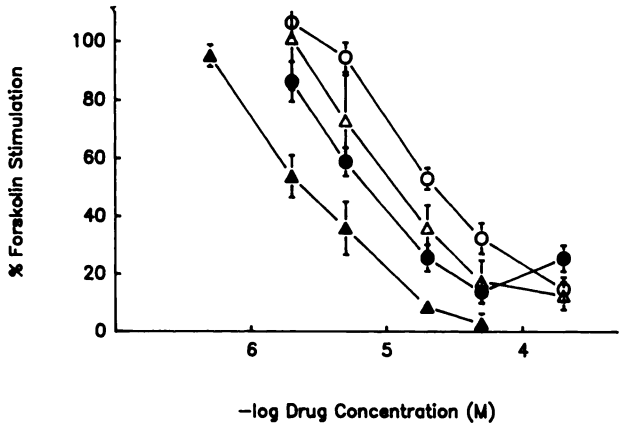

Fig. 2. Inhibition of forskolin-stimulated cyclic AMP accumulation in slices of rat cerebral cortex by different methylxanthines. Forskolin concentration was 0.5 μM. Drugs are 8-PST (▲), 7-BCT (●) theophylline (△), and caffeine (○). Each *point* is the mean ± standard error from duplicate determinations from three experiments. Average values for cyclic AMP accumulation were 0.12 ± 0.03 and 1.21 ± 0.12% conversion in the absence and presence of forskolin.

TABLE 1

Potencies of methylxanthines in inhibiting forskolin-stimulated cyclic AMP accumulation in slices of rat cerebral cortex

Each value is the mean ± standard error of four to six determinations.

Drug	IC_{50}	
	Against 0.5 μM forskolin	Against 10 μM forskolin
	μM	
8-PST	1.8 ± 0.7	6.9 ± 1.3
7-BCT	6.4 ± 0.5	ND[a]
Theophylline	9.8 ± 2.4	79 ± 35
Caffeine	21.0 ± 2.9	212 ± 34

[a] ND, not determined.

TABLE 2

Effect of Ro20 1724 on cyclic AMP responses to forskolin and caffeine

Experiments were conducted as described in Experimental Procedures. Each value is the mean ± standard error of three determinations.

	Conversion	
	Control	+200 μM Ro20 1724
	%	
Basal	0.43 ± 0.08	1.05 ± 0.04
Forskolin, 0.5 μM,	2.07 ± 0.21	4.29 ± 0.57
Forskolin + 200 μM caffeine	0.59 ± 0.37	2.12 ± 0.09

accumulation but did not affect the ability of caffeine to decrease the response to forskolin.

Synergistic interactions between forskolin and adenosine in increasing cyclic AMP accumulation. Because forskolin and adenosine have been reported to have synergistic interactions on cyclic AMP accumulation, the interactions of these two compounds were examined in cortical slices, by either addition of exogenous adenosine and/or blocking of the uptake of endogenous adenosine with dipyridamole (12). The effect of 10 μM dipyridamole on adenosine-stimulated cyclic AMP accumulation is shown in Fig. 3. Dipyridamole increased the cyclic AMP response to low (3–30 μM) concentrations of adenosine; however, this potentiation was overcome at higher adenosine concentrations, as would be expected. The interaction between dipyridamole and forskolin showed a different pattern (Fig. 3). Dipyridamole increased the cyclic AMP response to forskolin at all concentrations of forskolin examined (up to 40 μM). Much higher responses were obtained with forskolin than with adenosine, in either the presence or absence of dipyridamole.

The effect of adenosine on the response to forskolin (0.5 μM) in the presence or absence of dipyridamole (10 μM) is shown in Fig. 4. Addition of exogenous adenosine caused a concentration-dependent 8-fold increase in the cyclic AMP response to forskolin. The potency of adenosine in potentiating the forskolin

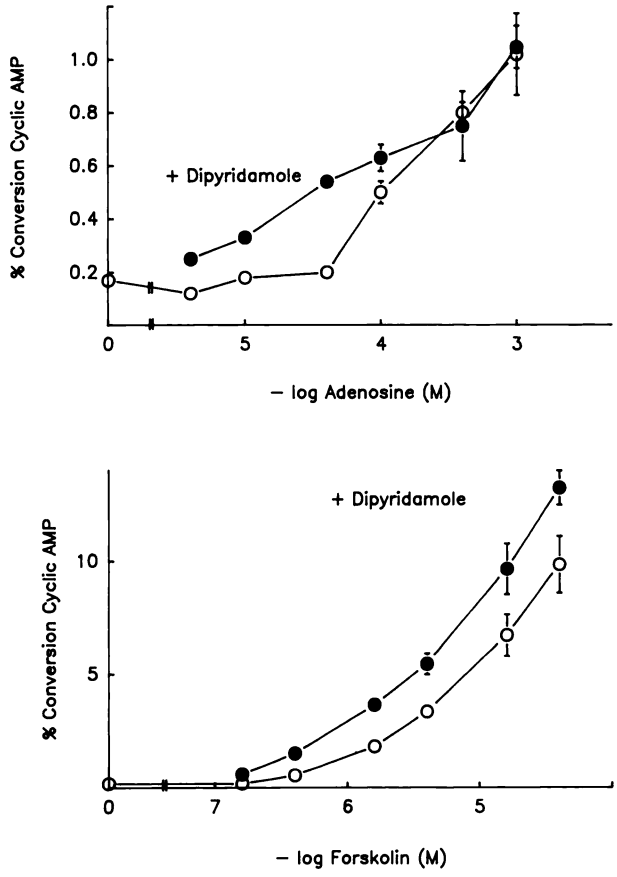

Fig. 3. Potentiation of cyclic AMP responses to adenosine and forskolin by dipyridamole. *Top*, slices of rat cerebral cortex were incubated with increasing concentrations of adenosine in the absence (○) or presence (●) of 10 μM dipyridamole. *Bottom*, slices of rat cerebral cortex were incubated with increasing concentrations of forskolin in the absence (○) or presence (●) of 10 μM dipyridamole. Each *point* is the mean ± standard error of duplicate or triplicate determinations from three experiments.

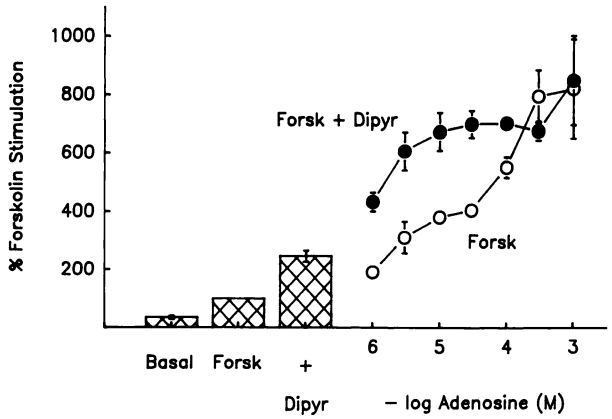

Fig. 4. Potentiation of forskolin-stimulated cyclic AMP accumulation by adenosine and dipyridamole. Slices of rat cerebral cortex were incubated with the indicated drugs, as described in Experimental Procedures. *Basal*, no drugs; *Forsk*, 0.5 μM forskolin; *+dipyr*, 0.5 μM forskolin plus 10 μM dipyridamole. The effects of increasing concentrations of adenosine were examined on the forskolin (0.5 μM) response in the absence (○) or presence (●) of 10 μM dipyridamole. Average percentage of conversion values were 0.18 ± 0.006 (basal), 0.64 ± 0.159 (forskolin alone), 4.24 ± .71 (forskolin plus 1 mM adenosine), and 4.43 ± 0.75 (forskolin plus 1 mM adenosine plus dipyridamole). Each value is the mean ± standard error of duplicate determinations from three experiments.

response was increased substantially by the presence of dipyridamole, although this compound did not alter the maximum potentiation observed.

Potencies of drugs in inhibiting cyclic AMP responses to forskolin and adenosine. Adenosine stimulates cyclic AMP accumulation by acting on adenosine A_2 receptors (5). If this action is involved in the effects of forskolin, the same compounds should inhibit responses to both forskolin and adenosine. The effects of caffeine, IBMX, and the nonxanthine adenosine receptor antagonist CGS 15943 on cyclic AMP responses to forskolin (0.5 μM) and adenosine (100 μM) are shown in Fig. 5. Both caffeine and CGS 15943 were about 20-fold more potent in blocking the response to forskolin than in blocking the response to adenosine. However, IBMX showed a different pattern. IBMX caused a concentration-dependent, virtually complete, inhibition of the adenosine response. At low concentrations, IBMX also caused a small inhibition of the forskolin response; however, the maximum inhibition observed was about 30% at a concentration of 20 μM. At higher concentrations, IBMX actually increased forskolin-stimulated cyclic AMP accumulation. IBMX had no significant effect on basal cyclic AMP accumulation, up to concentrations of 200 μM (data not shown). IBMX at 200 μM did not inhibit the forskolin response but almost completely inhibited the adenosine response. The same concentration of caffeine inhibited 85% of the forskolin response but only 25% of the adenosine response. Apparent IC_{50} values for each compound are listed in Table 3.

Potencies of drugs in inhibiting synergistic interactions between forskolin and adenosine. The effects of these drugs on synergistic responses to combinations of forskolin and adenosine are shown in Fig. 6. Addition of exogenous adenosine (3 μM) increased the forskolin (0.5 μM) response by about 3-fold. Inhibition of endogenous adenosine uptake by addition of dipyridamole (10 μM) caused a similar increase in the forskolin response (not shown). Both caffeine and CGS 15943 almost completely inhibited responses to both forskolin plus adenosine and forskolin plus dipyridamole. The potencies of these drugs

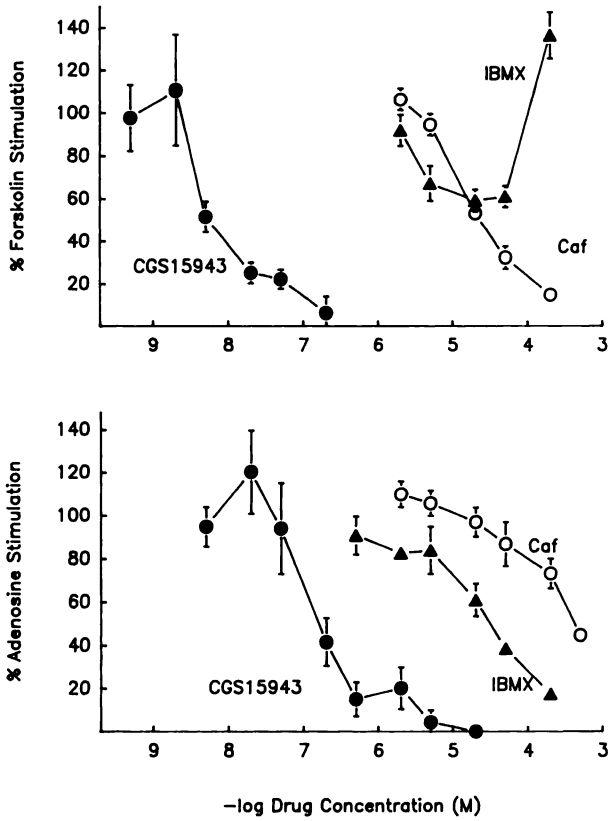

Fig. 5. Inhibition of forskolin- and adenosine-stimulated cyclic AMP accumulation by CGS 15943, IBMX, and caffeine. Slices of rat cerebral cortex were incubated with 0.5 μM forskolin (*top*) or 100 μM adenosine (*bottom*) and the indicated drug concentrations as described in Experimental Procedures. Each *point* is the mean ± standard error of duplicate or triplicate determinations from two separate experiments.

TABLE 3

Potencies of caffeine, IBMX, and CGS 15943 in inhibiting cyclic AMP responses to adenosine, forskolin, forskolin plus adenosine and forskolin plus dipyridamole

Cyclic AMP accumulation was measured in slices of rat cerebral cortex, as described in Experimental Procedures. Each value is the mean ± standard error of three independent determinations.

Stimulant	IC_{50}		
	Caffeine	IBMX	CGS 15943
		μM	
Adenosine, 100 μM	336 ± 50	30 ± 4.6	0.08 ± 0.033
Forskolin, 0.5 μM	21 ± 2.9	NC[a]	0.004 ± 0.001
Forskolin +3 μM adenosine	24 ± 1.7	16 ± 5.5	0.010 ± 0.004
Forskolin + 10 μM dipyridamole	31 ± 1.5	13 ± 3.0	0.005 ± 0.003

[a] NC, not calculated, because only a small inhibition was observed.

in inhibiting these responses were similar to those observed against forskolin alone (Table 3). IBMX also markedly decreased the response to both forskolin plus adenosine and forskolin plus dipyridamole, but this inhibition was not complete (Fig. 6). A substantial response, almost equivalent to that of forskolin alone, remained in the presence of 200 μM IBMX for both forskolin plus adenosine and forskolin plus dipyridamole.

The potencies of these compounds in inhibiting the adenosine- or dipyridamole-induced potentiation of the forskolin response was calculated as the difference between the response to drug combinations and the response to forskolin alone (Fig.

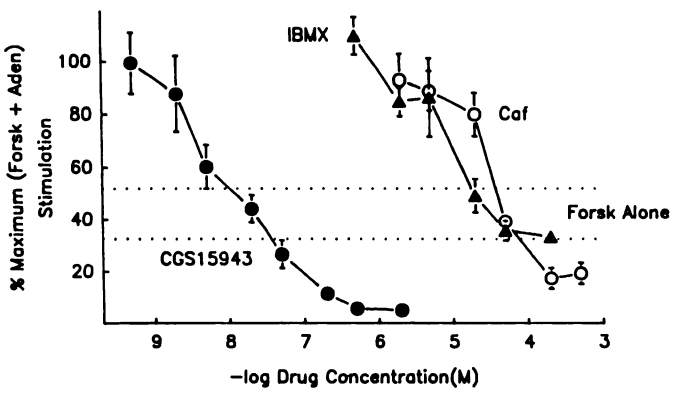

Fig. 6. Inhibition of synergistic interactions between forskolin (*Forsk*) and adenosine (*Aden*) on cyclic AMP accumulation by CGS 15943, IBMX, and caffeine (*Caf*). Slices of rat cerebral cortex were incubated with 0.5 μM forskolin, 3 μM adenosine, and the indicated drug concentrations, as described in Experimental Procedures. · · ·, The range of values observed with forskolin alone. Each *point* is the mean ± standard error of duplicate or triplicate determinations from three separate experiments.

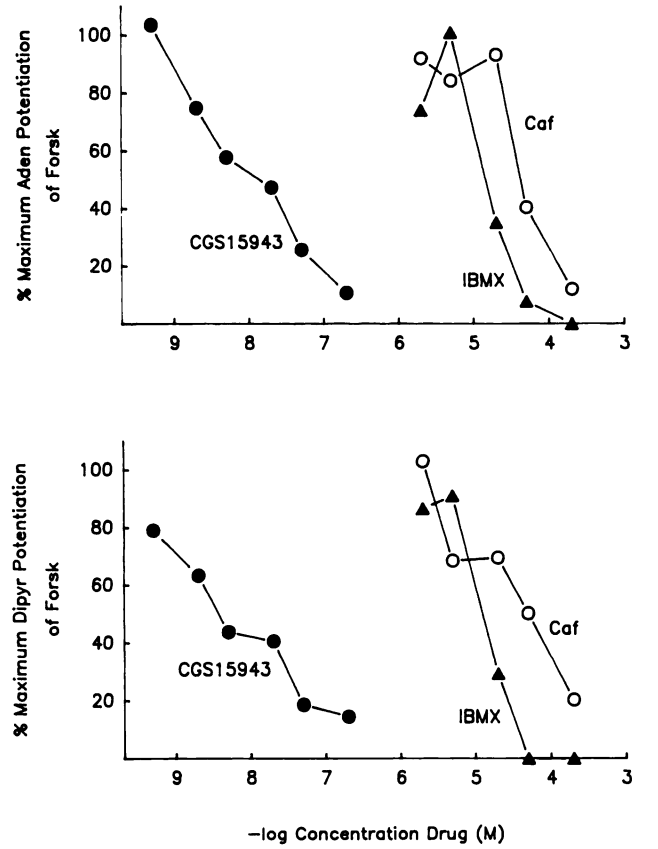

Fig. 7. Inhibition of the potentiation of the forskolin (*Forsk*) response caused by adenosine and dipyridamole. The inhibition of forskolin-stimulated cyclic AMP accumulation by each concentration of each drug was subtracted from the inhibition of the combined response to forskolin plus adenosine (*Aden*) (*top*) or forskolin plus dipyridamole (*Dipyr*) (*bottom*) to determine the inhibition of the potentiated response. Data are from Figs. 5 and 6. *Caf*, caffeine.

7). The order of potencies for inhibition of potentiation of both responses was similar to that for inhibition of the adenosine response (CGS 15943 > IBMX > caffeine), with all drugs inhibiting both potentiated responses by 80–100%. The pattern was clearly different from that observed with forskolin alone (see Fig. 5).

Effect of ADA on the stimulation of cyclic AMP accumulation by adenosine, forskolin, and KCl. To test the hypothesis that forskolin might be acting through release and/or potentiation of endogenous adenosine, we added ADA in an attempt to remove endogenous adenosine. The effects of increasing concentrations of exogenously added ADA on responses to adenosine (100 μM), forskolin (1 μM), and KCl (100 mM) are shown in Fig. 8. KCl was included because it increases cyclic AMP accumulation in guinea pig cortical slices through a mechanism that may involve endogenous adenosine (13). ADA blocked the cyclic AMP responses to all three compounds; however, it was most potent in blocking the response to adenosine. Approximately 200-fold more enzyme was needed to block an equivalent response to forskolin, and almost 2000-fold more enzyme was needed to block an equivalent response to KCl (Fig. 8, Table 4).

Specificity of inhibition by ADA. Blockade of responses to forskolin and KCl with high concentrations of ADA might be due to nonspecific effects of high enzyme concentrations or contaminants in the enzyme preparation. Therefore, the potent ADA inhibitor 2′-dCF (14, 15) was used to determine whether the inhibition of responses by ADA was due to specific breakdown of adenosine. Fig. 9 shows that the ability of ADA (0.5 units/ml) to block the cyclic AMP response to adenosine (100 μM) is completely reversed by 2′dCF, with an EC_{50} of about 30 nM. Although this same concentration of ADA causes only a small inhibition (20%) of the forskolin (1 μM) response, this is also reversed by 2′dCF with a similar potency (Fig. 9). In fact, 2′dCF (10–100 nM) caused a slight increase in the cyclic AMP response to forskolin (Fig. 9).

The concentrations of ADA necessary to inhibit responses to adenosine and forskolin in the presence of 2′-dCF are shown in Fig. 10. 2′dCF (1 μM) caused about a 200-fold increase in the concentration of ADA required to inhibit adenosine-stimulated cyclic AMP accumulation, and increasing the concentration of 2′dCF to 30 μM caused no further decrease in the potency of ADA. With respect to the forskolin response, 2′dCF (1 μM) slightly reduced the inhibition caused by low concentrations of ADA (0.05–0.5 units/ml) but had no effect on the concentration of ADA required to inhibit the forskolin response by 50% (Fig. 10, Table 4). A higher concentration of 2′dCF caused a further increase in the response to forskolin but caused less than a 2-fold increase in the concentration of ADA inhibiting the forskolin response by 50% (Table 3). The apparent reversal at lower concentrations of ADA may be due to a 2′dCF-induced potentiation of the response to forskolin (see Fig. 9).

Effect of ADA on the combined forskolin and adenosine responses. The effect of ADA on synergistic interactions between forskolin and adenosine was also determined (Fig. 11). ADA was substantially more potent in blocking the response to a combination of forskolin (0.5 μM) and exogenous adenosine (3 μM) than in blocking the response to a combination of forskolin (0.5 μM) and dipyridamole (10 μM) (Fig. 11). Almost 60-fold more ADA was required to inhibit the response to forskolin plus dipyridamole than that to forskolin plus adenosine (Table 4).

To determine whether dipyridamole could inhibit the effects of ADA, we examined the effect of dipyridamole (10 μM) on cyclic AMP responses to adenosine or forskolin plus adenosine, in the presence or absence of ADA (0.05 units/ml). Dipyridamole did not reverse the effects of ADA (data not shown).

Effect of caffeine and ADA on other cyclic AMP responses. The specificity of the effects of caffeine and ADA was examined by comparing their effects on cyclic AMP responses to forskolin with their effects on cyclic AMP responses to the β-adrenergic receptor agonist isoproterenol, alone and in combination with forskolin (Table 5). ADA almost completely inhibited the response to isoproterenol alone (10 μM) and to isoproterenol plus forskolin (0.5 μM). However, caffeine (200 μM) had no significant effect on either response (Table 5).

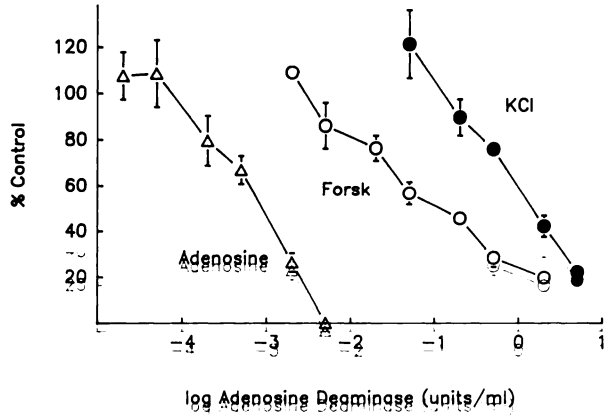

Fig. 8. Inhibition of cyclic AMP responses by ADA. Slices of rat cerebral cortex were incubated with adenosine (100 μM), forskolin (Forsk) (1 μM), or KCl (100 mM) and the indicated concentrations of ADA. Each point is the mean ± standard error of triplicate determinations from two or three experiments.

Discussion

We recently demonstrated that caffeine potently inhibits forskolin-stimulated cyclic AMP accumulation in slices of rat cerebral cortex (9). This effect occurred at concentrations of

TABLE 4

Concentrations of ADA causing 50% inhibition of cyclic AMP responses to adenosine, forskolin, forskolin plus adenosine, forskolin plus dipyridamole, and KCl

Cyclic AMP accumulation was determined in slices of rat cerebral cortex, as described in Experimental Procedures. Each value is the mean ± standard error of four to six determinations.

Stimulant	IC_{50}		
	Control	+1 μM 2′dCF	+30 μM 2′dCF
	units/ml		
Adenosine, 100 μM	0.0005 ± 0.00011	0.10 ± 0.025	0.13 ± 0.03
Forskolin, 1 μM	0.11 ± 0.026	0.11 ± 0.045	0.20 ± 0.01
Forskolin, 0.5 μM, + 3 μM adenosine	0.004 ± 0.001		
Forskolin, 0.5 μM, + 10 μM dipyridamole	0.26 ± 0.09		
KCl, 100 mM	1.12 ± 0.30		

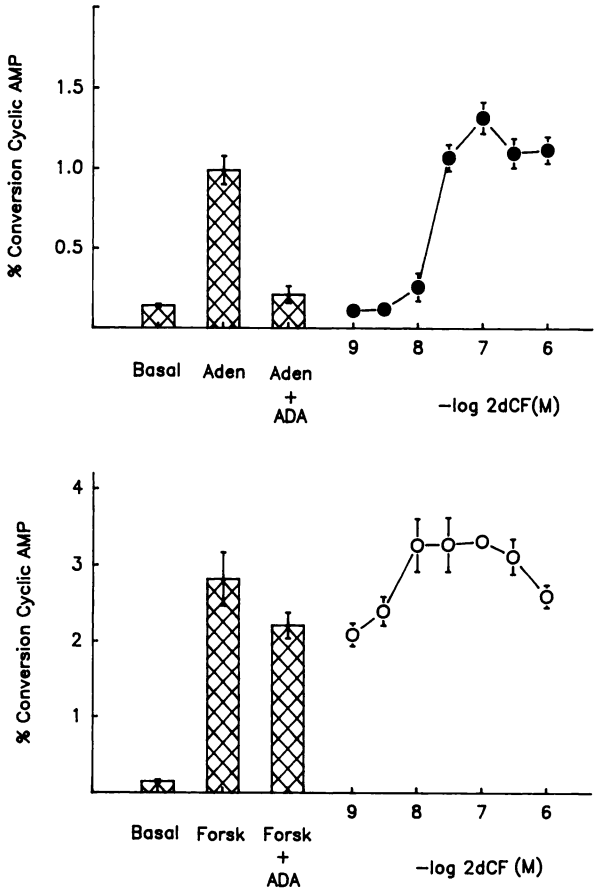

Fig. 9. Reversal of the effects of ADA by 2′dCF. Cyclic AMP accumulation in slices of rat cerebral cortex was determined as described in Experimental Procedures. *Top, Basal*, no drug; *Aden*, 100 μM adenosine; *Aden plus ADA*, 100 μM adenosine plus 0.5 units/ml ADA. (●) cyclic AMP accumulation in the presence of 100 μM adenosine plus 0.5 units/ml ADA, in the presence of increasing concentrations of 2′dCF. *Bottom, Basal*, no drug; *Forsk*, 1 μM forskolin; *Forsk plus ADA*, 1 μM forskolin plus 0.5 units/ml ADA. ○, Cyclic AMP accumulation in the presence of 1 μM forskolin plus 0.5 units/ml ADA, in the presence of increasing concentrations of 2′dCF. Each *point* is the mean ± standard error of duplicate determinations from two experiments.

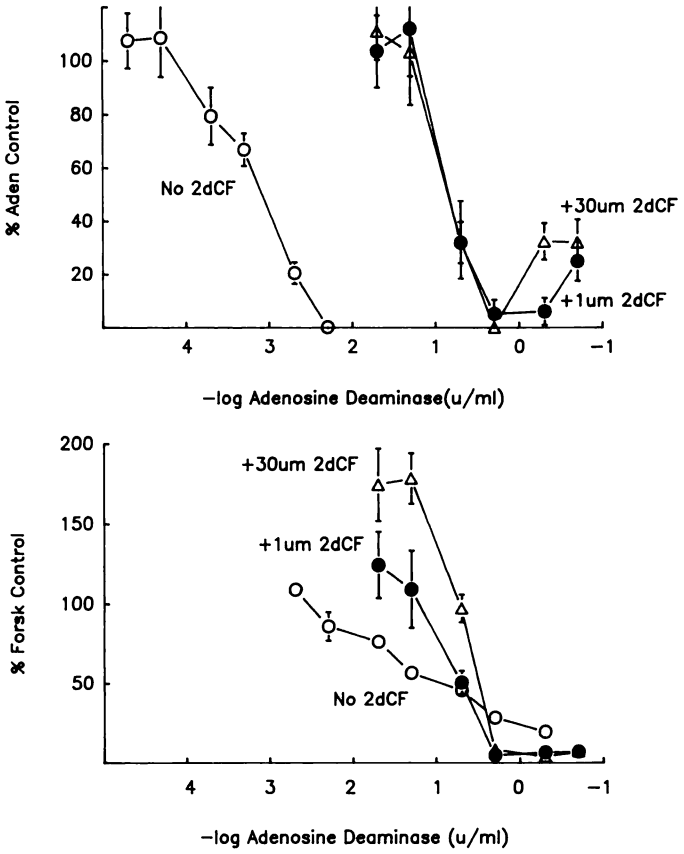

Fig. 10. Effect of 2′dCF on the potency of ADA in blocking cyclic AMP responses. Slices of rat cerebral cortex were incubated with 100 μM adenosine (*Aden*) (*top*) or 1 μM forskolin (*Forsk*) (*bottom*) and the indicated concentrations of ADA, in the absence (○) or presence of 1 μM (●) or 30 μM (△) 2′dCF. Each *point* is the mean ± standard error of duplicate of triplicate determinations from two or three experiments.

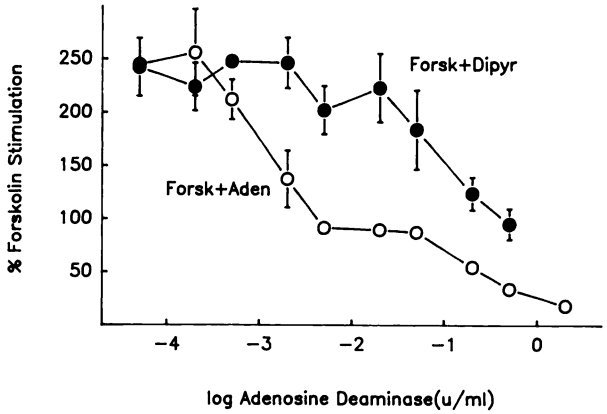

Fig. 11. Potency of ADA in blocking cyclic AMP responses to forskolin (*Forsk*) plus adenosine (*Aden*) or forskolin plus dipyridamole (*Dipyr*). Slices of rat cerebral cortex were incubated with 0.5 μM forskolin plus 100 μM adenosine (○) or 0.5 μM forskolin plus 10 μM dipyridamole (●) and the indicated concentrations of ADA. Each *point* is the mean ± standard error of duplicate determinations from two or three experiments.

caffeine that are associated with behavioral stimulation and was mimicked by theophylline, a behavioral stimulant, but not by IBMX, a behavioral depressant. These data suggested that this effect of caffeine might reflect some cellular action relevant to the behavioral properties of this compound.

Caffeine is widely thought to exert its behavioral stimulant actions by antagonizing adenosine receptors in brain (7). It is possible that forskolin stimulates cyclic AMP accumulation through a mechanism involving activation of adenosine A_2 receptors by endogenous adenosine in rat cortical slices and that the inhibition by caffeine might reflect blockade of obligatory adenosine receptor activation. The fact that IBMX, a potent antagonist at all known adenosine receptors, did not block the forskolin response argues against the involvement of adenosine. However, IBMX also potently inhibits cyclic AMP breakdown by 3′,5′-cyclic nucleotide phosphodiesterase (16), and an effect on this enzyme might mask a simultaneous inhibition of the forskolin response. We have, therefore, used several additional strategies to determine whether adenosine is involved in the cyclic AMP response to forskolin in rat cerebral cortex.

Several other methylxanthines mimicked the effect of caffeine, and the potencies of these compounds were similar to their potencies in blocking adenosine receptors (with the exception of IBMX). The fact that 8-PST, a charged compound that does not easily penetrate cells (17), was the most potent methylxanthine tested suggests that an extracellular site of action is involved in the effects of caffeine. Such an extracel-

TABLE 5

Specificity of caffeine and ADA inhibition of forskolin-stimulated cyclic AMP accumulation

Cyclic AMP accumulation was determined in slices of rat cerebral cortex, as described in Experimental Procedures. Each value is the mean ± standard error of four to eight determinations.

	Conversion	
	Basal	+0.5 μM Forskolin
	%	
Control	0.25 ± 0.05	2.22 ± 0.36
+0.2 units/ml ADA	ND[a]	0.50 ± 0.10
+200 μM Caffeine	ND	0.38 ± 0.06
+10 μM Isoproterenol	0.83 ± 0.06	6.95 ± 1.2
Isoproterenol + ADA	0.41 ± 0.09	1.05 ± 0.31
Isoproterenol + caffeine	0.61 ± 0.08	6.56 ± 1.8

[a] ND, not determined.

lular site could be cell-surface adenosine receptors, an external binding site for forskolin, or some other undefined site.

The effect of caffeine was observed at all concentrations of forskolin examined, although the degree of inhibition was inversely proportional to the concentration of forskolin. This suggests either that there is some type of surmountable "competition" between caffeine and forskolin or that caffeine inhibits a defined subset of the forskolin response that becomes proportionally less important at higher forskolin concentrations. Increasing the forskolin concentration by 20-fold (to 10 μM) caused a 5–10-fold reduction in the potency of several methylxanthines in blocking this response. The fact that these compounds are less potent in blocking the response to a higher forskolin concentration might suggest a competitive interaction between these two compounds. However, we found no effect of caffeine (200 μM) on direct activation of adenylate cyclase by forskolin in membrane preparations from rat cerebral cortex,[1] arguing against a direct interaction between caffeine and forskolin.

Although forskolin directly activates adenylate cyclase, it also potentiates the actions of receptors activating this enzyme through G_s. Data presented previously (1) and in this manuscript show that there are major potentiative interactions between forskolin and adenosine (presumably acting on an adenosine A_2 receptor) on cyclic AMP accumulation in slices of rat cerebral cortex. Inhibition of adenosine uptake with dipyridamole increased the response to adenosine and forskolin, both alone and in combination. Similar results were obtained when *p*-nitrobenzyl-6-thioguanosine was used to block adenosine uptake (data not shown). The effect of dipyridamole on the adenosine response, in either the presence or absence of forskolin, was overcome by increasing the concentration of adenosine, consistent with a competitive inhibition of adenosine uptake. Conversely, dipyridamole potentiated the effect of forskolin at all concentrations of forskolin examined. If forskolin were releasing adenosine in a concentration-dependent manner, the potentiation caused by dipyridamole should have been overcome at high concentrations of forskolin. The observed interaction is more consistent with a constant (low) amount of adenosine in the slices potentiated in a concentration-dependent manner by forskolin. Addition of dipyridamole would simply increase the endogenous adenosine concentration by a constant factor. In fact, high pressure liquid chromatographic analysis showed no significant effect of forskolin (10 μM) on adenosine or other adenine nucleotide levels in these slices (data not shown).

Adenosine and forskolin clearly have potentiative interactions in this system. If forskolin simply potentiates the action of endogenous adenosine, this could account for the greater potency of caffeine in inhibiting the response to forskolin (IC_{50} = 21 μM) than to 100 μM adenosine (IC_{50} = 336 μM). Because the concentration of endogenous adenosine would be much lower than that added exogenously, less caffeine would be needed to competitively antagonize the response. In this case, all competitive antagonists should be more potent in blocking the response to forskolin than to adenosine. We, therefore, compared the potencies of caffeine, IBMX, and the nonxanthine adenosine receptor antagonist CGS15943 in inhibiting the responses to forskolin, adenosine, and combinations of forskolin and adenosine or forskolin and dipyridamole. Strikingly, CGS15943 was also a potent inhibitor of the response to forskolin, adding support to the idea that the actions of forskolin are dependent on activation of adenosine receptors. In fact, CGS15943 was also 20-fold more potent in blocking the response to forskolin than to exogenous adenosine, similar to caffeine.

The actions of IBMX are interesting. IBMX was about 10-fold more potent than caffeine in blocking the cyclic AMP response to adenosine but it inhibited only a small portion (30%) of the response to forskolin at low concentrations and caused no inhibition of the forskolin response at higher concentrations. It is possible that the effects of IBMX on cyclic AMP breakdown obscure inhibition of the forskolin response. However, IBMX almost completely blocks the synergistic response to forskolin and either exogenous or endogenous adenosine essentially down to the level of the response to forskolin alone and then causes no further inhibition. This suggests that the response to forskolin is not primarily due to synergistic interactions between forskolin and endogenous adenosine, because such actions are potently blocked by IBMX.

We next attempted to directly define the role of adenosine in the forskolin response by eliminating endogenous adenosine. ADA inhibited the response to forskolin, suggesting that it may require endogenous adenosine. However, about 200-fold more enzyme was needed to block the response to forskolin than to block the response to a high concentration (100 μM) of adenosine. Because the endogenous adenosine concentration in the slice should be low, it is surprising that so much enzyme would be needed to eliminate it. The slope of the dose-response curve for ADA was also substantially steeper for inhibiting the response to adenosine than to forskolin, and the responses to both depolarization and the β-adrenergic receptor agonist isoproterenol were also inhibited by ADA. These data raise the possibility that this enzyme may have general depressant effects on the cyclic AMP system in these slices.

If the effects of ADA are specific and mediated by breakdown of adenosine, they should be blocked by 2′dCF (12, 13). In fact, 2′dCF did potently reverse the ADA-mediated inhibition of the adenosine response and slightly potentiated the response to forskolin. Although 2′-dCF (1 μM) caused a 200-fold increase in the concentration of ADA necessary to inhibit the response to adenosine, it did not significantly increase the concentration required to inhibit the response to forskolin. Increasing the concentration of 2′dCF by 30-fold did not further inhibit the

[1] S. Mante and K. P. Minneman, unpublished results.

actions of this enzyme. These observations suggest that the use of high concentrations of ADA can nonspecifically inhibit various responses. ADA decreases the forskolin response primarily in the high concentrations that nonspecifically decrease responsiveness. However, it is possible that ADA may not be able to gain access to the interior of the slice to deaminate endogenous adenosine, because ADA was 60-fold more potent against the combined forskolin and adenosine response than against the combined forskolin and dipyridamole response.

Overall, these results suggest that forskolin-stimulated cyclic AMP accumulation in rat cortical slices may have a small component (~30%) which is dependent on endogenous adenosine but that the largest portion of this response does not involve endogenous adenosine. This is supported primarily by the observations that IBMX and low concentrations of ADA cause only a small inhibition of the forskolin response, although they are both effective in blocking responses to combinations of adenosine and forskolin. Thus, although most of the forskolin response is not dependent on endogenous adenosine, it is potently inhibited by caffeine. It seems likely that this may reflect some as yet unknown action of caffeine on the brain. Although forskolin is not found naturally in the brain, the effect of caffeine on this response may reflect cellular actions that may be important in the behavioral actions of this compound. Studies on more defined cellular systems, such as primary cultures and continuous cell lines, will be necessary to clarify these effects of caffeine.

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