0026-895X/92/030587-11$03.00/0

MOLECULAR PHARMACOLOGY, 41:587-597

Cyclic AMP Differentiates Two Separate But Interacting Pathways of Phosphoinositide Hydrolysis in the DDT_1-MF_2 Smooth Muscle Cell Line

JOEL B. SCHACHTER and BARRY B. WOLFE

Departments of Pharmacology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104 (J.B.S.), and Georgetown University School of Medicine, Washington, DC 20007 (J.B.S., B.B.W.)

Received January 17, 1991; Accepted October 24, 1991

SUMMARY

The activation of adenosine A_1 receptors in DDT_1-MF_2 smooth muscle cells resulted in both the inhibition of agonist-stimulated cAMP accumulation and the potentiation of norepinephrine-stimulated phosphoinositide hydrolysis. Pharmacological analysis indicated the involvement of an A_1 adenosine receptor subtype in both of these responses. In the absence of norepinephrine, the activation of the adenosine receptor did not directly stimulate phosphoinositide hydrolysis. The adenosine receptor-mediated augmentation of norepinephrine-stimulated phosphoinositide hydrolysis was pertussis toxin sensitive and was selectively antagonized by agents that mimicked cAMP (8-bromo-cAMP) or raised cellular cAMP levels (forskolin). This initially suggested that cAMP might partially regulate the magnitude of the phospholipase C response to norepinephrine and that adenosine agonists might enhance the phospholipase C response by reducing cAMP levels. However, neither the reduction of cellular cAMP levels by other agents nor the inhibition of cAMP-dependent protein kinase was sufficient to replicate the action of adenosine receptor activation on phosphoinositide hydrolysis. Thus, in the presence of norepinephrine, adenosine receptor agonists appear to stimulate phosphoinositide hydrolysis via a pathway that is separate from, but dependent upon, that of norepinephrine. This second pathway can be distinguished from that which is stimulated by norepinephrine on the basis of its sensitivity to inhibition by both cAMP and pertussis toxin.

The mechanisms underlying the coupling of hormones, neurotransmitters, and autocoids to biochemical and physiological responses are currently a focus of intense scrutiny among laboratories studying a wide variety of cellular activation processes. In contrast to the diversity of cellular responses elicited by these chemical stimuli, the number of signal transduction mechanisms thought to mediate this coupling appears small, at the present time. Two such transduction mechanisms, the adenylyl cyclase and the PLC enzyme systems, have generated particular interest. Traditionally, these two systems have been described either as parallel independent pathways that regulate separate cellular functions or as mediators of opposing physiological functions that act separately upon downstream effector mechanisms in an inverse fashion. Recently, direct interactions between these two systems have been noted. Receptor coupling to adenylyl cyclase can be modulated by the activation of receptors coupled to PLC (1-3). Conversely, receptor coupling to PLC has been reported to be inhibited by the product of adenylyl cyclase, cAMP (4-9). Two second messengers are produced by PLC activation, diacylglycerol and inositol trisphosphate, which activate PKC and mobilize intracellular calcium, respectively (10). The activation of PKC has been shown to enhance adenylyl cyclase stimulation (3, 11-13), and cAMP can alter calcium storage or mobilization (14-18).

We have undertaken an examination of the role of cAMP in the regulation of PI hydrolysis in the DDT_1 smooth muscle cell line. This cell line is a particularly useful model system for this study because it contains a very high density of α_1-adrenergic receptors coupled to PI hydrolysis, as well as β_2-adrenergic receptors coupled to adenylyl cyclase activation (19, 20). We also sought to determine whether DDT_1 cells contained adenosine receptors coupled to the regulation of adenylyl cyclase. This seemed likely, because the cell line is derived from vas deferens, a tissue known to possess functional adenosine receptors (21, 22). The finding that DDT_1 cells respond to adenosine agonists by inhibiting cAMP accumulation provided us with a

This work was supported by grants from the National Institutes of Health (GM 31155, NS 26934, and AG 09884).

ABBREVIATIONS: PLC, phospholipase C; 8-Br-cAMP, 8-bromoadenosine cyclic 3′,5′-monophosphate; CPA, N^6-cyclopentyladenosine; CPCA, 5′-(*N*-cyclopropyl)carboxamidoadenosine; DMSO, dimethyl sulfoxide; G protein, heterotrimeric signal-transducing GTP-binding protein; G_i, GTP-binding protein linked to the inhibition of adenylyl cyclase; G_p, putative GTP-binding protein linked to the stimulation of phospholipase C; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NE, (−)-norepinephrine; PACPX, 1,3-dipropyl-8-(2-amino-4-chlorophenyl)xanthine; PI, phosphoinositide(s); PKC, protein kinase C; DDT_1, DDT_1-MF_2; PKA, protein kinase A; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate; ANOVA, analysis of variance.

system in which we could both raise and lower cAMP levels and determine the effects upon α_1 receptor-stimulated PLC activity. Our findings confirm observations from other laboratories that cAMP is inhibitory to PI hydrolysis (4–9). However, we find that PI hydrolysis in DDT_1 cells can be stimulated via two separate mechanisms, which can be differentiated from one another on the basis of their sensitivity to both cAMP and pertussis toxin. One mechanism, which is insensitive to pertussis toxin, is directly activated by α_1 receptors and shows little or no regulation by cAMP. The second mechanism, which is blocked by pertussis toxin pretreatment, is stimulated by adenosine agonists only when combined with NE and is inhibited by cAMP.

Materials and Methods

Cell culture. DDT_1 cells were originally obtained from Dr. James S. Norris (University of Arkansas). Cells were grown in suspension culture in Dulbecco's modified Eagle's medium containing 2.5% fetal bovine serum, 2.5% horse serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Spinner flasks (80 rpm) were kept at 37° in a humidified incubator containing 8% CO_2. Cells were seeded at 200,000–400,000 cells/ml and were utilized at 800,000–1,200,000 cells/ml.

Inositol monophosphate accumulation. PLC activity was measured as the accumulation of [^{3}H]inositol monophosphate in the presence of LiCl. The method of Berridge *et al.* (23) was modified in the following manner. Cells were washed free of growth medium and were incubated with [^{3}H]inositol (1 μCi/500,000 cells) at 37° for 3 hr, in a hooded, shaking, water bath gassed continuously with 95% O_2/5% CO_2. The incubation buffer consisted of 123 mM NaCl, 5 mM KCl, 1.3 mM $MgCl_2$, 1.4 mM KH_2PO_4, 26 mM $NaHCO_3$, 10 mM HEPES, pH 7.6, and 10 mM glucose. After incorporation of label, the cells were washed free of unincorporated [^{3}H]inositol, and 500,000 cells were added to each assay tube containing 10 mM LiCl and various drugs, in a final volume of 250 μl. This was incubated in the gassed shaking water bath for an additional 30 min before being terminated with 930 μl of $CHCl_3$/methanol (1:2). After the addition of 330 μl of $CHCl_3$ and of H_2O, the tubes were vortexed and centrifuged (1500 × *g* for 3 min) to separate the layers. Six hundred microliters of the upper (aqueous) layer were added to 2 ml of H_2O and then poured into a column containing 750 μl of a slurry of Dowex AG1-X8 (1:1 with H_2O). The resin was washed with a total of 25 ml of H_2O and eluted into counting vials with 1.75 ml of 0.2 M ammonium formate, 0.1 M formic acid. Three hundred microliters of the bottom (organic) layer of the reaction mixture were evaporated overnight and counted directly. Data are expressed as the percentage of conversion of PI to inositol monophosphates (100 times the cpm eluted from the column divided by the sum of cpm eluted from the column plus cpm in the organic layer) and are shown with basal activity subtracted, except where indicated.

cAMP accumulation. The accumulation of cAMP was measured using the method of Shimizu *et al.* (24), modified to mimic the conditions of the inositol monophosphate accumulation assay. Briefly, [^{3}H]adenine replaced [^{3}H]inositol and the incorporation was conducted for 90 min. After the unincorporated label was washed out, cells were incubated with drugs for 20 min. The reaction was stopped with 1 ml of 6.25% trichloroacetic acid. After centrifugation, [^{3}H]cAMP was isolated using the Dowex and alumina chromatography method of Salomon *et al.* (25). Data are expressed as percentage of conversion of ATP to cAMP (100 times the cpm eluted from the alumina divided by the sum of cpm from alumina plus cpm of ATP washing through the Dowex) and are shown with basal activity subtracted.

Binding of [^{3}H]prazosin. The relative amount of α_1-adrenergic receptors was defined by the binding of [^{3}H]prazosin to DDT_1 membranes. Membranes were prepared by hypotonic lysis at 0° for 15 min in 2.5 mM Tris, pH 7.4, followed by homogenization and centrifugation at 25,000 × *g* for 20 min. Two hundred micrograms of membranes were incubated at 37° for 90 min with 300 pM [^{3}H]prazosin, in 3 ml of 50 mM Tris, 10 mM $MgCl_2$, pH 7.4. The bound membranes were filtered over no. 30 glass fiber filters (Schleicher and Schuell) and washed with 10 ml of 5 mM Tris, 1 mM $MgCl_2$, 0.01% bovine serum albumin, pH 7.4, followed by scintillation counting. Nonspecific binding was defined by the addition of 50 μM phentolamine.

Materials. Adenosine agonists and antagonists were obtained from Research Biochemicals Inc. (Natick, MA) and were dissolved in DMSO at a concentration of 10 mM. The highest final concentration of DMSO used, 0.1%, had no effect upon basal or NE-stimulated PI hydrolysis or cAMP accumulation. Forskolin was from Calbiochem (San Diego, CA) and was dissolved in DMSO at 100 mM. Pertussis toxin (List Laboratories, Campbell, CA) and H-8 (Seikagaku America, St. Petersburg, FL) were dissolved in H_2O according to the package inserts. Tissue culture reagents were from GIBCO (Grand Island, NY), and radiochemicals were from New England Nuclear (Boston, MA). CEC, yohimbine, and clonidine were kindly provided by Dr. Kenneth J. Kellar (Department of Pharmacology, Georgetown University) and were dissolved in mixtures of DMSO and ethanol. All other chemicals were from Sigma, including NE, which was dissolved in 0.1 mM HCl at a concentration of 10 mM.

Statistics. The choice of statistical analysis depended upon the type of comparisons being made. For individual comparisons, Student's *t* test was used. For multiple comparisons, a one-way ANOVA was conducted, followed by one of two *post hoc* methods. Dunnett's test was used when multiple comparisons were made to a single control, and Newman-Keul's analysis was used for multiple intercomparisons. In all tests the minimum significance level was set at $p < 0.05$.

Results

Adenosine agonists potentiate NE-stimulated PI hydrolysis. DDT_1 cells showed robust stimulation of PI hydrolysis in response to NE (Fig. 1). The basal activity ranged between 1.5 and 2.5% of the incorporated counts (usually about 200,000 cpm). The response to NE was dose dependent, giving a maximum stimulation, at 10 μM NE, of 3–8% turnover of incorporated counts, after basal level subtraction. The effect on PI hydrolysis of modulating cAMP levels was investigated by adding isoproterenol, forskolin, or the adenosine analogue CPCA to a range of NE concentrations. Neither isoproterenol (100 μM) nor forskolin (30 μM) significantly affected NE-stimulated PI hydrolysis (data not shown); however, CPCA (10

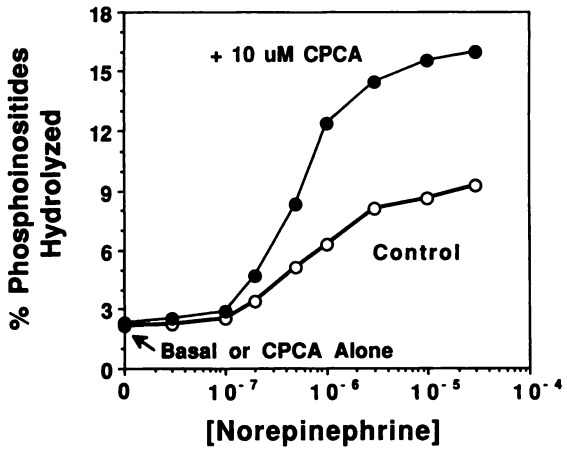

Fig. 1. An adenosine receptor agonist potentiates NE-stimulated PI hydrolysis. Inositol monophosphate accumulation was measured as described in Materials and Methods. CPCA (10 μM) was added at the same time as NE. Data are shown without subtraction of basal values and are the average of triplicate determinations. Two other similar experiments produced nearly identical results.

μM) enhanced NE-stimulated PI hydrolysis by approximately 2-fold throughout the dose-response curve for NE (Fig. 1).

NE stimulates PI hydrolysis via activation of α_{1b}-adrenergic receptors. NE is a nonselective agonist at adrenergic receptors. The potential role of various adrenergic receptor subtypes in NE-stimulated PI hydrolysis and in the potentiation by an adenosine agonist of that stimulation was investigated through the use of subtype-selective adrenergic agonists and antagonists (Fig. 2). The PI hydrolysis stimulation by NE or by NE plus the adenosine analogue CPA was completely blocked by the α_1-adrenergic antagonist prazosin but was unaffected by the α_2-adrenergic antagonist yohimbine. Consistent with these results, the α_2-adrenergic agonist clonidine had no effect in the absence or presence of CPA, whereas the α_1-adrenergic agonist phenylephrine did stimulate PI hydrolysis and this stimulation was potentiated by CPA. Phenylephrine was only a partial agonist, compared with NE, so combinations of phenylephrine with other subtype-selective agonists were tested to determine whether other adrenergic receptor subtypes might enhance the stimulation by phenylephrine. A recent report by Wilson and Minneman (26) demonstrated a synergistic interaction between the activation of α_1- and α_2-adrenergic receptors for the stimulation of PI hydrolysis in primary glial cell cultures. In DDT_1 cells the addition of the α_2-adrenergic agonist clonidine did not enhance phenylephrine-stimulated PI hydrolysis. A similar lack of effect was noted for the selective α_{1a} agonist methoxamine (27). Although methoxamine gave a small stimulation of PI hydrolysis by itself, it had no effect upon the stimulation by phenylephrine. The minor stimulation by methoxamine was potentiated by CPA, but the increase by the adenosine agonist was also minor. Stimulation by methoxamine and potentiation of that effect were only slightly increased in the presence of increased extracellular calcium (data not shown), suggesting that, in this cell line, PI hydrolysis is not activated by α_{1a} receptor-stimulated calcium influx (see Refs. 28 and 29 for an explanation).

Further delineation of the adrenergic receptor subtype involved in the stimulation of PI hydrolysis by NE was provided through the use of the α_{1b}-selective alkylating agent CEC (30, 31). CEC pretreatment resulted in the complete elimination of NE-stimulated PI hydrolysis (Fig. 3), regardless of whether stimulation was conducted in the presence or absence of either CPA or extracellular calcium. In parallel experiments, CEC-pretreated cells were homogenized and assayed for [^{3}H]prazosin binding. Under these conditions, CEC demonstrated the same potency for the reduction of prazosin binding as for the reduction of NE-stimulated PI hydrolysis; however, only about 80% of the binding was blocked. When cells were homogenized before CEC pretreatment, the potency of CEC was increased (i.e., the inactivation rate may be diffusion limited) and all of the prazosin binding was blocked. These data suggest that DDT_1 cells contain two populations of α_{1b}-adrenergic receptors. One population, which is susceptible to CEC inactivation in the intact cell, is coupled to PI hydrolysis. A second population is inaccessible to CEC in the intact cell but is inactivated by CEC in broken cells. This second population either is uncoupled from PI hydrolysis or is inaccessible to NE.

Effects of adenosine analogues are mediated via A_1 receptors. Adenosine is thought to influence cAMP synthesis through at least two different receptor subtypes (32, 33). An adenosine A_1 receptor has been defined by its ability to inhibit cAMP production and by its order of agonist binding affinities. A different order of agonist binding affinities defines the A_2 adenosine receptor, which is linked to the stimulation of cAMP production. We compared the nonselective adenosine agonist CPCA with the potent A_1-selective agonist CPA for their respective abilities to influence cAMP accumulation in DDT_1 cells. Neither compound, alone, increased basal cAMP accumulation (data not shown). In the presence of 50 μM forskolin, both compounds markedly inhibited cAMP accumulation. Fig. 4A shows that the A_1-selective agonist CPA is more potent than the nonselective agonist CPCA in the inhibition of cAMP accumulation. These data are indicative of the existence of a functional A_1 adenosine receptor in DDT_1 cells. Fig. 4B shows

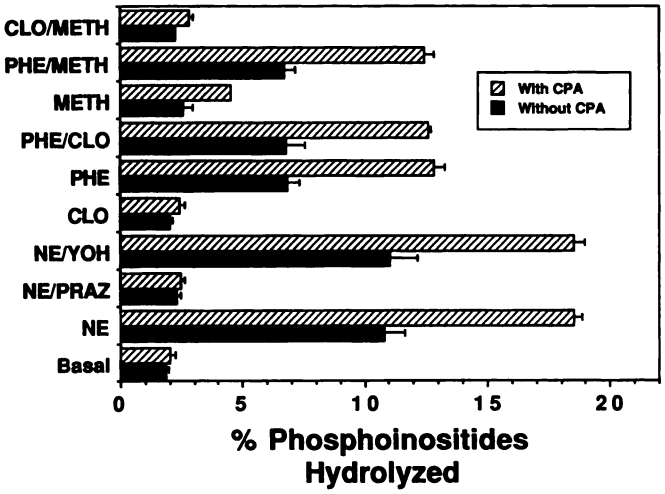

Fig. 2. Role of α-adrenergic receptor subtypes in NE-stimulated PI hydrolysis. Inositol monophosphate accumulation was measured in the presence and absence of 1 μM CPA. All drugs were added simultaneously with the lithium chloride. Drug concentrations were NE, 10 μM; prazosin (*PRAZ*), 100 nM; yohimbine (*YOH*), 100 nM; clonidine (*CLO*), 1 μM; methoxamine (*METH*), 100 μM; and phenylephrine (*PHE*), 100 μM. Data are the mean ± standard error of three experiments, performed in triplicate, and are shown with basal activity included.

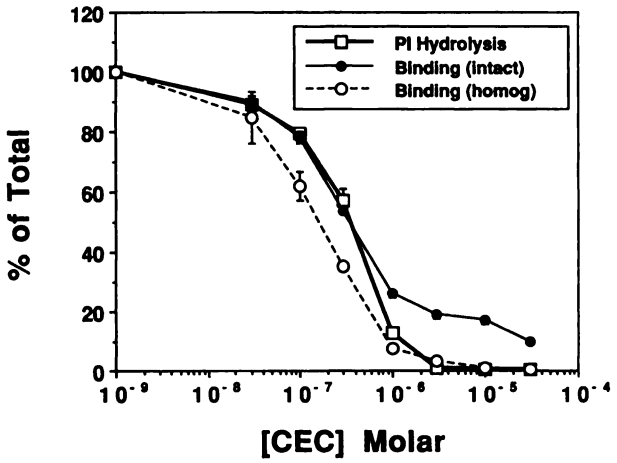

Fig. 3. Effect of the α_{1b}-selective alkylating agent CEC on [^{3}H]prazosin binding and NE-stimulated PI hydrolysis. Intact DDT_1 cells were preincubated for 40 min at 37° with various concentrations of CEC. After removal of CEC by centrifugation and two washes with buffer, cells were either assayed for NE-stimulated PI hydrolysis (□) or homogenized, centrifuged, and assayed for [^{3}H]prazosin binding (●). Another group of cells was homogenized before CEC treatment and then centrifuged and washed before [^{3}H]prazosin binding (○). Data are the mean ± standard error of four experiments.

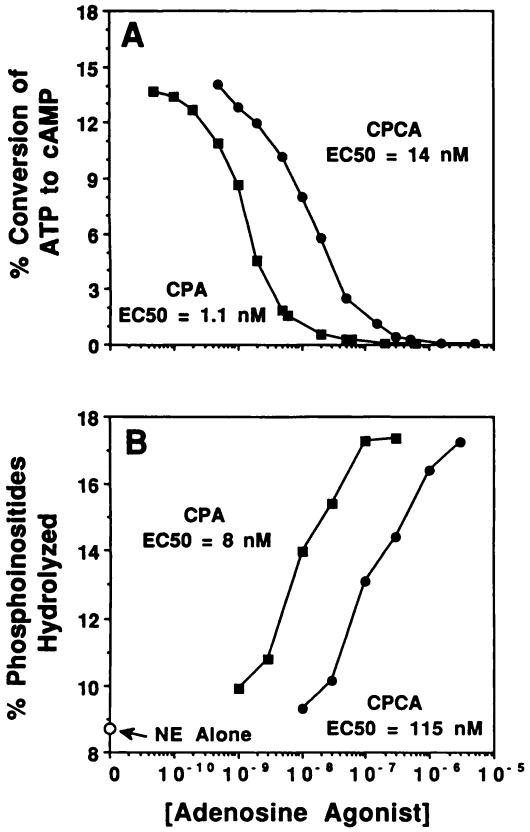

Fig. 4. Both an A_1-selective and a nonselective adenosine agonist potentiate NE-stimulated PI hydrolysis as well as inhibit forskolin-stimulated cAMP accumulation. A, cAMP accumulation was conducted as described in Materials and Methods, in the presence of 50 μM forskolin. Data shown are the averages of three experiments, conducted in duplicate. Basal activity was 0.18 ± 0.038% of incorporated counts and was subtracted from each point. Standard error for each point was <0.3%. Forskolin-stimulated cAMP accumulation was inhibited by 96–98% with either adenosine agonist. B, The potentiation of NE-stimulated PI hydrolysis by adenosine agonists was conducted as in Fig. 1, using 10 μM NE in all samples. Data are the average of three experiments, conducted in duplicate. Standard error for each point was <0.4%.

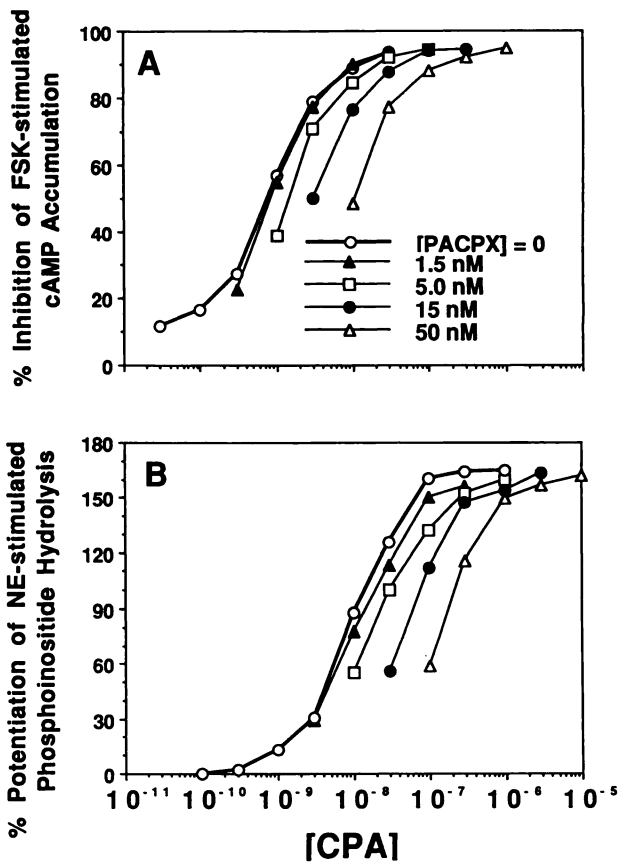

Fig. 5. The A_1-selective adenosine antagonist PACPX potently blocks the ability of CPA to inhibit forskolin-stimulated cAMP accumulation and to potentiate NE-stimulated PI hydrolysis. A, Data are expressed as percentage of inhibition of cAMP accumulation stimulated by 30 μM forskolin (*FSK*) (8.6 ± 0.2% of incorporated counts). Data shown are representative of three experiments conducted in triplicate. The standard error for each point was <3.5% of the expressed values. B, Data are expressed as the percentage of potentiation of PI hydrolysis stimulated by 10 μM NE (4.77 ± 0.13% of incorporated counts) and are representative of three experiments conducted in triplicate. PACPX had no effect upon forskolin-stimulated cAMP accumulation or upon NE-stimulated PI hydrolysis in the absence of CPA.

the potentiation by CPA and CPCA of NE-stimulated PI hydrolysis. Here again, the A_1-selective CPA is more potent than the nonselective CPCA, suggesting that this response may also be mediated via an A_1 adenosine receptor. Given that there are potential pitfalls to defining receptors by the potencies of agonists, a more rigorous association between the activation of an A_1 receptor and the potentiation of NE-stimulated PI hydrolysis was obtained through the comparison of antagonist K_i values. The ability of CPA to inhibit forskolin-stimulated cAMP accumulation was potently antagonized by the A_1-selective antagonist PACPX (Fig. 5A). Nanomolar concentrations of PACPX shifted the dose-response curve for CPA to the right. Schild analysis (34, 35) indicated a K_i value of 6 nM for PACPX (Fig. 6), which correlates well with its published affinity of 2.5 nM for the A_1 receptor in rat brain (36, 37). PACPX also shifted to the right the dose-response curve for the potentiation by CPA of PI hydrolysis (Fig. 5B). Schild analysis of these data (Fig. 6) revealed a K_i of 3.4 nM for PACPX, similar to its potency for blocking the effect of CPA on cAMP accumulation. Taken together, the data indicate that both the inhibition of forskolin-stimulated cAMP accumulation and the

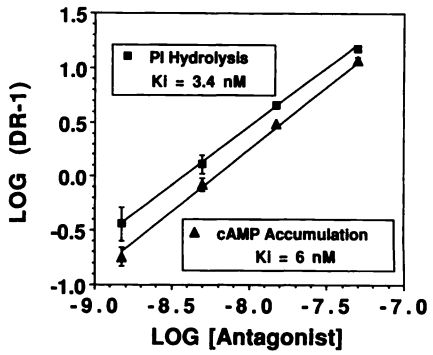

Fig. 6. Schild analysis comparing the ability of PACPX to block the effect of CPA on NE-stimulated PI hydrolysis and on forskolin-stimulated cAMP accumulation. Schild plots of the data in Fig. 5 were generated by comparing the dose ratios (*DR*) of CPA required to generate equivalent responses in the presence of various concentrations of PACPX. Values shown for K_i are the averages of three experiments.

potentiation of NE-stimulated PI hydrolysis are mediated by the A_1 subtype of adenosine receptor.

cAMP antagonizes the potentiation of PI hydrolysis. The similarity of the adenosine receptor pharmacology displayed in the potentiation of PI hydrolysis and in the inhibition of cAMP accumulation suggested that the two responses are likely to be mediated by the same population of adenosine receptors. Consequently, we asked whether the inhibition of cAMP accumulation might play a role in the potentiation of NE-stimulated PI turnover. Fig. 7A demonstrates the effect of exogenous addition of the membrane-permeable cAMP analogue 8-Br-cAMP on the potentiation of PI hydrolysis due to CPA. Whereas the NE-stimulated PI hydrolysis was not significantly affected by 8-Br-cAMP (Fig. 7A, *bar 4* versus *bar 3*), the potentiation due to the A_1 agonist was completely blocked (Fig. 7A, *bars 4* and *6*, compared with *bars 3* and *5*). To eliminate the possibility that 8-Br-cAMP was inhibiting the potentiation of PI hydrolysis by acting as an antagonist of the A_1 adenosine receptor, the ability of 8-Br-cAMP to affect CPA inhibition of cAMP accumulation was examined. The data in Fig. 7B demonstrate that CPA inhibited forskolin-stimulated cAMP accumulation with high efficacy (Fig. 7B, *bar 1* versus *bar 3*). The inhibition by CPA of cAMP accumulation was not affected by the addition of 8-Br-cAMP (Fig. 7B, *bar 7* versus *bar 3*), indicating that 8-Br-cAMP was not directly blocking the adenosine A_1 receptor.

To define more fully the relationship between cAMP accumulation and the potentiation of PI hydrolysis, paired experiments were performed examining the effects of CPA on these two functional assays. cAMP accumulation was modulated by conducting the experiments in the presence of either propranolol or forskolin or both (Fig. 8). NE, at a concentration of 10 μM, stimulated cAMP accumulation. This effect appears to be mediated via a β-adrenergic receptor, because propranolol (500 nM) completely blocked NE-stimulated cAMP accumulation (Fig. 8A). The effect of propranolol on cAMP accumulation was concomitant with a 25% enhancement of NE-stimulated PI hydrolysis (Fig. 8B), and propranolol further enhanced the potentiation of PI hydrolysis due to CPA. On the other hand, inclusion of forskolin (30 μM) significantly diminished the ability of CPA to potentiate PI hydrolysis (Fig. 8B). Forskolin had no effect upon NE-stimulated PI hydrolysis (Fig. 8B, at [CPA] = 0) but greatly enhanced cAMP accumulation in the presence of NE (Fig. 8A, at [CPA] = 0). CPA inhibited the cAMP accumulation due to forskolin plus NE, but not to the extent to which the adenosine analogue inhibited cAMP accumulation due to NE alone (Fig. 8A). In fact, even when the β-adrenergic receptor component of cAMP accumulation was blocked by propranolol, forskolin still prevented CPA from reducing cAMP accumulation all the way down to basal levels (Fig. 8A). Likewise, the combination of propranolol and forskolin strongly inhibited the potentiation by CPA of NE-stimulated PI turnover (Fig. 8B). This action of forskolin is likely due to its effect upon the cellular cAMP level, because the inactive analogue 1,9-dideoxyforskolin did not affect the potentiation of PI turnover (data not shown). Taken together, these results seem to support a role for cAMP in the regulation of NE-stimulated PI hydrolysis. Thus, whenever CPA was able to reduce cAMP to near-basal levels, NE-stimulated PI hydrolysis was enhanced. Conversely, whenever cAMP levels were maintained above basal, the potentiation of NE-stimulated PI hydrolysis by CPA was significantly diminished.

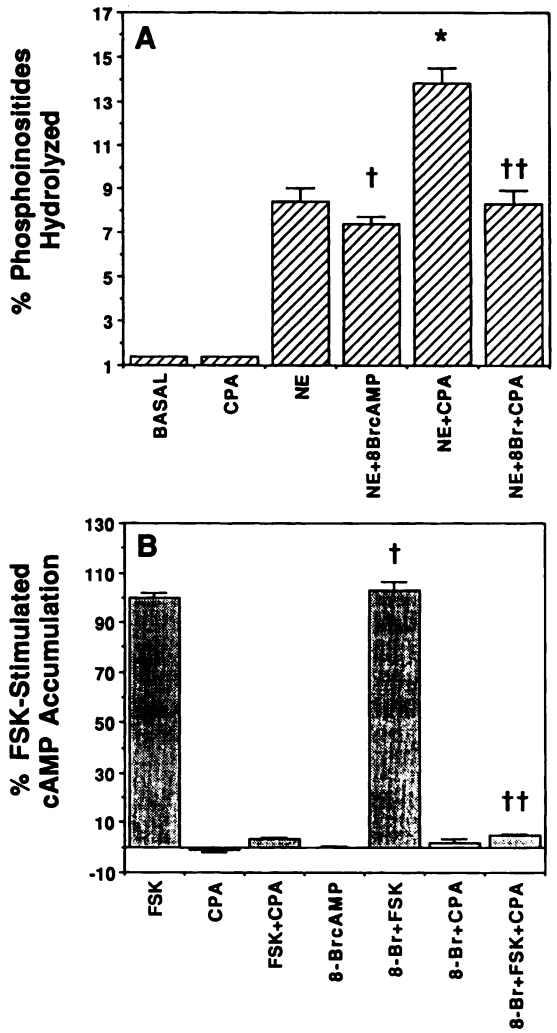

Fig. 7. 8-Br-cAMP reverses the CPA-mediated potentiation of NE-stimulated PI hydrolysis but does not block the ability of CPA to inhibit cAMP accumulation. A, PI hydrolysis was determined as described, except that 8-Br-cAMP (2 mM final concentration) was added 5 min before NE (10 μM) (*bar 4*) or NE plus CPA (10 μM and 1 μM respectively) (*bar 6*). Values are the average ± standard deviation of triplicate determinations and are typical of three to nine similar experiments. *, Significant difference ($p < 0.01$) from all other conditions; †, no significant difference ($p > 0.05$) from NE; ‡, no significant difference from either NE or NE plus 8-Br-cAMP, as determined by one-way ANOVA and Newman-Keul's test. B, 8-Br-cAMP was added 5 min before forskolin (*FSK*) (30 μM final concentration), and cAMP accumulation was determined as described. Drug concentrations were as in A. Data are the averages ± standard deviations of triplicate determinations. †, No significant difference from forskolin; ‡, no significant difference from forskolin plus CPA, as determined by Student's *t* test. Bars are numbered from the left side of the figure.

Effect of CPA on PI hydrolysis is not mediated solely through its inhibition of cAMP accumulation. Because the potentiation of PI hydrolysis by CPA was accompanied by a reduction in cellular cAMP levels, and because increases in cellular cAMP appeared to block the effect of CPA on PI hydrolysis, the hypothesis that NE-stimulated PLC activity is actively regulated by cAMP levels was considered. In accordance with this hypothesis, other agents that inhibit the cAMP accumulation should also enhance NE-stimulated PI hydrolysis.

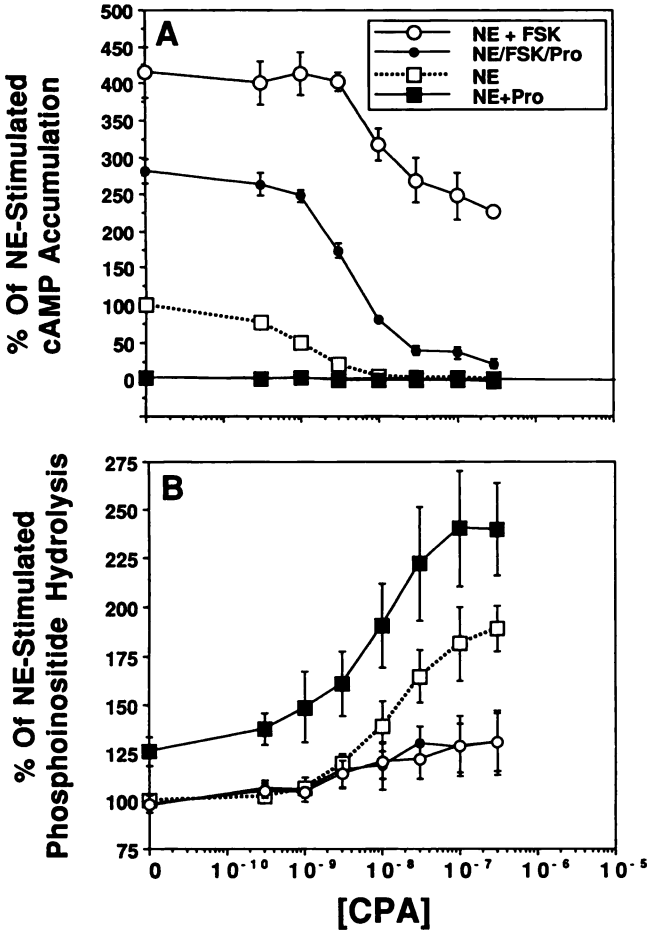

Fig. 8. Comparison of CPA dose-response characteristics for NE-stimulated cAMP accumulation (A) and potentiation of NE-stimulated PI hydrolysis (B) under conditions of enhanced or inhibited adenylyl cyclase activation. Drug concentrations were 10 μM NE, 500 nM propranolol (*PRO*), and 30 μM forskolin (*FSK*). Data from three separate paired experiments were combined by first expressing the data from each experiment as a percentage of the mean NE-stimulated value from that experiment. The average ± standard error of three experiments, performed in triplicate, is shown. Basal cAMP accumulation was 360 ± 51 cpm. In the presence of 3×10^{-7} M CPA (right-most point, greatest inhibition), the following data for cAMP accumulation were obtained: NE plus propranolol = 208 ± 25 cpm, NE = 406 ± 90 cpm, NE plus propranolol plus forskolin = 1693 ± 352 cpm, and NE plus forskolin = 18,827 ± 561 cpm. Both NE plus forskolin and NE plus propranolol plus forskolin were significantly different from basal ($p < 0.01$), whereas neither NE nor NE plus propranolol was significantly different from basal ($p > 0.05$), as determined by one-way ANOVA and Dunnett's test.

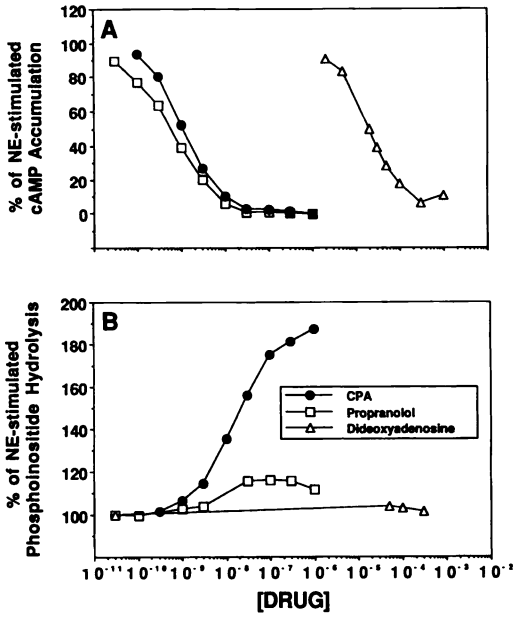

Fig. 9. Comparison of the effects of three inhibitors of NE-stimulated cAMP accumulation on cAMP accumulation (A) and PI hydrolysis (B). All assays contained 10 μM NE. The inhibitors used were propranolol, CPA, and 2′,3′-dideoxyadenosine. The averages of triplicate determinations are shown, from a representative experiment. NE-stimulated activity was 2695 ± 76 cpm, whereas basal activity amounted to 193 ± 28 cpm. In the presence of 300 μM dideoxyadenosine, the NE-stimulated activity was reduced to 325 ± 29 cpm. In the presence of 1 μM propranolol or 1 μM CPA, NE-stimulated activity was reduced to 211 ± 24 or 195 ± 11 cpm, respectively.

Fig. 9 compares the abilities of three agents to reduce NE-stimulated cAMP accumulation and to potentiate NE-stimulated PI hydrolysis. In addition to CPA, both the β-adrenergic receptor antagonist propranolol and the so-called P site adenylyl cyclase antagonist dideoxyadenosine (a compound with no action at the extracellular adenosine receptor) (38) were efficacious in the inhibition of NE-stimulated cAMP accumulation (Fig. 9A). On the other hand, dideoxyadenosine had no effect on, and propranolol only slightly enhanced, NE-stimulated PI hydrolysis (Fig. 9B). Although all three of these agents result in the inhibition of cAMP accumulation, they do so by three different modes of action. The differences in mode of action for these three agents may distinguish their differential abilities to affect PI hydrolysis. Alternatively, it is still possible that the relative efficacy of these three agents for potentiating PI hydrolysis is a direct consequence of their effects upon cellular cAMP concentrations but that the pertinent concentrations of cAMP are very close to the basal level and so are difficult to differentiate under these conditions. If, for example, CPA reduced the cellular cAMP concentration below the basal level, these assay conditions would likely not be sensitive enough to detect the difference, because basal activity represented <0.2% of incorporation. cAMP accumulation was, therefore, performed in the presence of the non-xanthine-based phosphodiesterase inhibitor Ro 20-1724, in order to measure the rate of cAMP synthesis in the absence of degradation. Under these conditions, basal activity was more than 5-fold higher (about 1% of incorporated counts), and so a stronger inhibition by CPA of cAMP accumulation (compared with propranolol) should have been observable, as a reduction below the basal level. Fig. 10 shows the effects of CPA and propranolol on both basal and NE-stimulated cAMP accumulation in the presence of 500 μM Ro 20-1724. In the absence of NE, CPA was able to reproducibly decrease cAMP accumulation below the basal level, whereas propranolol was without effect. Thus, in the presence of Ro 20-1724, this assay is sensitive enough to detect a reduction of cAMP accumulation below the basal value. In the presence of 10 μM NE, however, CPA was no more efficacious than propranolol for the inhibition of cAMP synthesis, and neither compound inhibited NE-stimulated activity below the basal level. Therefore, the differential efficacy of CPA and propranolol to enhance NE-stimulated PI hydrolysis is not explained by a difference in the rate of NE-stimulated cAMP synthesis in the presence of either compound.

Another way that CPA could conceivably reduce cellular cAMP levels below those generated in the presence of propran-

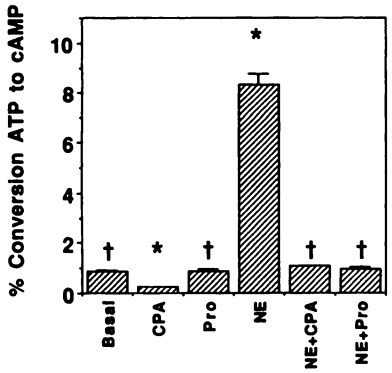

Fig. 10. Comparison of the inhibitory efficacy of propranolol and CPA on cAMP accumulation in the presence of a phosphodiesterase inhibitor. cAMP accumulation was measured in the presence of 500 μM Ro 20-1724 (to block phosphodiesterase without blocking adenosine receptors). After a 5-min preincubation of cells with the phosphodiesterase inhibitor, either 1 μM CPA or 500 nM propranolol (*Pro*) was added, with or without 10 μM NE. Data shown are the average ± standard error of three to six experiments. *, Significant difference from all other values ($p < 0.01$); †, no significant difference from other values designated by the same symbol (†) ($p > 0.05$), as determined by one-way ANOVA and Newman-Keul's test.

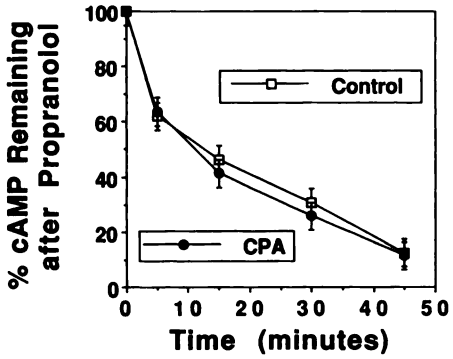

Fig. 11. Adenosine receptor activation does not affect cAMP phosphodiesterase activity. After preloading with [^{3}H]adenine, cells were stimulated with 10 μM NE for 20 min before the addition of either propranolol (500 nM) or propranolol plus CPA (1 μM). At various times the reaction was stopped by the addition of trichloroacetic acid, and the remaining [^{3}H]cAMP was measured. cAMP at time 0 was 4.03 ± 0.15% conversion of ATP to cAMP.

olol would be to enhance phosphodiesterase activity. The rate of cAMP degradation was measured by first increasing cAMP levels with NE and then blocking further production of cAMP with propranolol. Under these conditions, the addition of CPA did not appear to increase the rate of breakdown of cAMP (Fig. 11). Thus, in comparing the effects of CPA and propranolol upon NE-stimulated PI hydrolysis (Fig. 9) and upon NE-stimulated cAMP accumulation (Figs. 9–11), the difference in the effects of the drugs on PI hydrolysis does not appear to be explained by any difference in their effects on either cAMP synthesis or degradation. Hence, it is likely that CPA posseses an additional action, other than the inhibition of adenylyl cyclase, that accounts for its ability to potentiate PI hydrolysis.

To further test the hypothesis that a dramatic reduction or elimination of the actions of cAMP is all that is required for the CPA potentiation of NE-stimulated PI hydrolysis, we examined the effect of the PKA inhibitor H-8 on the potentiation by CPA of NE-stimulated PI hydrolysis and on the reversal by 8-Br-cAMP of the potentiation. In a dose-dependent manner, H-8 inhibited the ability of 8-Br-cAMP to reverse the potentiation of PI hydrolysis (Fig. 12). This is consistent with the action of H-8 to inhibit PKA in intact cells at these concentrations (39) and indicates that the effects of 8-Br-cAMP are mediated via the activation of PKA. The fact that H-8 increased NE-stimulated PI hydrolysis appears to support the hypothesis that cAMP regulates PLC activity. However, the observation that CPA still potentiates PI hydrolysis at concentrations of H-8 that completely block the effect of 2 mM 8-Br-cAMP (200 μM) indicates an action of CPA that does not involve effects upon cAMP-dependent protein kinase.

Potentiation of PI hydrolysis by CPA is pertussis toxin sensitive. An additional action of the adenosine receptor could be mediated through a transducer and effector mechanism completely different from that involved in the regulation of adenylyl cyclase. Alternatively, the same transducer might couple to more than one effector mechanism. To examine these possibilities, cells were treated with pertussis toxin, which results in a blockade of the action of G_i on adenylyl cyclase (40). Although pertussis toxin may also have other actions, the blockade of responses by this agent is frequently interpreted as implying a role for a G_i-like protein in the response. Because the potentiation by CPA of PI hydrolysis is inhibited by cellular cAMP and pertussis toxin treatment eliminates the ability of CPA to lower cAMP levels (data not shown), a blockade by pertussis toxin of the potentiation of NE-stimulated PI hydrolysis would not be surprising. To circumvent this trivial interpretation, the effect of CPA on pertussis toxin-treated cells was examined in the presence of 250 μM H-8. This concentration of H-8 blocked the ability of either 2 mM 8-Br-cAMP (Fig. 12) or 30 μM forskolin (data not shown) to inhibit the CPA-induced potentiation of PI hydrolysis. Under these conditions, then, an effect of pertussis toxin on the action of CPA should not be due to the fact that CPA can no longer decrease the cellular cAMP concentration. Fig. 13 demonstrates the blockade of the potentiation of PI hydrolysis (in the presence of 250 μM H-8) after a 12-hr pretreatment of cells with 100 ng/ml pertussis toxin. Thus, as much as pertussis toxin sensitivity can be considered a selective index, the potentiation by CPA of NE-stimulated PI hydrolysis appears to require a G_i-like protein.

Discussion

Potentiation of PI hydrolysis by adenosine agonists was first reported by Hollingsworth *et al.* (41) and by Hill and Kendall

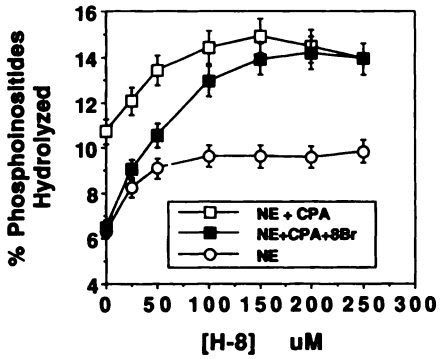

Fig. 12. Effect of a PKA inhibitor (H-8) on both the potentiation of PI hydrolysis by CPA and its reversal by 8-Br-cAMP. Drug concentrations were 1 μM CPA, 10 μM NE, and 2 mM 8-Br-cAMP (*8Br*). Data are the mean ± range of two experiments conducted in triplicate. 8-Br-cAMP had no effect upon NE-stimulated PI hydrolysis.

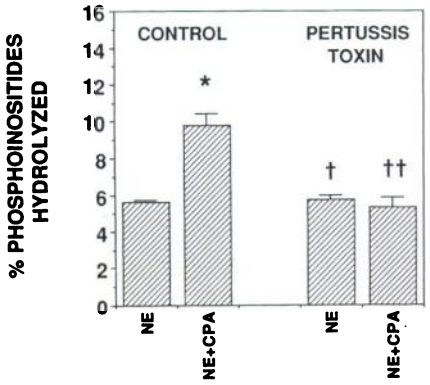

Fig. 13. Effect of pertussis toxin on the potentiation by CPA of NE-stimulated PI hydrolysis. This experiment was conducted in the presence of 250 μM H-8 to block the potential inhibitory effect of increased cAMP in pertussis toxin-treated cells. Drug concentrations were 10 μM NE and 1 μM CPA. Pertussis toxin (100 ng/ml) was added to cultures 12 hr before harvesting. These data are representative of three experiments. *, Significant difference from all other values ($p < 0.01$); †, no significant difference from control NE; ‡, no significant difference from pertussis toxin NE ($p > 0.05$), as determined by one-way ANOVA and Newman-Keul's test.

(42). Both groups observed the potentiation by adenosine agonists of histamine-stimulated PI hydrolysis in guinea pig cerebral cortical slices. More recently, El-Etr *et al.* (43) have reported that adenosine agonists potentiate both carbachol- and NE-stimulated PI hydrolysis in rat striatal slices and in mixed cultures of striatal neurons and glia. Two inherent problems in these studies limit the interpretation of the results. The use of brain slices or mixed cultures of cells cannot allow for the differentiation of intracellular from intercellular mechanisms. Furthermore, in these studies, the pharmacological delineation of the adenosine receptor mediating the enhanced responsiveness was inconclusive, due to the low drug potencies observed. Our studies, utilizing the clonal cell line DDT_1, have specifically addressed these two issues. Firstly, because the potentiation response occurs in a homogeneous population of cells, it would appear that the action of adenosine agonists to potentiate PI hydrolysis could be mediated via intracellular mechanisms, rather than requiring intercellular interactions. This does not, however, rule out the possibility that soluble factors might be released that could act as either autocoids or hormones. Such factors may explain the observation by El Etr *et al.* (43) of an apparent requirement for coculture of both neurons and glia in order to observe the potentiation of carbachol-stimulated PI hydrolysis. Secondly, the modulation of NE-stimulated PI hydrolysis produced by adenosine agonists and by a selective adenosine antagonist demonstrates a pharmacological profile that is consistent with the involvement of an A_1 subtype of adenosine receptor. We had noted previously the occurrence of second messenger responses that were consistent with the existence of adenosine A_1 receptors on DDT_1 cells (44), and these findings have been confirmed subsequently, using radioligand binding studies and photoaffinity labeling techniques (45, 46).

The EC_{50} values for the inhibition of NE-stimulated cAMP accumulation and for the potentiation of NE-stimulated PI hydrolysis produced by the A_1-selective agonist CPA and the nonselective agonist CPCA are summarized in Table 1 and are compared with published K_i values from radioligand binding studies using rat brain membranes. In both functional assays,

TABLE 1

Comparison of agonist EC_{50} values and antagonist K_i values from functional assays with published radioligand binding data

Values for functional assays are summarized from the data shown. Radioligand binding data are from Refs. 36, 37, and 59. The K_i value for the inhibition of [^{3}H] PD 116,948 binding by CPCA was not determined; however, NECA (5′-*N*-ethylcarboxamideadenosine), a compound exhibiting structure and pharmacology very similar to those of CPCA (36), had a K_i value of 90 nM in this study (59). CHA is N^6-cyclohexyladenosine.

Assay	CPA	CPCA	PACPX
cAMP accumulation	EC_{50} = 1.1 nM	EC_{50} = 14 nM	K_i = 6 nM
PI potentiation	EC_{50} = 8 nM	EC_{50} = 115 nM	K_i = 3.4 nM
A_1 receptor binding			
Agonist [^{3}H]CHA binding	K_i = 0.6 nM	K_i = 6.4 nM	K_i = 2.5 nM
Antagonist [^{3}H]PD 116,948 binding	K_i = 7.9 nM	ND[a]	K_i = 1.4 nM
A_2 receptor binding			
Agonist [^{3}H]NECA binding	K_i = 462 nM	K_i 13.4 nM	K_i = 92 nM

[a] ND, not done.

CPA demonstrates nanomolar potency and is about 14-fold more potent than CPCA, characteristic of the involvement of the A_1 adenosine receptor subtype. Interestingly, both compounds are about 8-fold more potent in the inhibition of cAMP accumulation than in the potentiation of PI hydrolysis. This could be due to at least three possible reasons. 1) The two responses could be mediated via two different adenosine receptor subtypes. 2) The inhibition of cAMP accumulation could have a large adenosine receptor reserve, compared with the coupling mechanism involved in the potentiation of PLC activity. 3) The potentiation of PI hydrolysis is not elicited in the presence of cAMP. Thus, the shifted dose-response curves in the two assays may be due to the fact that NE-stimulated cAMP levels may need to be reduced by >90% before the potentiation of PLC activation can occur.

The need to invoke the first two possible explanations may be due to the fact that the receptor(s) involved is (are) being defined in terms of agonist potencies. Because agonist potency can be increased by the existence of spare receptors, a more rigorous delineation of the receptor subtype involved in a functional study is accomplished by determining the K_i value of a selective antagonist via the method of Schild (34, 35). The A_1-selective antagonist PACPX was examined in both functional assays and was found to be nearly equipotent in blocking the effect of CPA on either function. Therefore, both of the functional responses to adenosine agonists appear to be mediated via the A_1 adenosine receptor subtype, and the differences in agonist potency are likely due either to spare receptors or to interactions between the two functional systems.

The third possibility is suggested by the following observations. 1) The mechanism by which CPA potentiates NE-stimulated PI hydrolysis requires the inhibition of cAMP accumulation, as evidenced by the fact that either 8-Br-cAMP (Fig. 7A) or forskolin (Fig. 8B) was able to block selectively the component of PI hydrolysis that was stimulated by the adenosine agonist CPA. 2) The concentration of CPA required to cause potentiation of PLC activity (>10 nM) was equal to or greater than the concentration that appeared to cause nearly complete inhibition of NE-stimulated cAMP accumulation (Fig. 8). These observations are consistent with the idea that the difference in the agonist potency in the two functional assays is due to the fact that CPA must decrease the cAMP

concentration to near-basal levels before the potentiation of PLC activity can occur. This suggestion is not well supported, however, by other data in Fig. 8. If it were so, then the addition of propranolol, which completely blocked NE-stimulated cAMP accumulation and so maintained cAMP at basal levels (Fig. 8A), should have shifted the dose-response curve for CPA-potentiated PLC activity to the left in Fig. 8B, which it did not. Additional attempts to shift the dose-response curve to the left in the presence of either H-8 or propranolol plus H-8 also proved negative (data not shown). These results suggest that, although cAMP levels must be decreased in order to observe the potentiation of NE-stimulated PI hydrolysis, this requirement does not account for the shifted dose-response curves for agonists when compared in the two functional assays. Therefore, the most likely of the three possible reasons suggested to explain the shifted dose-response curves is the occurrence of spare receptors for the inhibition of cAMP accumulation. The observation that the EC_{50} values in this assay compare well with the K_d values determined from radiolabeled agonist binding studies (Table 1) is probably coincidental, because agonist K_d values determined from radioligand binding studies in membranes are often subject to artifacts of the state of the receptor under *in vitro* conditions. What is more striking is that the EC_{50} value of CPA for the potentiation of PI hydrolysis is nearly identical to its published K_i value for the inhibition of radiolabeled antagonist binding (Table 1).

Although the inhibition of cAMP accumulation was required to observe potentiation of PLC, the reduction of cAMP accumulation alone was insufficient to cause the potentiation of NE-stimulated PI hydrolysis. This effect was not demonstrated by the adenylyl cyclase antagonist dideoxyadenosine and was only slightly induced by the β-adrenergic antagonist propranolol, both of which appeared to cause a decrease in cAMP comparable to that seen with CPA (Fig. 9). Additionally, CPA continued to potentiate PLC activity after blockade of PKA activity with H-8 (Fig. 12). Thus, CPA appears to possess two actions that combine to generate an enhancement of NE-stimulated PLC activity. One of these two actions, the inhibition of adenylyl cyclase activity, results in a reduction in the concentration of the endogenous inhibitor cAMP and is presumably mediated via the pertussis toxin-sensitive transducer protein G_i. The second action, although not mediated through the inhibition of adenylyl cyclase, is, nevertheless, pertussis toxin sensitive. This is consistent with the involvement of a G_i-like (or G_o-like) protein in this action as well. The interruption of either of these two actions is sufficient to block the CPA-stimulated potentiation of PI hydrolysis, without altering the NE-stimulated response. The fact that both cAMP and pertussis toxin selectively regulate the potentiation of PI hydrolysis, without significantly altering the response to NE, suggests that cAMP does not partially modulate the responsiveness of PLC to stimulation by NE but, instead, controls the full magnitude of the response of a second pathway, stimulated by CPA. This second pathway, although demonstrating independent regulation from the first pathway, is apparently dependent upon the simultaneous activation of both receptors, because adenosine agonists do not elicit PI hydrolysis in the absence of NE (Figs. 1, 2, and 7A). It is likely that some consequence of α_{1b}-adrenergic receptor activation plays an essential role in the mechanism of activation of the second pathway.

Our data agree with the observations of several other investigators who have suggested that cAMP negatively regulates PI hydrolysis (4–9). A common feature of these observations is that cAMP rarely inhibits PI hydrolysis by more than 30–50%. A second common feature is that the agonists used to stimulate PI hydrolysis in these studies (GTPγS, fluoride, carbachol, and thrombin) are known to activate both pertussis toxin-sensitive and -insensitive guanine nucleotide-mediated activities. In platelets, for example, thrombin both stimulates PLC and inhibits adenylyl cyclase (47, 48). Both cAMP and pertussis toxin cause a partial, but incomplete, inhibition of PI hydrolysis in response to thrombin (9, 47, 48), suggesting that, in platelets, thrombin may utilize more than one G protein to couple to PLC. The model system used in the present study (the DDT_1 cell line) has the advantage of being able to isolate the A_1 adenosine receptor-mediated inhibition of adenylyl cyclase from the α_{1b}-adrenergic receptor-coupled activation of PI hydrolysis. The results obtained through this system have revealed additional complexities in the regulation of PI hydrolysis by cAMP. A reason why cAMP only partially inhibits PI hydrolysis in the other systems may be explained by invoking the two-component model of PI hydrolysis, suggested by the present data. One component is thought to be due to the activation of a pertussis toxin-insensitive G protein, often referred to as G_p. The second component is due to the activation of a pertussis toxin-sensitive pathway, which may involve a G_i-like protein. [Whether this pertussis toxin-sensitive pathway utilizes the same G protein as that mediating the inhibition of adenylyl cyclase is unknown. Preparations of affinity-purified adenosine A_1 receptor from bovine brain contain α subunits from G_i and G_o, both of which are pertussis toxin substrates (49).] This second component is demonstrable only when a G_i-linked receptor is activated simultaneously with a G_p-linked receptor. The second, or G_i-mediated, component is completely blocked either by pertussis toxin or by cAMP, whereas the first, or G_p-mediated, component is insensitive to either agent.

We are not the first to suggest multiple pathways for the stimulation of PI hydrolysis. Gonzales and Crews (50) have demonstrated independent stimulation of PI hydrolysis in brain membranes, by either guanine nucleotides or by calcium. Stimulation of PI hydrolysis by calcium has also been demonstrated in permeabilized cells (50–52) and in liver plasma membranes (53). Given that α_1 receptor activation was reported to stimulate calcium influx in DDT_1 cells (54), one possible interpretation of the enhancement of NE-stimulated PI hydrolysis by CPA could be that CPA enhances NE-stimulated calcium influx and that this influx of calcium activates a calcium-sensitive PLC. Although still a possibility, this is deemed less likely, because of the fact that our PI hydrolysis experiments were conducted in the absence of added extracellular calcium. Still, CPA may regulate intracellular calcium concentration through its inhibition of adenylyl cyclase. Increased cAMP has been shown to decrease intracellular calcium mobilization in hepatocytes (55) and platelets (56) and to induce calcium sequestration in smooth muscle (57). A rise in intracellular calcium (resulting from the action of CPA to reduce the cAMP level), combined with the activation of a G_i-like protein, could potentially play a role in the recruitment of PLC enzymes that are not directly activated by NE alone. The role of calcium in this phenomenon is investigated in detail in the accompanying manuscript (58).

References

1. Chik, C. L., A. K. Ho, and D. C. Klein. α_1-Adrenergic potentiation of vasoactive intestinal peptide stimulation of rat pinealocyte adenosine 3′,5′-monophosphate and guanosine 3′,5′-monophosphate: evidence for a role of calcium and protein kinase-C. *Endocrinology* **122**:702–708 (1988).
2. Jakobs, K. H., Y. Watanabe, and S. Bauer. Interactions between the hormone-sensitive adenylate cyclase system and the phosphoinositide-metabolizing pathway in human platelets. *J. Cardiovasc. Pharmacol.* **8** (Suppl. 8):S61–S64 (1986).
3. Magistretti, P. J., and M. Schorderet. VIP and noradrenaline act synergistically to increase cyclic AMP in cerebral cortex. *Nature (Lond.)* **308**:280–282 (1984).
4. Yada, Y., S. Nagao, Y. Okano, and Y. Nozawa. Inhibition by cyclic AMP of guanine nucleotide-induced activation of phosphoinositide-specific phospholipase C in human platelets. *FEBS Lett.* **242**:368–372 (1989).
5. Akil, M., and S. K. Fisher. Muscarinic receptor-stimulated phosphoinositide turnover in human SK-N-SH neuroblastoma cells: differential inhibition by agents that elevate cyclic AMP. *J. Neurochem.* **53**:1479–1486 (1989).
6. Lazarowski, E. R., and E. G. Lapetina. Activation of platelet phospholipase C by fluoride is inhibited by elevation of cyclic AMP. *Biochem. Biophys. Res. Commun.* **158**:440–444 (1989).
7. Puurunen, J., M. J. Lohse, and U. Schwabe. Interactions between intracellular cyclic AMP and agonist induced inositol phospholipid breakdown in isolated gastric mucosal cells of the rat. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **336**:471–477 (1987).
8. Bianca, V. D., P. De Tongi, M. Grzeskowiak, L. M. Vicentini, and F. Di Virgilio. Cyclic AMP inhibition of phosphoinositide turnover in human neutrophils. *Biochim. Biophys. Acta* **886**:441–447 (1986).
9. Watson, S. P., R. T. McConnell, and E. G. Lapetina. The rapid formation of inositol phosphates in human platelets by thrombin is inhibited by prostacyclin. *J. Biol. Chem.* **259**:13199–13203 (1984).
10. Berridge, M. J. Inositol trisphosphate and diacylglycerol: two interacting second messengers. *Annu. Rev. Biochem.* **56**:159–193 (1987).
11. Bell, J. D., I. L. O. Buxton, and L. L. Brunton. Enhancement of adenylate cyclase activity in S49 lymphoma cells by phorbol esters. *J. Biol. Chem.* **260**:2625–2628 (1985).
12. Yoshimasa, T., D. R. Sibley, M. Bouvier, R. J. Lefkowitz, and M. Caron. Crosstalk between cellular signalling pathways suggested by phorbol-ester-induced adenylate cyclase phosphorylation. *Nature (Lond.)* **327**:67–70 (1987).
13. Sugden, D., and D. C. Klein. Activators of protein kinase C act at a postreceptor site to amplify cyclic AMP production in rat pinealocytes. *J. Neurochem.* **50**:149–155 (1988).
14. Supattapone, S., S. K. Danoff, A. Theibert, S. K. Joseph, J. Steiner, and S. H. Snyder. Cyclic AMP-dependent phosphorylation of a brain inositol trisphosphate receptor decreases its release of calcium. *Proc. Natl. Acad. Sci. USA* **85**:8747–8750 (1988).
15. Takuwa, Y., N. Takuwa, and H. Rasmussen. The effects of isoproterenol on intracellular calcium concentration. *J. Biol. Chem.* **263**:762–768 (1988).
16. Burgess, G. M., R. K. Dooley, J. S. McKinney, E. Nanberg, and J. W. Putney. Further studies on the interactions between the calcium mobilization and cyclic AMP pathways in guinea pig hepatocytes. *Mol. Pharmacol.* **30**:315–320 (1986).
17. Morgan, N. G., R. Charest, P. F. Blackmore, and J. H. Exton. Potentiation of α_1-adrenergic responses in rat liver by a cAMP-dependent mechanism. *Proc. Natl. Acad. Sci. USA* **81**:4208–4212 (1984).
18. Berridge, M. J. Cellular control through interactions between cyclic nucleotides and calcium. *Adv. Cyclic Nucleotide Protein Phosphorylation Res.* **17**:329–335 (1984).
19. Norris, J. S., D. J. Garmer, F. Brown, K. Popovich, and L. E. Cornett. Characteristics of an adenylate cyclase coupled β_2-adrenergic receptor in a smooth muscle cell line. *J. Recept. Res.* **3**:623–645 (1983).
20. Cornett, L. E., and J. S. Norris. Characterization of the α_1-adrenergic receptor subtype in a smooth muscle cell line. *J. Biol. Chem.* **257**:694–697 (1982).
21. Hedqvist, P., and B. B. Fredholm. Effects of adenosine on adrenergic neurotransmission; prejunctional inhibition and postjunctional enhancement. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **293**:217–223 (1976).
22. Iso, T. Effects of cyclic 3′,5′-AMP and related compounds on catecholamine-induced contraction of isolated guinea-pig vas deferens. *Jpn. J. Pharmacol.* **23**:717–721 (1973).
23. Berridge, M. J., R. M. C. Dawson, C. P. Downes, J. P. Heslop, and R. F. Irvine. Changes in the level of inositol phosphates after agonist-dependent hydrolysis of membrane phosphoinositides. *Biochem. J.* **212**:473–482 (1983).
24. Shimizu, H., J. W. Daly, and C. R. Creveling. A radioisotopic method for measuring the formation of adenosine 3′,5′-cyclic monophosphate in incubated slices of brain. *J. Neurochem.* **16**:1609–1619 (1969).
25. Salomon, Y., C. Londos, and M. Rodbell. A highly sensitive adenylate cyclase assay. *Anal. Biochem.* **58**:541–548 (1974).
26. Wilson, K. M., and K. P. Minneman. Synergistic interactions between α_1- and α_2-adrenergic receptors in activating ^{3}H-inositol phosphate formation in primary glial cell cultures. *J. Neurochem.* **56**:953–960 (1991).
27. Tsujimoto, G., A. Tsujimoto, E. Suzuki, and K. Hashimoto. Glycogen phosphorylase activation by two different α_1-adrenergic subtypes: methoxamine selectively stimulates a putative α_1-adrenergic receptor subtype (α_{1a}) that couples with Ca^{2+} influx. *Mol. Pharmacol.* **36**:166–176 (1989).
28. Han, C., K. M. Wilson, and K. P. Minneman. α_1-Adrenergic receptor subtypes and formation of inositol phosphates in dispersed hepatocytes and renal cells. *Mol. Pharmacol.* **37**:903–910 (1990).
29. Wilson, K. M., and K. P. Minneman. Different pathways of [^{3}H]inositol phosphate formation mediated by α_{1a}- and α_{1b}-adrenergic receptors. *J. Biol. Chem.* **265**:17601–17606 (1990).
30. Han, C., P. W. Abel, and K. P. Minneman. Heterogeneity of α_1-adrenergic receptors revealed by chlorethylclonidine. *Mol. Pharmacol.* **32**:505–510 (1987).
31. Minneman, K. P., C. Han, and P. W. Abel. Comparison of α_1-adrenergic receptor subtypes distinguished by chlorethylclonidine and WB 4101. *Mol. Pharmacol.* **33**:509–514 (1988).
32. van Calker, D., M. Muller, and B. Hamprecht. Adenosine regulates, via two different types of receptors, the accumulation of cyclic AMP in cultured brain cells. *J. Neurochem.* **33**:999–1005 (1979).
33. Londos, C., D. M. F. Cooper, and J. Wolff. Subclasses of external adenosine receptors. *Proc. Natl. Acad. Sci. USA* **77**:2551–2554 (1980).
34. Kenakin, T. P. The Schild regression in the process of receptor classification. *Can. J. Physiol. Pharmacol.* **60**:249–265 (1982).
35. Schild, H. O. The use of drug antagonists for the identification and classification of drugs. *Br. J. Pharmacol.* **2**:251–258 (1947).
36. Bruns, R. F., G. H. Lu, and T. A. Pugsley. Characterization of the A_2 adenosine receptor labeled by [^{3}H]NECA in rat striatal membranes. *Mol. Pharmacol.* **29**:331–346 (1986).
37. Daly, J. W., W. Padgett, M. T. Shamim, P. Butts-Lamb, and J. Waters. 1,3-Dialkyl-8-(*p*-sulfophenyl)xanthines: potent water-soluble antagonists for A_1- and A_2-adenosine receptors. *J. Med. Chem.* **28**:487–492 (1985).
38. Wolff, J., C. Londos, and G. H. Cook. Adenosine interactions with thyroid adenylate cyclase. *Arch. Biochem. Biophys.* **191**:161–168 (1978).
39. Tash, J. S., H. Hidaka, and A. R. Means. Axokinin phosphorylation by cAMP-dependent protein kinase is sufficient for activation of sperm flagellar motility. *J. Cell Biol.* **103**:649–655 (1986).
40. Ui, M. Islet-activating protein, pertussis toxin: a probe for functions of the inhibitory guanine-nucleotide regulatory component of adenylate cyclase. *Trends Pharmacol. Sci.* **5**:277–279 (1984).
41. Hollingsworth, E. B., R. A. De La Cruz, and J. W. Daly. Accumulations of inositol phasphates and cyclic AMP in brain slices: synergistic interactions of histamine and 2-chloroadenosine. *Eur. J. Pharmacol.* **122**:45–50 (1986).
42. Hill, S. J., and D. A. Kendall. Studies on the adenosine-receptor mediating the augmentation of histamine-induced inositol phospholipid hydrolysis in guinea-pig cortex. *Br. J. Pharmacol.* **91**:661–669 (1987).
43. El Etr, M., J. Cordier, J. Glowinski, and J. Premont. A neuroglial cooperativity is required for the potentiation by 2-chloroadenosine of the muscarinic-sensitive phospholipase C in the striatum. *J. Neurosci.* **9**:1473–1480 (1989).
44. Schachter, J. B., and B. B. Wolfe. The regulation of phosphatidylinositol turnover by cyclic AMP may involve products of arachidonic acid metabolism. *Soc. Neurosci. Abstr.* **15**:1007 (1989).
45. Vickram, R., W. W. Barrington, K. A. Jacobson, and G. L. Stiles. Demonstration of both A_1 and A_2 adenosine receptors in DDT$_1$ MF-2 smooth muscle cells. *Mol. Pharmacol.* **37**:149–156 (1990).
46. Gerwins, P., C. Nordstedt, and B. B. Fredholm. Characterization of adenosine A_1 receptors in intact DDT$_1$ MF-2 smooth muscle cells. *Mol. Pharmacol.* **38**:660–666 (1990).
47. Brass, L. F., M. J. Woolkalis, and D. R. Manning. Interactions in platelets between G proteins and the agonists that stimulate phospholipase C and inhibit adenylyl cyclase. *J. Biol. Chem.* **263**:5348–5355 (1988).
48. Brass, L. F., M. Laposata, H. S. Banga, and S. E. Rittenhouse. Regulation of the phosphoinositide hydrolysis pathway in thrombin-stimulated platelets by a pertussis toxin-sensitive guanine nucleotide-binding protein. *J. Biol. Chem.* **261**:16838–16847 (1986).
49. Munshi, R., and J. Linden. Co-purification of A_1 adenosine receptors and guanine nucleotide-binding proteins from bovine brain. *J. Biol. Chem.* **264**:14853–14859 (1989).
50. Gonzales, R. A., and F. T. Crews. Differential regulation of phosphoinositide phosphodiesterase activity in brain membranes by guanine nucleotides and calcium. *J. Neurochem.* **50**:1522–1528 (1988).
51. Fisher, S. K., L. M. Domask, and R. M. Roland. Muscarinic receptor regulation of cytoplasmic calcium concentrations in human SK-N-SH neuroblastoma cells: calcium requirements for phospholipase C activation. *Mol. Pharmacol.* **35**:195–204 (1989).
52. McDonough, P. M., D. Goldstein, and J. H. Brown. Elevation of cytoplasmic calcium concentration stimulates hydrolysis of phosphatidylinositol bisphosphate in chick heart cells: effect of sodium channel activators. *Mol. Pharmacol.* **33**:310–315 (1988).
53. Uhing, R. J., H. Jiang, V. Prpic, and J. H. Exton. Regulation of a liver plasma membrane phosphoinositide phosphodiesterase by guanine nucleotides and calcium. *FEBS Lett.* **188**:317–320 (1985).
54. Reynolds, E. E., and G. R. Dubyak. Activation of calcium mobilization and calcium influx by *alpha*$_1$-adrenergic receptors in a smooth muscle cell line. *Biochem. Biophys. Res. Commun.* **130**:627–632 (1985).
55. Morgan, N. G., P. F. Blackmore, and J. H. Exton. Modulation of the α_1-

adrenergic control of hepatocyte calcium redistribution by increases in cyclic AMP. *J. Biol. Chem.* **258**:5110–5116 (1983).
56. Feinstein, M. B., J. J. Egan, R. I. Sha'afi, and J. White. The cytoplasmic concentration of free calcium in platelets is controlled by stimulators of cyclic AMP formation (PGD_2, PGE_1, forskolin). *Biochem. Biophys. Res. Commun.* **113**:598–604 (1983).
57. Berridge, M. J. The interaction of cyclic nucleotides and calcium in the control of cellular activity. *Adv. Cyclic Nucleotide Res.* **6**:1–98 (1975).
58. Schachter, J. B., J. K. Ivins, R. N. Pittman, and B. B. Wolfe. Competitive regulation of phospholipase C responses by cAMP and calcium. *Mol. Pharmacol.* **41**:577–586 (1992).
59. Bruns, R. F., J. H. Fergus, E. W. Badger, J. A. Bristol, L. A. Santay, J. D. Hartman, S. J. Hays, and C. C. Huang. Binding of the A_1-selective adenosine antagonist 8-cyclopentyl-1,3-dipropylxanthine to rat brain membranes. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **335**:59–63 (1987).

Send reprint requests to: Barry B. Wolfe, Department of Pharmacology, Georgetown University School of Medicine, 3900 Reservoir Rd. NW, Washington DC 20007.