0026-895X/92/030577-10$03.00/0

MOLECULAR PHARMACOLOGY, 41:577-586

Competitive Regulation of Phospholipase C Responses by cAMP and Calcium

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Received August 16, 1991; Accepted December 6, 1991

SUMMARY

DDT_1-MF_2 smooth muscle cells demonstrated a robust phospholipase C response to norepinephrine, as detected by inositol phosphate accumulation. A selective A_1-adenosine receptor agonist, cyclopentyladenosine, caused only a minor stimulation of phospholipase C, which was eliminated in the absence of added extracellular calcium. The simultaneous addition of norepinephrine and cyclopentyladenosine resulted in a synergistic increase in phosphoinositide hydrolysis either in the absence or in the presence of external calcium. In the presence of external calcium and a calcium ionophore, the adenosine agonist caused a significant stimulation of phosphoinositide hydrolysis without the addition of norepinephrine. Influx of extracellular calcium through voltage-sensitive calcium channels did not appear to be required to observe an effect of cyclopentyladenosine, because neither calcium channel antagonists (nifedipine, verapamil, and $LaCl_3$) nor a chelator of extracellular calcium (ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid) were able to alter the degree of potentiation of norepinephrine-stimulated phosphoinositide hydrolysis due to the adenosine agonist. On the other hand, buffering of intracellular calcium concentration with the membrane-permeant calcium chelator quin2 blocked the potentiation. This blockade of potentiation by quin2 was reversed by the addition of extracellular calcium. Agents that stimulated cAMP production or membrane-permeable analogues of cAMP also blocked the action of the adenosine agonist to potentiate norepinephrine-stimulated phosphoinositide hydrolysis. This effect of cAMP was less pronounced in the presence of elevated extracellular calcium and was abolished in the presence of a calcium ionophore. When norepinephrine-stimulated calcium transients were quantitated using fura-2 fluorescence, a reduction in the amplitude of the calcium response was observed in the presence of forskolin. Conversely, both the amplitude and the duration of the calcium response were enhanced by the addition of the adenosine agonist. The results of these studies suggest that the mechanism by which adenosine receptors enhance the stimulation of phosphoinositide hydrolysis is dependent upon a rise in intracellular Ca^{2+} concentration resulting from the simultaneous activation of α_1-adrenergic receptors. The results further suggest that cAMP inhibits this mechanism by decreasing the norepinephrine-stimulated rise in intracellular Ca^{2+} concentration.

α_1-Adrenergic receptors are thought to initiate a number of biochemical responses through the activation of PLC, leading ultimately to the activation of cellular responses such as transmitter release and smooth muscle contraction. PLC cleaves membrane PI to yield diacylglycerols, which can activate PKC, and inositol phosphates such as inositol-1,4,5-trisphosphate, which can mobilize intracellular calcium stores (1). The modulation of α_1 receptor-stimulated PLC activity by the actions of other hormones or pharmacological agents might be expected to result in altered tissue responses to α_1 agonists. For example, pretreatment of smooth muscle cells with phorbol esters, which activate PKC (2), results in a decrease of receptor-stimulated PLC activity (3) and also results in a decreased contractile response of smooth muscle to stimulation by α_1 agonists (4).

Studies of smooth muscle preparations have demonstrated a modulatory role for adenosine on α_1 receptor-stimulated contraction. Although the most commonly observed response of vascular smooth muscle to adenosine is relaxation (see Ref. 5), a number of other smooth muscle preparations, including the guinea pig vas deferens, exhibit minimal responses to adenosine alone but demonstrate a significantly enhanced contractile response to α_1-adrenoceptor agonists in the presence of aden-

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

ABBREVIATIONS: PLC, polyphosphoinositide-specific phospholipase C; 8-Br-cAMP, 8-bromoadenosine cyclic 3′,5′-monophosphate; CPA, N^6-cyclopentyladenosine; G protein, heterotrimeric signal-transducing GTP-binding protein; Ins-P_3, inositol trisphosphate(s); NE, (−)-norepinephrine; PI, phosphoinositide(s); PKC, calcium- and phospholipid-dependent protein kinase; TPA, 12-*O*-tetradecanoyl-phorbol-13-acetate (also referred to as phorbol 12-myristate 13-acetate); EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; AM, acetoxy methyl ester; GTPγs, guanosine-5′-*O*-(3-thio)triphosphate; PKA, protein kinase A; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; $[Ca^{2+}]_o$, extracellular free Ca^{2+} concentration.

osine (6). The mechanism by which adenosine acts to potentiate α_1 receptor-stimulated smooth muscle contraction is unknown but could conceivably involve effects upon PLC activation. We have previously reported (7, 8) that the selective A_1 adenosine agonist CPA potentiates NE-stimulated PI hydrolysis in the DDT_1-MF_2 smooth muscle cell line derived from hamster vas deferens. We noted that the PLC response to the combination of NE and CPA appeared to consist of two components. One component was due to the activation of α_1-adrenoceptors by NE. This activity was insensitive to cAMP or pertussis toxin. A second component of PI hydrolysis, stimulated by the combination of CPA and NE, was due to the action of CPA at adenosine A_1 receptors, although CPA did not stimulate PI hydrolysis in the absence of NE. This second component was selectively inhibited by 8-Br-cAMP or forskolin and was eliminated by pertussis toxin pretreatment.

These studies have investigated why α_1-adrenoceptor activation is required in order to demonstrate A_1 adenosine receptor-stimulated PI hydrolysis. The results strongly suggest that PLC activation by CPA requires a simultaneous rise in $[Ca^{2+}]_i$, which is provided by α_1 receptor activation. Given this finding, we have proceeded to investigate the relationship between the effects of cAMP and calcium in this response. The data indicate that cAMP and calcium have opposing actions on the secondary, CPA-stimulated, component of the PLC response.

Materials and Methods

Cell culture. Suspension cultures of DDT_1-MF_2 cells were grown in Dulbecco's modification of Eagle's medium containing 2.5% fetal bovine serum, 2.5% horse serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine. Spinner flasks (80 rpm) were maintained in a humidified atmosphere of 8% CO_2, in a 37° incubator. Cells were fed by dilution every 2–3 days and were used at densities of $0.9–1.2 \times 10^6$ cells/ml, as assessed by using a Coulter counter.

Inositol phosphate accumulation. Inositol monophosphate accumulation was monitored as described previously (8). 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 the cpm eluted plus the cpm in the organic layer) and are shown with the basal activity subtracted.

Production of inositol polyphosphates was measured using modifications of the method for measurement of inositol monophosphate. The number of cells was increased from 5×10^5 to 6×10^6/tube, and the amount of [^{3}H]inositol was increased proportionately. Because LiCl has been reported to enhance the accumulation of inositol polyphosphates (9, 10), cells were preincubated with 20 mM LiCl for 20 min before the addition of agonists. These reactions were terminated by the addition of 100 μl of 10% perchloric acid (w/v), which increases the recovery of inositol polyphosphates, relative to that of aqueous/organic extraction under neutral conditions (11). The acid extracts were allowed to remain for 20 min at 4° before being neutralized with 625 μl of 150 mM KOH. After another 20 min at 4°, the samples were centrifuged for 10 min at $1500 \times g$. The supernatants were diluted with 5 ml of water and were poured over Dowex AG1-X8 columns as described (8). Inositol phosphates were eluted from these columns by a modification of the method of Berridge *et al.* (12), and the procedure was validated with the use of standards. [^{3}H]Inositol was washed out with 20 ml of water. Glycerophosphoinositol was eluted with 20 ml of 25 mM ammonium formate, 2 mM sodium tetraborate. Inositol monophosphates were eluted with 18 ml of 200 mM ammonium formate, 100 mM formic acid. The inositol bisphosphate fraction was eluted with 18 ml of 400 mM ammonium formate, 100 mM formic acid. Ins-P_3 were eluted with 18 ml of 1000 mM ammonium formate, 100 mM formic acid. Data are shown with basal values subtracted.

Measurement of extracellular free calcium. The calcium concentration of the buffer used in the PI hydrolysis experiments ($[Ca^{2+}]_o$) was measured using the free acid forms of the fluorescent calcium indicator dyes quin2 and fluo-3. The free acid forms were generated from the AM derivatives by alkaline hydrolysis in 10 mM NaOH. The solutions were neutralized with HCl and buffered with 10 mM HEPES. Aliquots of these solutions were added to polystyrene cuvettes containing 2 ml of the Kreb's buffer described above, to give final concentrations of 250 nM for quin2 and 200 nM for fluo-3. Fluorescence measurements were made on a Perkin Elmer LS-5B luminescence spectrophotometer, using excitation and emission wavelengths of 339 and 492, respectively, for quin2 and 490 and 530, respectively, for fluo-3. F_{min} was defined in the presence of 2 mM EGTA, and F_{max} was defined in the presence of 10 mM $CaCl_2$ for quin2 or 25 mM $CaCl_2$ for fluo-3. The free concentration of calcium in the buffer was calculated according to the equation $[Ca^{2+}] = K_d (F - F_{min})/(F_{max} - F)$ (Ref. 13), using the K_d values of 126 nM for quin2 and 450 nM for fluo-3.

Measurement of intracellular free calcium. $[Ca^{2+}]_i$ was determined using the fluorescent calcium indicator dye fura-2 (14). Cells were incubated for 90 min at room temperature with 0.5 μM fura-2/AM in Leibowitz's L-15 medium. The loaded cells were washed and then resuspended in L-15 medium at 37° for 15 min, to allow for hydrolysis of the AM. An aliquot (25 μl) of these cells was dropped onto a coverslip affixed to the bottom of a 35-mm tissue culture dish through which a 1-cm hole had been drilled. Cells were allowed to settle for 5 min and then were carefully diluted with 4.5 ml of F-12 medium modified as follows. Phenol red was omitted and sodium bicarbonate was replaced by an equimolar amount of sodium chloride. The medium was buffered to pH 7.4 with 20 mM HEPES and was further supplemented with 100 μg/ml bovine serum albumin, 5 μg/ml insulin, 10 μg/ml transferrin, 30 nM selenium, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, and 500 nM propranolol. Temperature was maintained at 37 ± 0.2° with a Leiden tissue culture chamber.

Drug applications were performed by rapid addition of a 500-μl aliquot of prewarmed F-12 medium containing a 10× concentration of drug(s). This method of drug addition resulted in rapid mixing while minimizing temperature fluctuations. Quantification of intracellular calcium concentration was performed using a digital imaging fluorescence microscope, previously described in detail (15). A few cells (11 of 103) demonstrated unusually high or low basal calcium values (greater than twice or less than half the average) and unusually poor responses to agonist stimulation. These cells were omitted from the final analysis.

Materials. Compounds were obtained from the following sources: fluorescent calcium indicators were from Molecular Probes (Eugene, OR), TPA and forskolin were from Calbiochem (San Diego, CA), CPA and phentolamine were from Research Biochemicals Inc. (Natick, MA), and tissue culture reagents were from GIBCO (Grand Island, NY). All other compounds were from Sigma (St. Louis, MO).

Results

Potentiation by CPA of PI hydrolysis is not specific for α_1-adrenergic receptor-mediated activation of PLC. As reported in the accompanying paper (8), the selective A_1 adenosine agonist CPA had no effect upon PI hydrolysis in DDT_1-MF_2 cells in the absence of added calcium (basal, 2.16 ± 0.09%; CPA-treated, 2.23 ± 0.05% hydrolysis). Under the same conditions, CPA was able to enhance the stimulation by NE of PI hydrolysis (Fig. 1). The effect of CPA did not appear to be specific for α_1-adrenergic receptor-stimulated PI hydrolysis, because CPA also potentiated ATP-stimulated PLC activity. ATP did not appear to have a synergistic effect with NE, because the stimulation by the combination of ATP plus NE

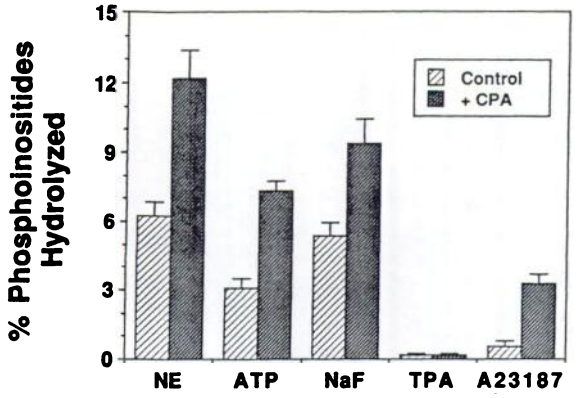

Fig. 1. An A_1-selective adenosine receptor agonist potentiates receptor- and post-receptor-stimulated PI hydrolysis. Accumulation of inositol monophosphates was conducted in nominally calcium-free buffer, as described in Materials and Methods. Drug concentrations were 10 μM NE, 200 μM ATP, 10 mM NaF (plus 10 μM $AlCl_3$), 100 nM TPA, and 10 μM A23187. The phorbol ester TPA was also tested at 10 and 1000 nM, with similar results. Each agonist was tested in the absence and the presence of 1 μM CPA. Values shown are the means ± standard errors of six experiments, each conducted in triplicate.

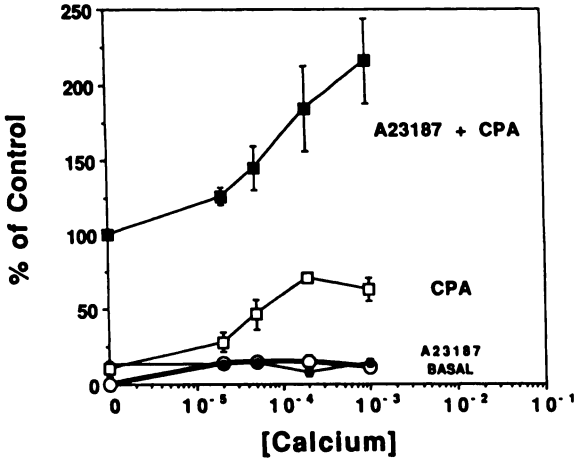

Fig. 2. Under conditions of increased calcium influx, CPA is able to directly stimulate PI hydrolysis without concomitant activation by another agonist. The effect of extra- and intracellular calcium concentration on basal and CPA-stimulated PI hydrolysis was examined in the absence or the presence of 10 μM A23187, with or without 1 μM CPA. Basal activity in the absence of added calcium (2.63%) has been subtracted from all points. Data are the mean ± standard error of three experiments, each conducted in triplicate, and are expressed relative to the control stimulation by A23187 with CPA (without added calcium) for each experiment. Activity due to A23187 with CPA was 3.15 ± 1.15% in the absence of added calcium and was 6.37 ± 1.17% in the presence of 1 mM added extracellular calcium.

was equal to the sum of the stimulations by each of the individual compounds (data not shown).

CPA also potentiated PI hydrolysis generated by sodium fluoride (in the presence of aluminum chloride). Although the mechanism by which fluoride stimulates PI hydrolysis is not completely understood, it has been suggested that, by analogy with the action of aluminum fluoride to stimulate adenylyl cyclase activity via the activation of G_s, aluminum fluoride may also activate other G proteins, such as the one(s) coupled to PLC (16, 17). Thus, adenosine receptor activation appears to enhance α_1 receptor stimulation of PI hydrolysis at a site distal from the α_1 receptor. This enhancement could involve one of the consequences of NE-stimulated PLC activation, PKC activation or increased intracellular calcium concentration.

Phorbol esters, such as TPA, are known to activate PKC directly (2). Calcium ionophores, such as A23187, can increase $[Ca^{2+}]_i$ either by allowing the influx of calcium from the extracellular environment or by releasing internal stores of calcium (see Ref. 18). TPA did not stimulate PI hydrolysis, either in the absence or in the presence of CPA (Fig. 1). No apparent role for PKC in the potentiation phenomenon was discerned in additional experiments examining the effects of acute or chronic treatments with either TPA or the PKC antagonist staurosporine (data not shown). Experiments in which the $[Ca^{2+}]_i$ was raised with the calcium ionophore A23187 suggested that $[Ca^{2+}]_i$ might play a role in the potentiation phenomenon. Whereas 10 μM A23187 alone caused only a very slight stimulation of PI hydrolysis, the combination of CPA with the ionophore resulted in a much larger stimulation (Fig. 1).

Mechanism by which CPA acts to enhance PI hydrolysis is contingent upon intracellular calcium and is sensitive to but not dependent upon extracellular calcium. The addition of extracellular calcium had little effect upon basal activity either with or without addition of the calcium ionophore (Fig. 2). As indicated above, CPA had no effect in the absence of added calcium but caused a small stimulation of PI hydrolysis upon addition of external calcium (20 μM to 1 mM). In the presence of both calcium and A23187, CPA caused a stimulation of PI hydrolysis similar in magnitude to the effect of the adenosine agonist on NE-stimulated PI hydrolysis. Thus, the effect of this ionophore on CPA-stimulated PI hydrolysis demonstrates a distinct calcium dependence, suggesting, first, that the effect of A23187 on PLC is specific to its action as a calcium ionophore and, second, that the ability of CPA to stimulate PI hydrolysis is contingent upon the $[Ca^{2+}]_i$.

The relationship between $[Ca^{2+}]_o$ and the potentiation of NE-stimulated PI hydrolysis by CPA was also examined. Stimulation of PLC activity by NE demonstrated a complex dependence upon $[Ca^{2+}]_o$ (Fig. 3A). Generally, NE-stimulated PLC activity was reduced under conditions of low calcium and increased with increasing $[Ca^{2+}]_o$; however, the relationship was distinctly multiphasic. Unexpectedly, at calcium concentrations of 2–5 mM, NE-stimulated PI hydrolysis declined toward values seen in the absence of added extracellular calcium. The addition of CPA to NE resulted in enhanced stimulation of PI hydrolysis at all calcium concentrations tested. Although the absolute amount of enhancement by CPA was greater at higher calcium concentrations (between 30 μM and 1 mM), the degree of enhancement, as assessed by the percentage of increase of the NE-stimulated value, was constant at all calcium concentrations (Fig. 3B).

Potentiation by CPA of NE-stimulated PI hydrolysis did not appear to require influx of extracellular calcium through voltage-sensitive calcium channels, because no calcium was added to the buffer used to generate the data in Fig. 1. Additionally, the calcium channel antagonists nifedipine (Fig. 4A) and verapamil (data not shown) had no significant effect upon the action of CPA in the absence or presence of added extracellular calcium. The inorganic calcium channel blocker $LaCl_3$ was also ineffective at a concentration (30 μM) that was reported to block calcium influx into these cells (19, 20). (Higher concentrations of $LaCl_3$ formed a pink precipitate in the buffer, which did not alter the degree of potentiation by CPA but did inhibit NE-stimulated activity either in the absence or in the presence of extracellular calcium.) Whereas the addition of 1 mM calcium

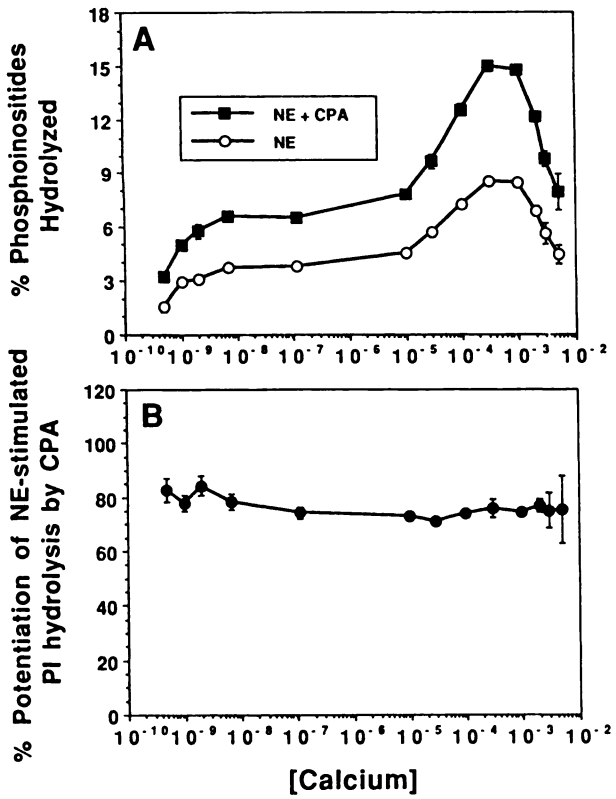

Fig. 3. Effects of extracellular calcium concentration on PI hydrolysis stimulated by NE with or without CPA. A, the $[Ca^{2+}]_o$ of nominally calcium-free Krebs buffer was measured with the free acid forms of quin2 and fluo-3, as described in Materials and Methods. Measurements with quin2 indicated $[Ca^{2+}]_o$ = 121 ± 14.5 nM (three experiments) and those with fluo-3 indicated 109 ± 13.1 nM (three experiments). The values for stimulation of PI hydrolysis in nominally calcium-free buffer were, therefore, indicated at a $[Ca^{2+}]_o$ of 115 nM. Calcium concentrations above this nominally free concentration were generated by the addition of calcium chloride. Calcium concentrations below the nominally free level were generated by the addition of EGTA (1, 3, 5, and 10 μM), and the free calcium concentrations were calculated using the program FRION-3, written by Dr. Suresh Joseph, University of Pennsylvania. The mean ± standard deviation is shown from a representative experiment (one of three) using 10 μM NE, without or with 1 μM CPA. B, The data from A were replotted as the percentage of potentiation of NE stimulation due to the addition of CPA.

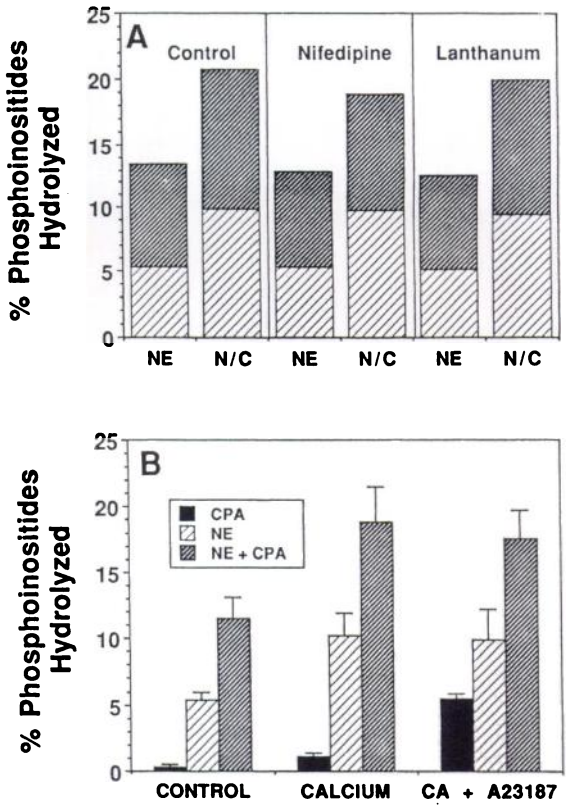

Fig. 4. Differential effects of increased intracellular or extracellular calcium upon CPA-stimulated and NE-stimulated PI hydrolysis and lack of sensitivity to calcium channel blockers. A, PI hydrolysis was measured in the absence (*bottom bars*) or presence (*top bars*) of 1 mM $CaCl_2$, in response to 10 μM NE alone (*NE*) or with 1 μM CPA (*N/C*). Nifedipine or $LaCl_3$ was added to final concentrations of 1 μM and 30 μM, respectively. These data are from a representative experiment. The ranges of the values of triplicate determinations were <9% of the values shown. Two additional experiments gave qualitatively similar results. B, PI hydrolysis in response to 1 μM CPA, 10 μM NE, or the combination of NE plus CPA was examined in nominally calcium-free buffer (*CONTROL*) or in the presence of 1 mM calcium (*CALCIUM*) or 1 mM calcium plus 10 μM A23187 (*CA + A23187*). Data are the means ± standard errors of three to five experiments.

increased NE-stimulated PI hydrolysis, addition of A23187 to the calcium-containing buffer did not further increase NE-stimulated activity but did, again, enhance the stimulation due to CPA alone (Fig. 4B). Under these conditions, the activities of CPA and of NE were approximately additive.

Fig. 5 shows the results of experiments in which the concentration of intracellular free calcium was reduced via the use of the calcium chelator quin2/AM (21). After preincubation, the cells were washed to remove extracellular quin2/AM, and PI hydrolysis was measured in response to stimulation by NE with or without CPA. In the absence of added extracellular calcium, preincubation with quin2/AM blocked the potentiation by CPA of NE-stimulated PLC activity. The idea that quin2 was blocking potentiation through its buffering of $[Ca^{2+}]_i$ was confirmed by the demonstration that the blockade by quin2 was overcome by the addition of extracellular calcium.

Potentiation by CPA of PI hydrolysis is regulated by cAMP in a calcium-sensitive manner. In the absence of added extracellular calcium, forskolin caused a dose-dependent inhibition of the potentiation due to CPA (Fig. 6), while having no effect upon the stimulation due to NE (data not shown). This effect of forskolin was likely due to its stimulation of adenylyl cyclase, because an inactive analogue, 1,9-dideoxyforskolin, did not inhibit potentiation. In the presence of added extracellular calcium, forskolin continued to inhibit the potentiation, but not to the extent occurring in the absence of extracellular calcium. These data suggested an antagonistic relationship between cAMP and calcium in this CPA-stimulated response.

One previously reported action of cAMP on smooth muscle cells is the reduction of $[Ca^{2+}]_i$, due to both extrusion and sequestration by membrane-bound calcium pumps (see Refs. 18 and 22). cAMP may also reduce the release of intracellular calcium stores by decreasing the sensitivity of the Ins-P_3 receptor (23, 24). The reduction of $[Ca^{2+}]_i$ has been considered as a possible mechanism accounting for the relaxant effect of cAMP on smooth muscle tone (see Refs. 18 and 25). The presence of a calcium ionophore could be expected either to counter the effect of activated calcium pumps or to mimic the release of intracellular calcium stores, thus preventing a reduction of $[Ca^{2+}]_i$ by cAMP. In the absence of added calcium, both forsko-

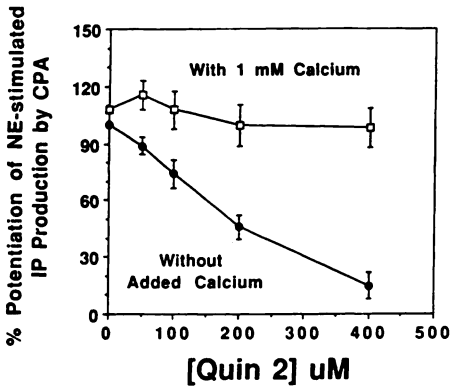

Fig. 5. The buffering of $[Ca^{2+}]_i$ with quin2 blocks the potentiation of NE-stimulated PI hydrolysis by CPA. Quin2/AM was incubated with cells for 3 hr during the incorporation of *myo*-[^{3}H]inositol. Extracellular quin2 was removed by dilution and centrifugation, followed by two washes with nominally calcium-free buffer. Inositol phosphate accumulation in response to NE and to NE plus CPA was measured without and with the addition of 1 mM $CaCl_2$, and the percentage of potentiation due to CPA was plotted. Whereas the percentage of potentiation by CPA was greatly reduced by preincubation with quin2/AM, the stimulation by NE was affected to a lesser extent. Thus, 200 μM quin2/AM reduced NE-stimulated PI hydrolysis by 18.7 ± 3.3%, but the CPA-induced potentiation of the NE-stimulated value under this condition was 56 ± 2.6% lower than the potentiation of the NE-stimulated value under control conditions (four experiments).

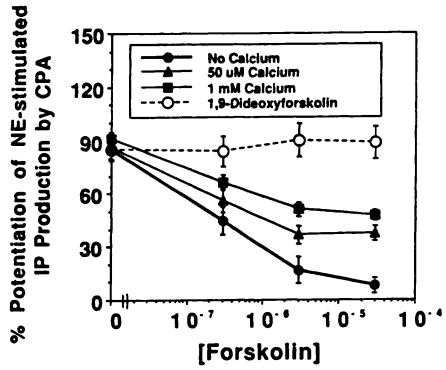

Fig. 6. The efficacy of forskolin to block the CPA-induced potentiation of PI hydrolysis is reduced in the presence of extracellular calcium. Forskolin was added at the same time as either 10 μM NE or 10 μM NE plus 1 μM CPA, in nominally calcium-free buffer or in buffer containing either 50 μM or 1 mM $CaCl_2$. The inactive analogue 1,9-dideoxyforskolin was tested in nominally calcium-free buffer. Data shown are the mean ± standard error of three experiments and are expressed as the percentage of potentiation of NE-stimulated PI hydrolysis due to the addition of CPA. Neither forskolin nor 1,9-dideoxyforskolin had a significant effect upon NE stimulation.

lin and 8-Br-cAMP selectively abolished the potentiation by CPA of NE-stimulated PI hydrolysis (Fig. 7A), without affecting the response to NE alone (data not shown). However, when a calcium ionophore was added (again in the absence of added extracellular calcium), neither agent was capable of significantly inhibiting the potentiation (Fig. 7B).

Calcium and cAMP also modulate inositol polyphosphate metabolism and calcium mobilization. The studies described above utilized the measurement of inositol monophosphate accumulation as an index of PLC activity. In the process of smooth muscle contraction, PLC activity results both in calcium mobilization by Ins-P_3 and in the activation of PKC by diacyglycerols. These two phenomena have been linked to the rapid (or phasic) and to the prolonged (or tonic) com-

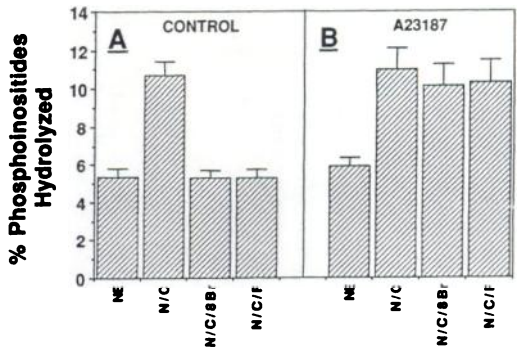

Fig. 7. The action of cAMP to block the CPA-induced potentiation of PI hydrolysis is eliminated via the addition of the calcium ionophore A23187. These experiments were conducted in nominally calcium-free buffer and were conducted either in the absence (A) or in the presence (B) of ionophore A23187. *N/C*, 10 μM NE plus 1 μM CPA; *8Br*, 2 mM 8-BrcAMP; *F*, 30 μM forskolin. The calcium ionophore A23187 was used at 10 μM. Data are the mean ± standard error of four experiments.

ponents of smooth muscle contraction, respectively (see Refs. 26 and 27, but see also Refs. 28 and 29). The regulation by CPA and cAMP of inositol monophosphate production would be expected to affect the generation of diacylglycerols from PI, but the data described thus far do not indicate whether Ins-P_3 and calcium mobilization are also affected. Fig. 8 shows the time course of inositol polyphosphate production. Both in the absence and in the presence of added calcium, CPA enhanced the NE-stimulated accumulation of inositol mono-, bis-, and trisphosphates. Accumulation of all three inositol phosphates was enhanced in the presence of extracellular calcium at all time points subsequent to the 10-sec point. Accumulation of inositol monophosphates was linear over 30 min in both the presence and the absence of calcium (note the change in scale of the *x*-axis after 300 sec). Accumulation of inositol bisphosphates and Ins-P_3 demonstrated more complex behavior. In the presence of added extracellular calcium, inositol polyphosphate accumulation was biphasic, being rapid for the first 10 sec and then slower over the next 30 min (1800 sec). In the absence of added calcium, NE-stimulated inositol polyphosphate accumulation was qualitatively similar to that seen in the presence of calcium; however, the addition of CPA induced a rise in inositol polyphosphates at 10 sec that actually exceeded the value seen at 1 min.

The effect of increased cellular cAMP on the potentiation of inositol polyphosphates was assessed at the 10-sec time point, corresponding to the early peak of Ins-P_3 production observed in the absence of added extracellular calcium (Fig. 9). The addition of CPA, again, resulted in an increase of product formation over the values obtained for NE alone. Forskolin, added 5 min before NE, did not affect NE-stimulated inositol polyphosphate production; however, the response of all three inositol phosphates to the combination of NE and CPA was inhibited by the addition of forskolin.

The effects of CPA and forskolin on the early production of Ins-P_3 suggested that these agents might be capable of modulating the calcium response to NE in these cells. Accordingly, changes in intracellular free calcium were assessed using the fura-2 fluorescence technique (14). Fluorescence changes in individual cells were monitored by computer-enhanced video microscopy, and the data were combined to generate Fig. 10, *left*. Under the conditions used for these studies, $[Ca^{2+}]_i$ in quiescent cells ranged from approximately 150 to 350 nM,

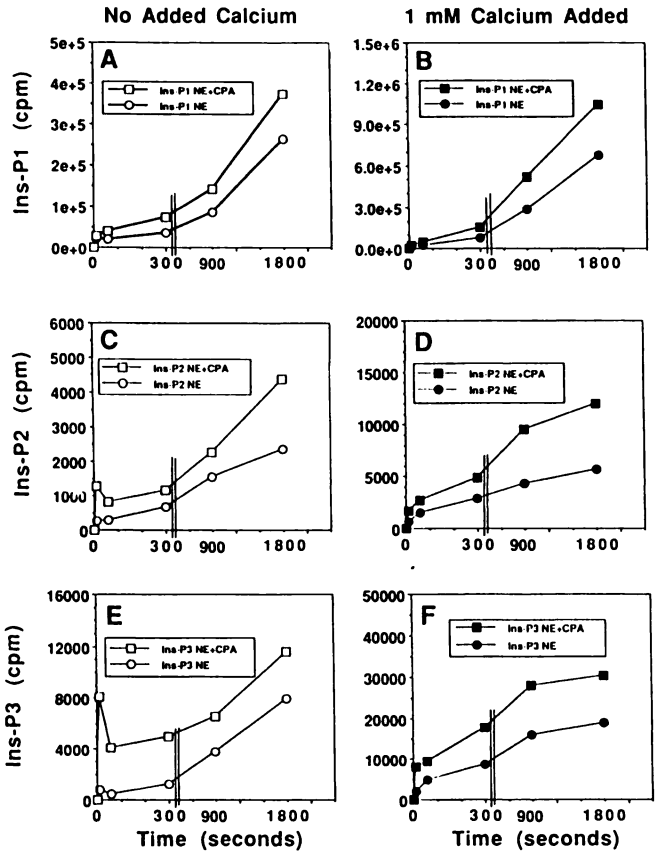

Fig. 8. Effects of CPA on NE-stimulated inositol polyphosphate production in the absence and presence of extracellular calcium. Inositol polyphosphate production was measured as described in Materials and Methods. Drug concentrations were 10 μM NE and 1 μM CPA. Reactions were terminated at the following time points: 0, 10, 60, 300, 900, and 1800 sec. Data are shown from a representative experiment. Note the different scales used in each panel. A and B, Inositol monophosphate (*Ins-P1*); C and D, inositol bisphosphate (*Ins-P2*); E and F, Ins-P_3 accumulation. The data in A, C, and E were generated in nominally calcium-free buffer, whereas the data in B, D, and F were generated in buffer containing 1 mM calcium.

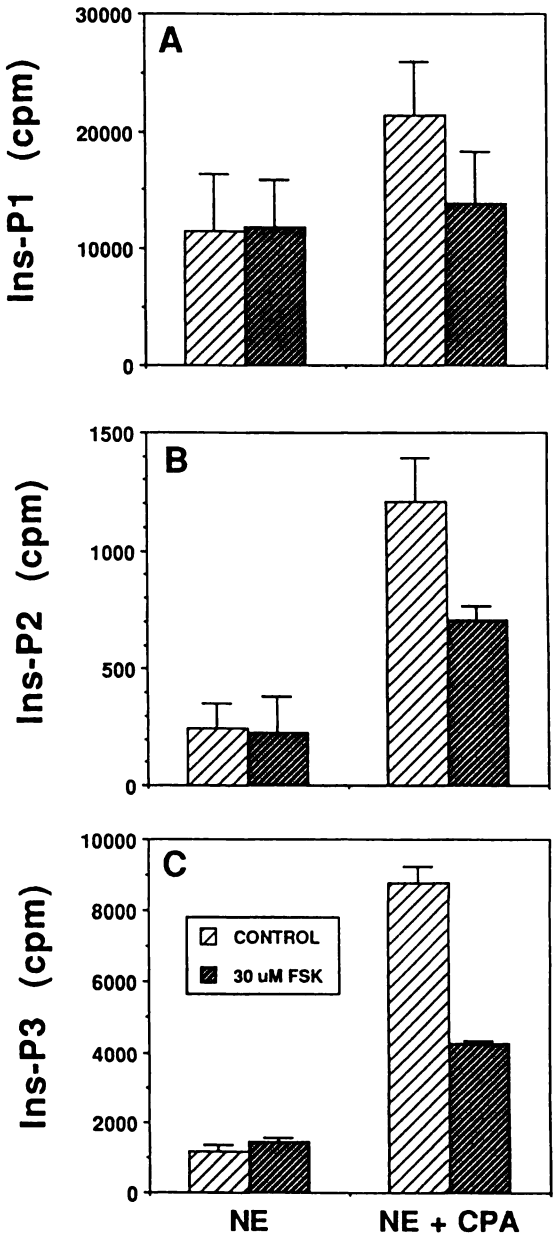

Fig. 9. Effect of forskolin (*FSK*) on inositol polyphosphate production in the first 10 sec after stimulation. Drug concentrations were as in Fig. 7. Data are the mean ± standard error of three experiments. The following basal values were subtracted from the data as shown: inositol monophosphate (*Ins-P1*), 16,841 ± 815 cpm; inositol bisphosphate (*Ins-P2*), 1038 ± 111 cpm; Ins-P_3, 5898 ± 170 cpm.

averaging about 250 nM. Addition of 10 μM NE resulted in an immediate (within 5 sec) increase in $[Ca^{2+}]_i$. The increased $[Ca^{2+}]_i$ decayed rapidly over the next 2 min and then plateaued or decayed slowly over the following 3 min. The addition of CPA to NE resulted in a calcium peak of greater amplitude than in the absence of CPA. The plateau phase, beginning at 2 min after addition of CPA plus NE and remaining stable through the next 3 min, was nearly as high as the peak value seen in response to NE alone. On the other hand, the addition of forskolin 5 min before NE resulted in a decreased peak $[Ca^{2+}]_i$ value, as well as the apparent elimination of a plateau phase in which $[Ca^{2+}]_i$ was maintained above basal values. The differences between the basal and peak calcium levels for individual cells are plotted as histograms in Fig. 10, *right*. Stimulation by NE resulted in a wide distribution of responses, which demonstrated a modal increase of 75 nM. The responses to NE plus CPA occurred over a similar range, but the mode appeared to be shifted to the right and there was a greater percentage of highly responsive cells. Conversely, the addition of forskolin to NE both shifted the mode to the left and decreased the range of responses by virtually eliminating the occurrence of highly responsive cells.

Action of CPA on PI hydrolysis requires the simultaneous and continuous presence of NE. If NE stimulation provides the calcium necessary for CPA to activate PLC, then two questions are immediately suggested. 1) Does CPA require the increased $[Ca^{2+}]_i$ that occurs during the peak of the NE response or is the $[Ca^{2+}]_i$ of the plateau phase sufficient? 2) Because CPA is able to stimulate Ins-P_3 production and calcium mobilization when added simultaneously with NE, is the continued presence of NE necessary or will the increased $[Ca^{2+}]_i$, once made available, continue to fuel the CPA-stimulated pathway? These questions are addressed in Table 1. In this experiment, agents were added at times 0, 10, and 20 min and the assay was stopped at 30 min. Phentolamine (50 μM) was used

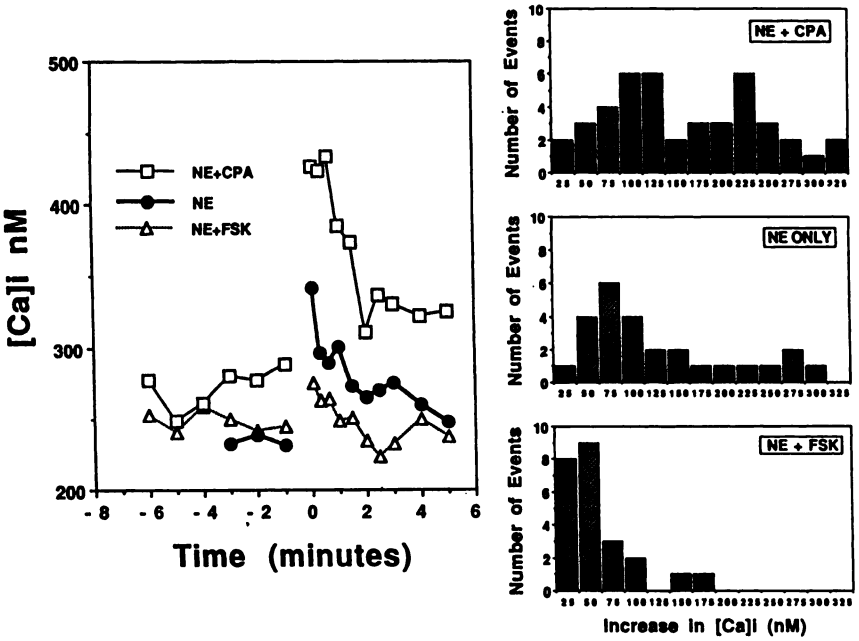

Fig. 10. Measurement of $[Ca^{2+}]_i$ in single cells with fura-2. $[Ca^{2+}]_i$ was determined as described in Materials and Methods and in Ref. 15. Drugs were added at time 0 and measurements were made at 5, 20, 40, 60, 90, 120, 150, 180, 240, and 300 sec. *Left*, data were obtained from six experiments and are the means from 26 cells for stimulation by NE, from 43 cells for stimulation by NE in the presence of 2 μM CPA (added 3–5 min before NE), and from 24 cells for stimulation by NE in the presence of 30 μM forskolin (*FSK*) (added 3–5 min before NE). *Right*, data are histograms indicating the magnitude of the increase in $[Ca^{2+}]_i$ for individual cells at 5 sec after the addition of NE in the presence of CPA (*top*), no other drug (*middle*), or forskolin (*bottom*).

TABLE 1

Requirement for simultaneous stimulation by NE and CPA and lack of requirement for the NE-stimulated calcium transient in the action of CPA

[^{3}H]Inositol-loaded cells were preincubated for 20 min with 40 mM LiCl and then drug additions were made at 10-min intervals, as indicated. All assays were terminated at 30 min. —, Addition of buffer only. The drug concentrations were 10 μM NE, 1 μM CPA, and 50 μM phentolamine. All drugs, as well as buffer additions, contained 40 mM LiCl, and the NaCl concentration was reduced by an equal amount to account for this. These data are the mean ± standard deviation of triplicate determinations and are representative of at least four experiments. The basal value of 1.76% was subtracted from each condition shown in the table. This basal value was unaffected by phentolamine or CPA.

Row	Addition			PI hydrolysis
	Time 0	10 min	20 min	
				%
1	NE	Phentolamine	—	1.15 ± 0.06
2	—	—	NE	1.16 ± 0.09
3	NE	CPA + phentolamine	—	1.35 ± 0.06
4	NE	—	—	3.74 ± 0.13
5	NE + CPA	Phentolamine	—	2.71 ± 0.08
6	—	—	NE + CPA	2.76 ± 0.11
7	CPA	—	NE	2.66 ± 0.19
8	NE	CPA	—	6.54 ± 0.18
9	NE + CPA	—	—	8.31 ± 0.48

to discontinue the α-adrenergic stimulation by NE. The efficacy of this agent is demonstrated by comparing Table 1, rows 1, 2, and 4. Table 1, row 3, indicates that CPA has no effect if NE stimulation is blocked at the same time that CPA is added. This implies that NE-stimulated metabolites such as inositol phosphates, diacylglycerol, or phosphatidic acid (which have accumulated during the 10-min stimulation with NE in the presence of LiCl) are not capable of supporting the action of CPA. Table 1, rows 5, 6, and 9, demonstrate that, even if CPA is present during the peak of the calcium transient, the addition of phentolamine stops the production of inositol phosphates. Table 1, row 7, shows that the action of CPA to potentiate NE stimulation does not change over the time of the assay, suggesting both that CPA does not cause the accumulation of metabolites that influence NE-stimulated PLC activity and that the CPA effect is stable over time. Table 1, row 8, indicates that CPA is still able to potentiate NE stimulation after the calcium response to NE has diminished to the plateau phase. The data in this table indicate that adenosine receptor activation must occur simultaneously with α_1 receptor activation to cause potentiation of inositol phosphate accumulation and that the action of CPA, although requiring the NE-stimulated calcium response, does not require the $[Ca^{2+}]_i$ that occurs during the peak of the calcium transient.

Discussion

The objective of these investigations was to examine possible mechanisms by which adenosine is able to enhance NE-stimulated physiological responses in certain smooth muscles. A selective A_1 adenosine receptor agonist, CPA, was observed to modulate the stimulation of the PLC transducer mechanism in a smooth muscle model system, the DDT_1-MF_2 cell line. The action of CPA to enhance α_1-adrenergic receptor-stimulated PLC activation in a smooth muscle cell line is consistent both with the ability of adenosine agonists to enhance α_1-adrenergic receptor-mediated smooth muscle contraction (6) and with a role for PLC as a biochemical transducer of receptor-stimulated smooth muscle contraction. The observation that the CPA-induced potentiation of PI hydrolysis was not specific for PLC stimulation by α_1-adrenergic receptors but also occurred when CPA was added during stimulation with ATP or fluoride (Fig. 1) suggested that the interaction between the adenosine and adrenergic receptors occured distally to the receptor proteins.

At least two possibilities could be envisioned to account for the action of CPA on NE-stimulated PI hydrolysis. Either 1) the overall sensitivity of PLC to stimulation could be enhanced by an action of CPA, as has been suggested to occur after stimulation of certain growth factor receptors (30, 31), or 2) one or more of the products of NE-stimulated PLC activation could be utilized by the adenosine receptor-mediated signal transduction mechanism to allow CPA to stimulate the same (or a different) pool of PLC. These two possibilities are not mutually exclusive and both could conceivably be involved.

Two sequelae of PLC stimulation, PKC activation and mo-

bilization of intracellular calcium, were mimicked through the use of a phorbol ester and a calcium ionophore. The lack of effect of the PKC agonist TPA or of the antagonist staurosporine in any aspect of CPA action on PLC suggests that PKC may not play a significant role in this particular response. Data from the use of the calcium ionophore A23187 suggest that calcium mobilization could play a role in enabling CPA to modulate PLC activity. The observation that the buffering of $[Ca^{2+}]_i$ with quin2 is able to block the potentiation of NE-stimulated PLC and that this block is reversed by the addition of calcium (Fig. 5) suggests that increased calcium is not merely sufficient to allow for an action of CPA on PLC. Rather, the α_1 receptor-stimulated rise in $[Ca^{2+}]_i$ is a necessary component in the mechanism by which CPA acts to potentiate NE-stimulated PLC.

Although the data in Fig. 4A imply that influx of extracellular calcium through voltage-sensitive channels is not necessary for the action of CPA, the data in Fig. 3A indicate some role for extracellular calcium in regulating the sensitivity of PLC to agonist activation. Interestingly, although the magnitude of the CPA effect is also correlated with calcium concentration, the degree of potentiation (expressed as percentage of potentiation) is relatively constant (Fig. 3B). Again, this could reflect a fixed effect of CPA on the responsiveness of PLC to stimulation. Alternatively, it could be accounted for by additivity of NE- and CPA-stimulated components of PLC activity (Fig. 4B), acting together in a coordinated fashion in which NE stimulation provides the rise in $[Ca^{2+}]_i$ required by the CPA-stimulated pathway. In this model, the magnitude of the PLC response to NE both would be determined by and would further determine the available $[Ca^{2+}]_i$ for use by the CPA-stimulated pathway. It is possible that NE stimulation opens plasma membrane calcium channels that are insensitive to organic calcium channel blockers but contribute to the rise in $[Ca^{2+}]_i$ and, therefore, to the stimulation of PLC by CPA. However, it is clear that CPA can potentiate NE-stimulated PLC activity at $[Ca^{2+}]_o$ values that are too low for influx down a concentration gradient to be expected (Fig. 3A, *left*). Thus, the increase in $[Ca^{2+}]_i$ provided by NE, in the absence of added calcium, is most likely derived from release of intracellular stores. It is also possible that the increased stimulation seen in the presence of 30 μM to 1 mM calcium may be accounted for by the refilling of depleted intracellular calcium storage sites. Calcium-dependent mechanisms for the regulation of calcium storage and release could also account for the unusual concentration-response curve seen in Fig. 3A, whereas calcium influx down a concentration gradient would not. A model of "capacitive" calcium entry, whereby extracellular calcium must first enter an intracellular calcium storage compartment(s) before being released into the cytoplasm of nonexcitable cells, has been proposed by Putney (32) and by others (33, 34). Some of the details of this model have recently been modified (35, 36). These studies suggest that the uptake of extracellular calcium is regulated by the calcium level of the intracellular storage sites. If such mechanisms are active in DDT_1-MF_2 cells, then the $[Ca^{2+}]_i$ may be buffered to an extent that, over the range of 10 nM to 10 μM, extracellular calcium has little effect upon $[Ca^{2+}]_i$.

In direct contrast to the effects we observe in DDT_1-MF_2 cells, some other cells demonstrate a decrease in receptor-stimulated PI hydrolysis in response to activation of the adenosine A_1 receptor (for reviews, see Refs. 37 and 38). A mechanism that has been suggested to account for the A_1 receptor-mediated decrease in PI hydrolysis is a decrease in $[Ca^{2+}]_i$ due to reduced activity of voltage-sensitive calcium channels. Adenosine A_1 receptors have been linked to increased potassium conductance in several cell types, and the resulting hyperpolarization might result in reduced calcium influx through voltage-sensitive channels. The DDT_1-MF_2 cell line appears to differ from these other systems, in that voltage-sensitive calcium channels do not appear to play a significant role in NE-stimulated PI hydrolysis [based upon the data in Fig. 4A and the lack of effect of high potassium buffer (data not shown) and further supported by the report of Nelemans *et al.* (39), who did not detect the presence of voltage-sensitive calcium channels during patch-clamp studies on DDT_1-MF_2 cells]. Furthermore, the calcium requirement for activation of PLC in these cells is at least partially satisfied by the intracellular calcium stores; thus, small decreases of calcium influx would be expected to play a less significant role in the regulation of PLC activity in DDT_1-MF_2 cells than in cells that depend upon voltage-sensitive calcium channels for their source of increased $[Ca^{2+}]_i$.

Our examinations of the effects of CPA on PI hydrolysis have relied upon inositol monophosphate accumulation as a measure of PLC activity. Suggestions have been made that inositol monophosphate can be produced as a direct product of PLC action on PI (40–42). Recently, however, a very elegant examination of the metabolism of inositol phosphate isomers in SK-N-SH cells by Fisher *et al.* (43) supported previous assertions (44–46) that, by far, the predominant portion of inositol monophosphates were derived from the release of Ins-P_3 via PLC action on phosphatidylinositol-4,5-bisphosphate. We asked whether the action of CPA on NE-stimulated inositol monophosphate production was also manifest in the production of Ins-P_3, a physiological stimulator of calcium mobilization.

The results of measurements of Ins-P_3 (Fig. 8), particularly at the shorter time points, revealed distinguishing details about the respective mechanisms by which CPA and NE activate PLC. The similarity of the time course of Ins-P_3 production (particularly in the absence of extracellular calcium) with that of calcium mobilization (Fig. 10), combined with the calcium sensitivity of agonist-stimulated PLC activation (Fig. 3), suggests the possibility that the agonist-stimulated rise in $[Ca^{2+}]_i$ may enhance agonist-stimulated PLC activation in a positive feedback mechanism. The possibility of this type of positive feedback was previously suggested by Fisher *et al.* (47). Such a mechanism could account for the large early peak seen in the Ins-P_3 response to the combination of CPA plus NE. This coincides with the time when the $[Ca^{2+}]_i$ is also peaking (Fig. 10). Because the data in Fig. 10 indicate that CPA enhances the magnitude and duration of the rise in $[Ca^{2+}]_i$ stimulated by NE, one potential mechanism that ought to be considered is that CPA might enhance NE-stimulated PLC activity by maintaining $[Ca^{2+}]_i$ at a higher level than that obtained in the presence of NE alone. In this scenario, the presence of increased $[Ca^{2+}]_i$ would enhance the sensitivity of PLC to stimulation by NE. Although such a mechanism might partially account for the effect of CPA on NE-stimulated PI hydrolysis in the absence of added calcium, it is unlikely to explain the enhancement seen in the presence of added calcium. The data in Fig. 4B indicate that, in the presence of extracellular calcium, the ionophore A23187 does not further enhance NE-stimulated

PLC activity, whereas CPA does. Thus, in the presence of 1 mM extracellular calcium, the sensitivity of PLC to stimulation by NE is maximal, and any action of CPA to further raise $[Ca^{2+}]_i$ is not likely to account for the potentiation of NE-stimulated PI hydrolysis seen under these conditions.

Certain characteristics of the Ins-P_3 response to NE differ from those seen with the combination of CPA plus NE (Fig. 8). For example, the early time point for NE-stimulated Ins-P_3 production (10 sec) does not demonstrate the same dramatic peak value as that seen in the presence of CPA. In the presence of forskolin a second difference between the Ins-P_3 response to NE and that to CPA plus NE becomes apparent. Forskolin pretreatment reduces the magnitude of the calcium transient response to NE (Fig. 10). Although forskolin is able to reduce the effect of CPA on NE-stimulated Ins-P_3 production, it does not affect the Ins-P_3 response to NE alone (Fig. 9). The lack of an effect of forskolin upon NE-stimulated Ins-P_3 production, as well as the reversibility of the forskolin effect upon CPA-stimulated PLC activity by the action of a calcium ionophore (Fig. 7), suggests that forskolin is not acting directly to reduce the sensitivity of PLC to receptor activation. A mechanism of PLC phosphorylation and inactivation was previously suggested to account for the action of cAMP on PI turnover in C6Bu1 cells (48). The present data from DDT_1-MF_2 cells indicate that forskolin reduces the amount of free calcium made available by NE stimulation. A similar finding was reported by Campbell *et al.* (49), who observed that forskolin pretreatment inhibited bradykinin-stimulated PI hydrolysis and calcium mobilization in NG108-15 cells. These authors noted that forskolin pretreatment of intact cells did not alter the sensitivity of calcium-stimulated PLC activity in membranes prepared from these cells. However, this finding does not rule out the possibility that cAMP might alter the calcium sensitivity of receptor- or G protein-stimulated PLC activity. Misaki *et al.* (50) demonstrated an inhibition of GTPγS-stimulated Ins-P_3 production by cAMP-dependent protein kinase in membranes from HL-60 cells. No effect was seen upon calcium-stimulated PLC activity, suggesting an effect of PKA on the functional coupling of a G protein to PLC in their preparation.

Differences in the cAMP sensitivity of the Ins-P_3 response, as well as in the nature of the peak of Ins-P_3 production occurring during the calcium transient, are indicative of some mechanistic difference between the activation of PLC by NE or by CPA. Although both the NE- and the CPA-mediated components of PLC activity are calcium sensitive, the calcium concentration dependence for the two components may differ. If, for example, the calcium requirement for activation of the NE-stimulated component of PLC activity was saturated at a $[Ca^{2+}]_i$ that is lower than that occurring during the peak of the calcium transient, then the NE-stimulated Ins-P_3 production would not be expected to peak during the calcium transient and would not be expected to be enhanced by the addition of an ionophore. If, on the other hand, the calcium requirement of the CPA-stimulated pathway was not saturated at the $[Ca^{2+}]_i$ occuring during the transient, then the activity of this pathway would be proportional to $[Ca^{2+}]_i$ and, therefore, would be expected to demonstrate a peak response during the NE-stimulated calcium transient, as well as responding to the addition of the calcium ionophore. Additionally, this would account for the relative lack of activity of CPA in the absence of either NE or ionophore and for the sensitivity of this pathway to reduction of the calcium levels by forskolin.

As stated above, the possibility was considered that CPA was acting merely to enhance the sensitivity of NE-stimulated PLC activity. However, the differential sensitivities of the NE- and the CPA-stimulated components of PLC activity to calcium, and to cAMP deemed this possibility unlikely. What appears more likely is that the increased $[Ca^{2+}]_i$, provided by the actions of NE, enables the manifestation of the otherwise latent CPA-stimulated pathway. The pertussis toxin sensitivity of CPA-stimulated PI hydrolysis (8) suggested the possible role, in this pathway, of a different G protein than the pertussis toxin-insensitive G protein that mediates NE-stimulated PLC. These new data suggest that there may be differential calcium requirements for the activation of PI hydrolysis by CPA and by NE and support previous suggestions that there are multiple pathways for the stimulation of PLC activity (8, 51–54).

The ability of cAMP to modulate the calcium response to NE (Fig. 10) and the PLC response to CPA (Figs. 6, 7, and 9) further supports an essential role for $[Ca^{2+}]_i$ in the differential regulation of NE-stimulated responses by agents that alter cellular cAMP levels or that act at adenosine receptors. $[Ca^{2+}]_i$ may thus be considered to be a central regulatory factor that coordinates the magnitude of the PLC response to the simultaneous activation of multiple receptors.

Acknowledgments

The authors would like to thank Dr. Robert Yasuda for volunteering his advice and for many helpful discussions during the course of these studies.

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