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Protein Kinase C Activators Sensitize Cyclic AMP Accumulation by Intact 1321N1 Human Astrocytoma Cells

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SUMMARY

Pretreatment of 1321N1 human astrocytoma cells with phorbol 12-myristate-13-acetate or other activators of protein kinase C led to 2.5- to 5-fold increases (sensitization) in subsequent stimulation by forskolin of intracellular cyclic AMP accumulation. These compounds caused much smaller or no increases in receptor-mediated stimulation of cyclic AMP accumulation induced by isoproterenol and by prostaglandin E_1. Carbachol and histamine, agonists acting at receptors coupled to polyphosphoinositide turnover in these cells, induced less sensitization of subsequent stimulation by forskolin but greater sensitization of stimulation by isoproterenol and by prostaglandin E_1. The specificities of various analogs of phorbol 12-myristate-13-acetate, for induction of sensitization of forskolin stimulation were consistent with involvement of protein kinase C. The effects of protein kinase inhibitors and of down-regulation of protein kinase C activity also indicated involvement of protein kinase C in sensitization of forskolin stimulation, although additional mechanisms are likely to be involved in sensitization of isoproterenol stimulation. Neither pertussis toxin pretreatment nor inclusion of isobutylmethylxanthine during assays of cyclic AMP accumulation were able to prevent or mimic these sensitization phenomena, suggesting that the primary site of modification responsible for sensitization is neither the inhibitory guanine nucleotide-binding protein nor cyclic AMP phosphodiesterase. Sensitization was only observed in assays with intact cells. These results, together with those from our previous study describing protein kinase C-mediated desensitization of broken cell adenylate cyclase activity, indicate that activation of protein kinase C leads to multiple changes in the receptor-stimulated adenylate cyclase signal transduction pathway of these cells.

Cellular responsiveness is subject to regulation based on the prior history of the cell in terms of exposure to various stimuli. The most widely characterized of these adaptive changes is the decreased responsiveness that often occurs following exposure to activating stimuli, referred to as agonist-induced desensitization. Alternatively, adaptive increases in responsiveness can occur following exposure to inhibitory stimuli, either due to desensitization of the inhibitory pathways or due to compensatory increases, or "sensitization," of stimulatory pathways. The cellular and molecular mechanisms involved in these regulatory changes in cellular responsiveness are the subject of much current research.

Two of the major signal transduction mechanisms mediating cellular responses to external stimuli are those involving cAMP (1, 2) and PPI hydrolysis products (3, 4) as second messengers. The cellular effects of cAMP elevation are mediated primarily by activation of cAMP-dependent protein kinase, whereas those of PPI hydrolysis are thought to result from both activation of protein kinase C by diacylglycerol and elevation of intracellular Ca^{2+} concentration by inositol trisphosphate. Adaptive changes have been demonstrated for both the cAMP and PPI hydrolysis signal transduction pathways following activation by their respective stimuli, and there is also evidence for the occurrence of regulatory interactions between these two signal transduction pathways (3–6). In the present studies, we have investigated the effects of protein kinase C and of PPI-coupled receptor agonists on activity of the cAMP regulatory pathway in 1321N1 human astrocytoma cells, a well characterized system in terms of agonist-induced desensitization of β-adrenergic receptors and adenylate cyclase (7).

Phorbol esters and related compounds have been widely used to investigate the role of protein kinase C in regulation of receptors and signal transduction pathways (3–6). We previously reported that treatment of cells with activators of protein kinase C led to desensitization of β-adrenergic receptor-stimulated adenylate cyclase activity in membrane preparations from

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ABBREVIATIONS: PPI, polyphosphoinositide; PMA, phorbol 12-myristate-13-acetate; H7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine; IBMX, 3-isobutyl-1-methylxanthine; DMEM, Dulbecco's modified Eagle's medium; HEPES, 4-(2-hydroxyethyl)-1-piperazinesulfonic acid; DMSO, dimethylsulfoxide; G_i, inhibitory guanine nucleotide-binding protein; G_s, stimulatory guanine nucleotide-binding protein.

1321N1 cells (8). There was no change in stimulation by forskolin, an agent that is thought to activate directly the catalytic unit of adenylate cyclase (9). In the present studies, we found very different effects of protein kinase C activators on cAMP accumulation measured in intact 1321N1 cells, namely a marked increase in cAMP accumulation stimulated by forskolin with little or no change in β-adrenergic receptor-mediated stimulation. The mechanisms involved in this sensitization phenomenon observed in intact cell assays and the reasons for the different results obtained in assays with broken cell preparations were, therefore, investigated.

1321N1 cells also express muscarinic cholinergic receptors coupled to the activation of PPI hydrolysis (10). This in turn leads to a decrease in cAMP levels due to the activation of Ca^{2+}-stimulated cAMP phosphodiesterase (11, 12). Meeker and Harden (13) reported that pretreatment of these cells with the muscarinic agonist carbachol led to an increase in subsequent stimulation of cAMP accumulation measured in intact cells. We, therefore, included in our studies an investigation of the possible involvement of protein kinase C in mediating the sensitizing effects of carbachol on the cAMP regulatory system in these cells.

Experimental Procedures

Materials. The following chemicals were purchased from Sigma Chemical Co. (St. Louis, MO): PMA, 4-*O*-methyl-PMA, α- and β-phorbol didecanoate, carbachol, histamine, isoproterenol, H7, IBMX, and prostaglandin E_1. Forskolin was purchased from Calbiochem (San Diego, CA), pertussis toxin from List Biologicals Laboratories, Inc. (Campbell, CA), staurosporine from Kyowa Hakko USA (New York, NY), and [^{3}H]adenine from ICN Radiochemicals (Irvine, CA). Cholera toxin was a gift from R. Finkelstein (University of Missouri, Columbia, MO). Tissue culture medium and trypsin were from GIBCO (Grand Island, NY). Serum was either from GIBCO, Biocell (Carson, CA), or Cell Culture Laboratories (Cleveland, OH).

Cell culture. 1321N1 human astrocytoma cells were grown in monolayer culture at 37° in a humidified atmosphere containing 8% CO_2. Growth medium was low glucose DMEM supplemented with 5% fetal bovine serum. Confluent monolayers were removed from tissue culture flasks with 0.05% trypsin and plated at a density of 20,000 cells/cm^2 in plastic tissue culture dishes. Experiments were performed on cells 4 or 5 days after plating (just confluent).

Assays of intracellular cAMP accumulation. cAMP accumulation in intact cells was determined by the method of Shimizu (14). Growth medium was aspirated and the cell sheets were washed once with 2 ml of DMEM buffered to pH 7.4 with 20 mM HEPES (DMEM-HEPES). Cells were then incubated for 1 hr at 37° in 1 ml of DMEM-HEPES containing 2 μCi of [^{3}H]adenine. Pretreatment drugs or the appropriate vehicle were included during this prelabeling step. Prelabeling medium and pretreatment drugs were aspirated and the cells were washed and then incubated for 2 min at 37° in DMEM-HEPES in the absence or presence of various agents to stimulate cAMP accumulation. This stimulation medium was then aspirated and 1 ml of 5% trichloroacetic acid was added to stop further reactions and to extract cellular nucleotides. [^{3}H]cAMP and [^{3}H]ATP were then separated by sequential chromatography over Dowex and alumina columns, essentially as described (11). Radioactivity in the column eluates was determined by liquid scintillation counting in 10 ml of BudgetSolve. Accumulation of [^{3}H]cAMP was then expressed as percentage of conversion of [^{3}H]ATP to [^{3}H]cAMP ([^{3}H]cAMP/[^{3}H]cAMP + [^{3}H]ATP). Incorporation of ^{3}H into the ATP fraction was typically 500,000 cpm/dish, and this value was not markedly altered by any of the pretreatments. In most experiments DMSO was included in all pretreatments. However, similar effects of carbachol and histamine were observed in the absence of DMSO as well.

Data analysis. Computerized curve-fitting of dose-response curves was performed by nonlinear regression using GraphPAD (GraphPAD Software, San Diego, CA). Statistical analysis of the significance of changes induced by the various treatments was by analysis of variance, using the Number Cruncher Statistical System (NCSS, Kaysville, UT).

Results

Characterization of sensitization. The effects of pretreating 1321N1 cells with various agents that activate protein kinase C on subsequent stimulation of intracellular cAMP accumulation were determined (Table 1). In agreement with the previous study by Meeker and Harden (13), pretreatment of these cells with the muscarinic receptor agonist carbachol led to an approximately 2-fold increase in intracellular cAMP accumulation stimulated by the β-adrenergic receptor agonist isoproterenol. Receptor-mediated stimulation of cAMP accumulation by prostaglandin E_1 and direct stimulation of adenylate cyclase by forskolin were sensitized to a similar extent. These cells also express histamine receptors coupled to PPI hydrolysis (15), and pretreatment of cells with histamine led to changes in subsequent stimulation of cAMP accumulation similar to, but consistently smaller than, those induced by carbachol (Table 1). Dose-response curves for sensitization of isoproterenol and forskolin stimulation by carbachol and histamine were determined in separate experiments, and these results are summarized in Table 2.

PMA and mezerein are structurally dissimilar but both lead to direct activation of protein kinase C (independent of stimulation of PPI hydrolysis). Pretreatment of 1321N1 cells with either of these agents led to an approximately 4-fold sensitization of forskolin stimulation of cAMP accumulation (Table 1). The effects of these direct activators of protein kinase C on receptor-mediated stimulation induced by isoproterenol and by prostaglandin E_1 were variable over the course of these studies, ranging from the small decrease in activity seen for prostaglandin E_1 stimulation in Table 1 to approximately 50% increase in activity, as seen for isoproterenol stimulation in Table 1. In most experiments PMA induced no significant change in stimulation by isoproterenol (cf. Figs. 3–5). A possible explanation for this variability is presented in the Discussion. No consistent changes in isoproterenol stimulation were observed in additional experiments varying the time of pretreatment with PMA, the concentration of PMA used during the pretreatment, or the concentration of isoproterenol used to stimulate cAMP accumulation (data not shown).

In additional experiments, the effects of pretreating cells with either carbachol or PMA on cAMP accumulation stimulated by cholera toxin and by the various combinations of cholera toxin, isoproterenol, and forskolin were determined (Table 3). As previously demonstrated in a variety of systems (9), the combination of isoproterenol plus forskolin induced markedly greater cAMP accumulation in control cells than was observed with either agent alone. Cholera toxin pretreatment led to an 8-fold stimulation of cAMP accumulation. Stimulation by isoproterenol and by isoproterenol plus forskolin were similar in the absence or presence of cholera toxin pretreatment. However stimulation by forskolin plus cholera toxin was markedly greater than with either agent alone.

Exposure to carbachol before assay sensitized stimulation by isoproterenol and by forskolin, as in the previous experiments, but stimulation by the combination of isoproterenol plus for-

TABLE 1

Sensitization of intact cell cAMP accumulation

Cells were pretreated for 60 min at 37° with 1% DMSO (control), 100 μM carbachol, 100 μM histamine, 1 μM PMA, or 1 μM mezerein. Cells were washed and cAMP accumulation was then determined in the absence (basal) or presence of 10 μM isoproterenol, 10 μM prostaglandin E_1, or 30 μM forskolin. Stimulation is expressed as fold of basal activity in control cells, and values are the means ± standard errors of three experiments, each performed in triplicate. Values in parentheses represent the extent of sensitization expressed as fold of the stimulation by the same agent observed in control cells. The basal value for control cells was 0.014 ± 0.003% conversion.

Assay	Fold stimulation of cAMP accumulation				
	Control	Carbachol	Histamine	PMA	Mezerein
Basal	1.0	0.7 ± 0.1	0.7 ± 0.1[a]	0.9 ± 0.1	1.2 ± 0.1
Isoproterenol	54 ± 7	111 ± 17 (2.1)[b]	94 ± 11 (1.7)[b]	77 ± 10 (1.4)[a]	72 ± 16 (1.3)
Prostaglandin E_1	29 ± 4	53 ± 8 (1.8)[b]	42 ± 5 (1.4)[b]	24 ± 3 (0.8)	24 ± 3 (0.8)
Forskolin	15 ± 3	33 ± 4 (2.2)[b]	27 ± 4 (1.8)[a]	58 ± 12 (3.9)[b]	64 ± 13 (4.3)[b]

[a] Significantly different from the corresponding value in control cells, $p < 0.05$.
[b] Significantly different from the corresponding value in control cells, $p < 0.01$.

TABLE 2

Concentration dependence of sensitization of intact cell cAMP accumulation

Cells were incubated for 60 min at 37° in the absence or presence of varying concentrations of the indicated pretreatment drugs. Cells were washed and cAMP accumulation stimulated by 10 μM isoproterenol and by 30 μM forskolin was then determined. The concentrations of pretreatment drugs inducing half-maximal sensitization (EC_{50}) and the fold sensitization were then determined by computerized curve-fitting using GraphPAD. Values are means ± standard error estimates from the indicated number (*n*) of experiments, each performed in duplicate or triplicate.

Drug	EC_{50}	Fold sensitization	*n*
Isoproterenol stimulation			
Carbachol	2.8 ± 1.0 μM	2.0 ± 0.1	8
Histamine	1.5 ± 0.7 μM	1.5 ± 0.0	6
Forskolin stimulation			
Carbachol	2.2 ± 0.8 μM	2.0 ± 0.1	8
Histamine	0.5 ± 0.1 μM	1.8 ± 0.0	7
PMA	4.7 ± 2.4 nM	2.6 ± 0.1	7
β-Phorbol didecanoate	4.6 ± 1.3 nM	2.2 ± 0.1	3
Mezerein	8.3 ± 2.8 nM	3.4 ± 0.1	3
4-*O*-Methyl-PMA	3.1 ± 0.9 μM	[a]	2
α-Phorbol didecanoate	ND[b]	1.1 ± 0.1	

[a] The curve for 4-*O*-methyl-PMA did not reach a plateau at the highest concentrations tested, so the maximal effect for purposes of curve fitting was forced to be the same as that for PMA in the same experiments.
[b] ND, not determined.

skolin was not increased following carbachol pretreatment. Carbachol pretreatment also sensitized stimulation by cholera toxin and by cholera toxin plus isoproterenol. Stimulation by cholera toxin plus forskolin increased to a much smaller extent, and there was no increase in stimulation by the combination of cholera toxin, isoproterenol, and forskolin.

Exposure to PMA before assay sensitized forskolin stimulation by 5-fold, with only minimal increases in stimulation by isoproterenol and by the combination of isoproterenol plus forskolin. PMA pretreatment induced marked sensitization of stimulation by cholera toxin and by cholera toxin plus isoproterenol. Stimulation by cholera toxin plus forskolin and by the combination of cholera toxin, isoproterenol, and forskolin also were sensitized in PMA-pretreated cells, but to a much smaller extent.

Dose-response curves for isoproterenol stimulation were compared in control cells and in cells sensitized by pretreatment with carbachol (Fig. 1). Sensitization was clearly due to an increase in maximal stimulation with no significant change in the potency of isoproterenol. Similar experiments were performed for sensitization of forskolin stimulation by carbachol and by PMA (Fig. 1). Although solubility of forskolin prevented determination of the maximal stimulation, the results for both carbachol and PMA were consistent with increases in the magnitude of stimulation with little change in the potency of forskolin, inasmuch as marked sensitization was observed even at the highest concentrations of forskolin tested. In all cases sensitization occurred rapidly, with half-maximal effects observed by 5 min and maximal effects by 60 min (data not shown).

Evidence for protein kinase C involvement in sensitization. Dose-response curves for sensitization of forskolin stimulation by PMA and related compounds were determined, and the results are summarized in Table 2. Half-maximal sensitization by PMA, which is a potent activator of protein kinase C, occurred at a concentration of about 5 nM. 4-*O*-Methyl-PMA, a structural analog of PMA that is much less potent as an activator of protein kinase C, also induced sensitization but was markedly less potent, with half-maximal sensitization occurring at a concentration of 3 μM. The β-isomer

TABLE 3

Sensitization of intact cell cAMP accumulation stimulated by isoproterenol, forskolin, and cholera toxin alone and in combination

Cells were preincubated for 1 hr in the absence or presence of 100 ng/ml cholera toxin and were then pretreated for an additional 60 min at 37° in the presence of 1% DMSO (control), 100 μM carbachol, or 1 μM PMA. Stimulation of cAMP accumulation was then determined in the absence (basal) or presence of 10 μM isoproterenol and/or 30 μM forskolin. Values for stimulation are expressed as fold of basal activity in control cells and values for sensitization (in parentheses) are expressed as fold of the stimulation by the same agents observed in control cells. Values are means from three experiments, each performed in triplicate.

Assay	Fold stimulation of cAMP accumulation		
	Control	Carbachol	PMA
Isoproterenol	41 ± 8	102 ± 25 (2.5)[a]	54 ± 7 (1.3)
Forskolin	9 ± 1	20 ± 2 (2.2)[a]	48 ± 7 (5.3)[a]
Isoproterenol + forskolin	199 ± 43	223 ± 35 (1.1)	232 ± 35 (1.2)
Cholera toxin	8 ± 1	17 ± 3 (2.1)[a]	44 ± 3 (5.6)[a]
Cholera toxin + isoproterenol	29 ± 4	84 ± 7 (2.9)[a]	143 ± 14 (5.0)[a]
Cholera toxin + forskolin	85 ± 12	112 ± 15 (1.3)	222 ± 37 (2.6)[a]
Cholera toxin + isoproterenol + forskolin	222 ± 38	199 ± 30 (0.9)	376 ± 72 (1.7)[a]

[a] Significantly different from the corresponding value in control cells, $p < 0.05$.

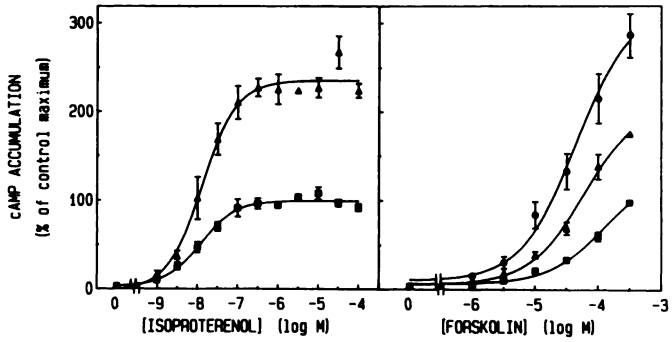

Fig. 1. Dose-response curves for stimulation of cAMP accumulation in control and sensitized cells. Cells were pretreated for 60 min at 37° in the absence (■) or presence of 100 μM carbachol (▲) or 1 μM PMA (●). Cells were washed and cAMP accumulation stimulated by the indicated concentrations of isoproterenol (*left*) or forskolin (*right*) was then determined. Values are expressed as percentage of maximal stimulation in control cells and are the means ± standard errors from three (*left*) or four (*right*) experiments, each performed in duplicate or triplicate. The curves were computer-generated using GraphPAD. The computer-generated values for EC_{50} and apparent maximal stimulation, respectively, were as follows: control cells, isoproterenol stimulation, 12 ± 2 nM, 100%; carbachol-pretreated cells, isoproterenol stimulation, 13 ± 2 nM, 235 ± 5%; control cells, forskolin stimulation, 120 ± 24 μM, 132 ± 11%; carbachol-pretreated cells, forskolin stimulation, 59 ± 9 μM, 209 ± 10%; and PMA-pretreated cells, forskolin stimulation, 44 ± 9 μM, 318 ± 17%.

of phorbol didecanoate, which is a potent activator of protein kinase C, induced an extent of sensitization similar to that seen with PMA, with half-maximal effect observed at a concentration of 5 nM. The α-isomer, which is weak or ineffective for protein kinase C activation, was essentially ineffective for inducing sensitization of forskolin stimulation as well. These results suggest that activation of protein kinase C can lead to sensitization of forskolin stimulation of intracellular cAMP accumulation in 1321N1 cells.

The effects of protein kinase C inhibitors on sensitization of isoproterenol- and forskolin-stimulated cAMP accumulation were determined (Fig. 2). Inclusion of either H7 (16) or staurosporine (17) during the pretreatment with DMSO in control cells did not alter stimulation of cAMP accumulation by either isoproterenol or forskolin (Fig. 2, legend). Inclusion of staurosporine during pretreatment with PMA caused 72% inhibition of sensitization of forskolin stimulation, whereas H7 inhibited PMA-induced sensitization of forskolin stimulation by 48%. Carbachol-induced sensitization of forskolin stimulation was inhibited 49% by staurosporine and 42% by H7. Carbachol-induced sensitization of isoproterenol stimulation was inhibited only 17% by staurosporine and 15% by H7.

To address further the involvement of protein kinase C in sensitization of cAMP accumulation, the effects of down-regulation of protein kinase C on subsequent sensitization were determined (Fig. 3). Cells were incubated for 18 hr with 10 μM PMA, conditions similar to those previously shown to cause almost complete down-regulation of protein kinase C in these cells (18). In separate experiments, we confirmed that this treatment reduced protein kinase C activity to approximately 10% of that in control cells.[3] Down-regulation of protein kinase C reduced the absolute values of basal and isoproterenol-stimulated cAMP accumulation in nonsensitized cells by about 50%, whereas forskolin-stimulated cAMP accumulation was in-

[3] C. C. Franklin and M. L. T., unpublished observations.

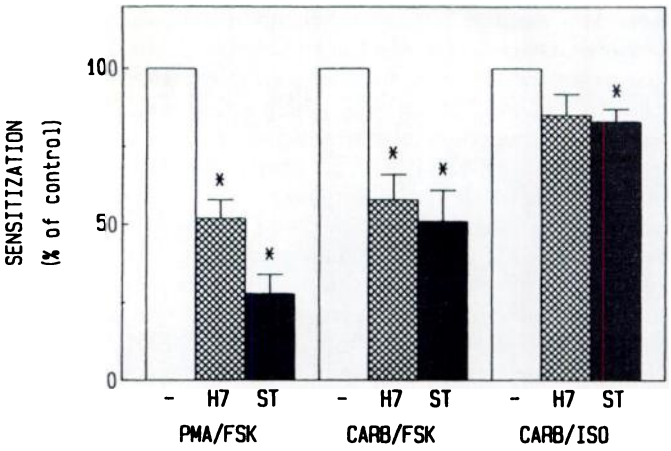

Fig. 2. Effects of protein kinase inhibitors on sensitization. Cells were pretreated for 60 min at 37° in the absence or presence of 300 μM H7 or 1 μM staurosporine (*ST*) to inhibit protein kinase activity and either 100 μM carbachol (*CARB*) or 1 μM PMA to induce sensitization. Protein kinase inhibitors were added 5 min before sensitizing agents. Cells were washed and cAMP accumulation stimulated by 10 μM isoproterenol (*ISO*) and by 30 μM forskolin (*FSK*) was then determined. Values are expressed as percentage of the sensitization in control cells pretreated in the absence of inhibitor and are the means ± standard errors from four to six experiments performed in triplicate. The percentage of conversion values for isoproterenol stimulation in nonsensitized cells were as follows: control, 0.49 ± 0.03; H7, 0.48 ± 0.04; and ST, 0.51 ± 0.04. The percentage of conversion values for forskolin stimulation in nonsensitized cells were as follows: control, 0.22 ± 0.02; H7, 0.21 ± 0.03; and ST, 0.23 ± 0.07. The 100% values for sensitization in the absence of inhibitors were as follows: carbachol/isoproterenol, 2.17-fold; carbachol/forskolin, 2.11-fold; and PMA/forskolin, 5.28-fold. The *asterisk* indicates that the value in the presence of inhibitor was significantly different from the value in the absence of inhibitor, $p < 0.05$.

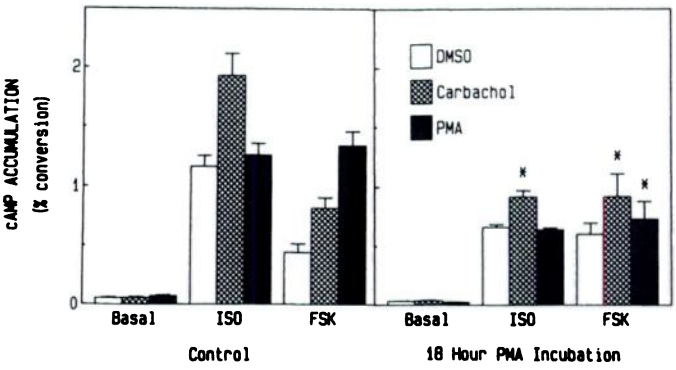

Fig. 3. Effects of protein kinase C down-regulation on sensitization. Cells were incubated for 18 hr at 37° in the absence (control) or presence of 10 μM PMA. Cells were then pretreated for 60 min at 37° in the absence or presence of 100 μM carbachol or 1 μM PMA to induce sensitization. Stimulation of cAMP accumulation by 10 μM isoproterenol (*ISO*) and by 30 μM forskolin (*FSK*) was then determined. Values are expressed as percentage of conversion and are the means ± standard errors from three experiments performed in triplicate. The values for fold sensitization in control cells and cells preincubated for 18 hr with PMA, respectively, were as follows: carbachol sensitization of isoproterenol stimulation, 1.65 ± 0.04 and 1.39 ± 0.07; PMA sensitization of isoproterenol stimulation, 1.10 ± 0.17 and 0.96 ± 0.05; carbachol sensitization of forskolin stimulation, 1.89 ± 0.11 and 1.51 ± 0.09; and PMA sensitization of forskolin stimulation, 3.12 ± 0.15 and 1.17 ± 0.06. The *asterisk* indicates that the fold sensitization in cells preincubated for 18 hr with PMA was significantly less than that in control cells, $p < 0.05$.

creased by about 35%. The fold stimulation by isoproterenol in down-regulated cells was similar to that in control cells and that by forskolin was about twice that in control cells. Down-regulation of protein kinase C almost completely eliminated the sensitization of forskolin stimulation induced by PMA, reduced sensitization of forskolin stimulation induced by carbachol by about 40%, and similarly reduced sensitization of isoproterenol stimulation induced by carbachol by about 40% (see values in Fig. 3 legend). These results suggest that protein kinase C is involved in sensitization in each of these cases, although additional mechanisms are likely to be involved in the sensitization phenomena induced by carbachol.

Possible mechanisms of sensitization. Phosphodiesterase inhibitors were not routinely included in the cAMP accumulation assays. Studies with the phosphodiesterase inhibitor IBMX (12) were performed to determine whether changes in phosphodiesterase activity might be responsible for the observed sensitization of cAMP accumulation (data not shown). Inclusion of IBMX during the stimulation phase of the assay increased both isoproterenol- and forskolin-stimulated cAMP accumulation, although the effects of IBMX are small in these cells (12). Sensitization of isoproterenol stimulation by carbachol pretreatment and of forskolin stimulation by both carbachol and PMA pretreatment were still observed in the presence of this maximally effective concentration of inhibitor, suggesting that decreased activity of phosphodiesterase is not the mechanism of sensitization.

To address the possibility that sensitization might result from decreased activity of G_i, the effects of pertussis toxin on sensitization were determined (Fig. 4). Pretreatment with pertussis toxin under conditions similar to those previously shown to inactivate function of G_i in these cells (19) did not alter basal or isoproterenol-stimulated cAMP accumulation but did cause an approximately 50% reduction in forskolin stimulation. Although this effect of pertussis toxin has not been further investigated, a similar effect was noted in related studies with HT29 human colonic adenocarcinoma cells (20). The fold sensitization of isoproterenol stimulation induced by carbachol pretreatment and of forskolin stimulation induced by both carbachol and PMA pretreatment were not significantly different between control and pertussis toxin-pretreated cells (see values in Fig. 4 legend). Because pertussis toxin neither mimicked nor prevented sensitization, decreases in inhibitory effects mediated by G_i are not likely to be the major mechanism of sensitization in these cells.

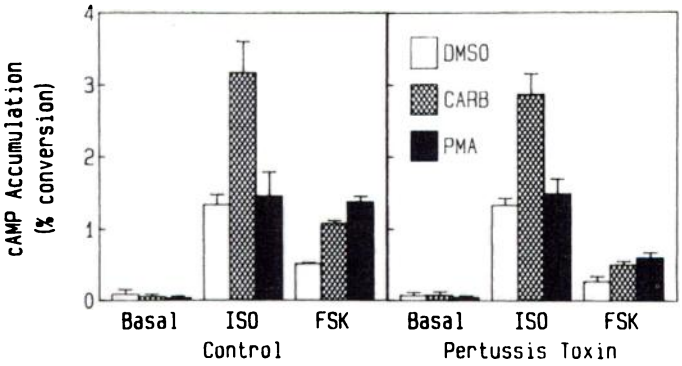

Fig. 4. Effects of pertussis toxin on sensitization. Cells were incubated for 20 hr in the absence or presence of 100 ng/ml pertussis toxin. Cells were then pretreated for 60 min at 37° in the absence or presence of 100 μM carbachol (*CARB*) or 1 μM PMA and finally stimulation of cAMP accumulation by 10 μM isoproterenol (*ISO*) and by 30 μM forskolin (*FSK*) was determined. Values are expressed as percentage of conversion and are the means ± standard errors from three experiments performed in triplicate. The values for fold sensitization in control and pertussis toxin-pretreated cells, respectively, were as follows: carbachol sensitization of isoproterenol stimulation, 2.39 ± 0.15 and 2.15 ± 0.06; PMA sensitization of isoproterenol stimulation, 1.06 ± 0.13 and 1.12 ± 0.06; carbachol sensitization of forskolin stimulation, 2.15 ± 0.07 and 1.95 ± 0.23; and PMA sensitization of forskolin stimulation, 2.76 ± 0.17 and 2.39 ± 0.42. In none of these cases was the value in pertussis toxin-treated cells significantly different from that in control cells, $p < 0.05$.

In our previously published study (8), we found that pretreatment of cells with PMA led to an approximately 50% decrease in isoproterenol-stimulated adenylate cyclase activity in broken cell preparations, with little or no change in forskolin- or fluoride-stimulated activities. In additional experiments to date, including studies with various Mg^{2+} concentrations present during the assay (21), we have been unable to retain consistently either carbachol- or PMA-induced sensitization in assays of adenylate cyclase activity in broken cell preparations. Meeker and Harden (13) also reported that carbachol-induced sensitization was not retained in broken cell adenylate cyclase assays.

Discussion

These studies demonstrate that, in 1321N1 human astrocytoma cells, activation of protein kinase C leads to an increase in the ability of a variety of agents to increase intracellular cAMP accumulation. Thus, direct activators of protein kinase C, such as PMA, β-phorbol didecanoate, and mezerein all increased forskolin stimulation approximately 4-fold, whereas analogs of these compounds that are weak or ineffective as protein kinase C activators, such as 4-*O*-methyl-PMA and α-phorbol didecanoate, did not induce sensitization at comparable doses. The protein kinase inhibitors staurosporine and H7 inhibited the sensitization of forskolin stimulation induced by PMA. Finally, treatment of cells to down-regulate protein kinase C almost completely eliminated the sensitization of forskolin stimulation induced by PMA.

The effects of PMA pretreatment on stimulation of intracellular cAMP accumulation by isoproterenol and by prostaglandin E_1, agonists acting through receptors coupled to adenylate cyclase, were different from those on stimulation by forskolin, which is thought to interact primarily with the catalytic unit of adenylate cyclase. PMA induced only a small, or in many experiments no, increase in isoproterenol stimulation, in spite of inducing marked sensitization of forskolin stimulation. Surprisingly, in these assays of cAMP accumulation by intact cells, we also did not observe the PMA-induced desensitization of isoproterenol stimulation that was observed in our previous study using broken cell adenylate cyclase assays (8). To explain these results, we propose that activation of protein kinase C leads to two distinct modifications of the receptor-coupled adenylate cyclase system of these cells, 1) a desensitization of receptor-mediated stimulation, presumably due to modification of the receptors themselves, and 2) a sensitization of catalytic activity, presumably due to modification of the adenylate cyclase catalytic unit or of its interaction with G_s. This second modification is either unstable to cell lysis or is otherwise masked in broken cell adenylate cyclase assays. Thus, sensitization of forskolin stimulation is observed in intact cell assays but not in broken cell assays. In intact cell assays of stimulation

by isoproterenol, the sensitization of activity of the catalytic unit is balanced by a similar extent of desensitization of receptor function, thus leading to the observed lack of any consistent sensitization of receptor-mediated stimulation in intact cells. In broken cell assays, the sensitization of catalytic activity is lost, thus revealing the desensitization of receptor function. The PMA-induced desensitization of receptor-mediated stimulation in broken cell assays has been less consistently observed than the PMA-induced sensitization of forskolin stimulation observed in intact cell assays. Based on the model above, this variability in the extent to which the receptor desensitization modification occurs could also account for the variability in the effects on receptor-mediated stimulation observed in the intact cell assays. Evidence for multiple and opposing effects of phorbol esters on intact cell cAMP accumulation in S49 cells has been presented recently (21, 22), although the pattern of effects observed in these cells was somewhat different from those presented here.

Carbachol and histamine, agents that act at receptors coupled to PPI turnover and subsequent activation of protein kinase C, also caused sensitization of forskolin stimulation, although the extent of sensitization (approximately 2-fold) was only about half of that observed with the direct activators. Although protein kinase C activation is almost certainly responsible for the PMA-induced sensitization, additional mechanisms must also be involved in the sensitization induced by carbachol and histamine. There are both quantitative and qualitative differences between the sensitization induced by carbachol and histamine and that induced by PMA. The smaller extent of sensitization of forskolin stimulation observed with carbachol and histamine than with PMA can probably be accounted for by the fact that activation of protein kinase C by carbachol and histamine will be less pronounced and of shorter duration due to rapid metabolism of diacylglycerol, the endogenous activator of protein kinase C. It is more difficult to explain the sensitization of receptor-mediated stimulation of cAMP accumulation induced by carbachol and histamine based on protein kinase C activation, because activation of protein kinase C with PMA did not sensitize receptor-mediated stimulation. Further evidence for involvement of additional mechanisms in this particular aspect of sensitization comes from the fact that neither protein kinase inhibitors nor protein kinase C down-regulation were able to inhibit carbachol-mediated sensitization as effectively as they inhibited PMA-induced sensitization.

We would propose that an additional mechanism, perhaps related to increased intracellular Ca^{2+}, is involved in the sensitization induced by carbachol and histamine. This proposed second mechanism either could directly increase receptor-mediated stimulation by altering the receptors or the guanine nucleotide-binding protein or, alternatively, could inhibit the protein kinase C-mediated desensitization of receptor function so that the sensitization of catalytic activity detected in assays of forskolin stimulation is now also observed in assays of receptor-mediated stimulation. Additional studies will be required to identify this second process and to investigate the possible role of Ca^{2+}-mediated changes in the overall mechanism of sensitization.

Recent studies in a variety of cell types have described effects of phorbol esters or PPI-coupled receptor agonists on cAMP formation that may be related to the sensitization phenomenon we have studied in 1321N1 cells. However, the experimental protocols employed and the patterns of sensitization observed have been quite variable. In many of these studies, phorbol esters or PPI-coupled receptor agonists were included during the assays of cAMP formation rather than as a pretreatment, and the effects were described as "synergism" or "potentiation" rather than as sensitization. Nonetheless, it seems likely that similar mechanisms may be involved. In addition to studies using only phorbol esters and other direct activators of protein kinase C (21–33), these phenomena have also been reported to be induced by vasopressin (34–36), angiotensin (37, 38), histamine (39–41), α_1-adrenergic agonists (39, 42–46), and muscarinic agonists (13, 39, 47). Sensitization during the assay may account for the increase in forskolin-stimulated cAMP accumulation recently observed in studies with cloned M1 and M4 muscarinic receptors (48).

A variety of mechanisms have been proposed to explain these sensitization phenomena. Based primarily on the effects of pertussis toxin, changes in G_i function have been postulated to be the mechanism in some cases (24, 29, 32, 34, 35) but not in others (28, 30, 33). Other studies have suggested G_s or the adenylate cyclase catalytic unit as the site of modification (23, 25, 31, 33). Changes in prostaglandins (45), adenosine (40), and cellular Ca^{2+} (36, 40, 41) have also been postulated to be involved in some cases.

In addition to sensitization induced by protein kinase C activators and PPI-coupled receptor agonists, sensitization has also been observed following pretreatment with agonists acting at receptors coupled to inhibition of adenylate cyclase, such as opiates, somatostatin, α_2-adrenergic agonists, and muscarinic receptor agonists in some tissues (49). In these cases, sensitization can be viewed more as an adaptive response of the cells to maintain cAMP levels in the presence of prolonged inhibition of adenylate cyclase rather than as an example of cross-talk between receptor signalling pathways. The sensitization induced by carbachol in 1321N1 cells combines elements of both of these views of sensitization. Although muscarinic receptors in these cells are coupled to PPI turnover rather than to inhibition of adenylate cyclase as in many tissues, one of the consequences of stimulation of PPI turnover in these cells is a decrease in cellular cAMP levels due to activation of a Ca^{2+}-stimulated phosphodiesterase (10–12). The increase in stimulation of cAMP accumulation observed following longer term exposure to carbachol could thus be viewed as a homeostatic mechanism to balance the increased phosphodiesterase activity.

Because carbachol initially decreases cAMP levels in these cells through activation of phosphodiesterase activity, adaptive decreases in phosphodiesterase activity would appear to be a likely mechanism for the observed sensitization of cAMP accumulation. This mechanism would also explain our failure to observe sensitization in broken cell adenylate cyclase assays, because these assays are carried out in the presence of phosphodiesterase inhibitor and excess cAMP. However, inhibition of phosphodiesterase activity with IBMX neither mimicked nor prevented carbachol- or PMA-induced sensitization, suggesting that this is not the mechanism involved. The previous study of carbachol-induced sensitization reported similar results and demonstrated more directly that carbachol did not induce changes in phosphodiesterase activity (13). Changes in G_i activity also appear not to be the mechanism for sensitization in 1321N1 cells, based on the lack of effect of pertussis toxin on sensitization. Thus, changes in the rate of synthesis of cAMP

due to modification of either the catalytic unit of adenylate cyclase or G_s are the most likely mechanisms for the sensitization observed in this study.

In summary, our results demonstrate that both activation of receptors coupled to PPI turnover and direct activation of protein kinase C can lead to increased stimulation of cAMP accumulation in 1321N1 cells. Multiple mechanisms appear to be involved in the overall process of sensitization. Based on our studies to date, it seems unlikely that either decreased phosphodiesterase activity or decreased G_i activity is responsible for the sensitization observed. Modification of the activity of the catalytic unit of adenylate cyclase, presumably via protein kinase C-mediated phosphorylation, seems the most likely mechanism for the sensitization of forskolin stimulation, although this modification is not retained or detected in broken cell assays. Additional mechanisms, perhaps related to intracellular Ca^{2+} mobilization, are required to explain the sensitization induced by carbachol and histamine. The goal of future studies will be to delineate further the pathways and specific molecular modifications involved in these sensitization phenomena.

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