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Muscarinic Receptor-Stimulated Phosphoinositide Turnover in Human SK-N-SH Neuroblastoma Cells: Differential Inhibition by Agents that Elevate Cyclic AMP

*Mayada Akil and †Stephen K. Fisher

*Neuroscience Laboratory and Departments of *Psychiatry and †Pharmacology, University of Michigan, Ann Arbor, Michigan, U.S.A.*

Abstract: The possibility that an increased intracellular concentration of cyclic AMP (cAMP) can regulate the extent of muscarinic receptor-stimulated phosphoinositide (PPI) turnover in the human neuroblastoma cell line SK-N-SH was examined. Addition of either forskolin (or its water-soluble analog, L-85,8051), theophylline, isobutylmethylxanthine, or cholera toxin, agents that interact with either the catalytic unit of adenylate cyclase, cAMP phosphodiesterase, or the guanine nucleotide binding protein linked to adenylate cyclase activation, resulted in a 45–181% increase in cAMP concentration and a 27–70% inhibition of carbachol-stimulated inositol phosphate release. Through the use of digitonin-permeabilized cells, the site of inhibition was localized to a step at, or distal to, the guanine nucleotide binding protein that regulates phospholipase C activity. In contrast, when intact SK-N-SH cells were exposed to prostaglandin E_1, the ensuing increases in cAMP were not accompanied by an inhibition of stimulated PPI turnover. These differential effects of increased cAMP concentrations on stimulated PPI turnover may reflect the compartmentation of cAMP within SK-N-SH cells. **Key Words:** Phosphoinositide—Cyclic AMP—Muscarinic receptor—Human neuroblastoma, SK-N-SH—Forskolin—Cholera toxin. **Akil M. and Fisher S. K.** Muscarinic receptor-stimulated phosphoinositide turnover in human SK-N-SH neuroblastoma cells: Differential inhibition by agents that elevate cyclic AMP. *J. Neurochem.* **53,** 1479–1486 (1989).

The hydrolysis of inositol lipids and regulation of adenylate cyclase activity constitute two of the major signal transduction pathways in both neural and non-neural cells. The possibility that cross-talk exists between these two signaling pathways has been suggested and evidence for cyclic AMP (cAMP)-mediated inhibition of stimulated phosphoinositide (PPI) hydrolysis has been obtained in some nonneural tissues (Nishizuka, 1986; Della Bianca et al., 1986; Jakobs et al., 1986; Neylon and Summers, 1988; Madison and Brown, 1988). However, little information exists to indicate whether a similar regulation of PPI turnover by cAMP also occurs in neural tissues.

In the present study, we have utilized the human neuroblastoma cell line SK-N-SH to determine whether elevated intracellular concentrations of cAMP result in an inhibition of receptor-stimulated PPI turnover. These cells appear well suited to such a study because they are homogeneous and possess receptors that are linked to the regulation of adenylate cyclase activity (Yu et al., 1986, 1988; Baron and Siegel, 1988). Furthermore, SK-N-SH neuroblastomata possess a high density of muscarinic cholinergic receptors (mAChRs) that are effectively coupled to PPI turnover, both in intact and permeabilized cells (Fisher and Snider, 1987; Fisher, 1988; Fisher et al., 1989). Our studies indicate that the addition of selective agents that elicit an increased intracellular concentration of cAMP independent of receptor activation also results in an inhibition of mAChR-stimulated PPI turnover in SK-N-SH cells.

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Address correspondence and reprint requests to Dr. S. K. Fisher at Neuroscience Laboratory, University of Michigan, 1103 E. Huron St., Ann Arbor, MI 48104-1687, U.S.A.

Abbreviations used: 8BcGMP, 8-bromo-cyclic GMP; cAMP, cyclic AMP; CT, cholera toxin; dBcAMP, 2-*O*-dibutyryl cyclic AMP; FSK, forskolin; G_i, inhibitory guanine nucleotide binding protein; G_p, guanine nucleotide binding protein that regulates phospholipase C activity; G_s, stimulatory guanine nucleotide binding protein; GTPγS, guanosine-5′-*O*-(3-thiotriphosphate); IBMX, 3-isobutyl-1-methylxanthine; IP, inositol phosphate(s) (inositol mono-, bis-, tris-, and tetrakisphosphates); IP_1, inositol monophosphate; L-85,8051, 7β-deacetyl-7β-(γ-*N*-methylpiperazino)butyryl forskolin; mAChR, muscarinic acetylcholine receptor; NMS, *N*-methylscopolamine; PGE_1, prostaglandin E_1; PPI, phosphoinositide (phosphatidylinositol, phosphatidylinositol 4-phosphate, and phosphatidylinositol 4,5-bisphosphate); SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Using digitonin-permeabilized cells, we demonstrate that the inhibition can be localized to a site that is either at, or distal to, the guanine nucleotide binding protein (G_p) that regulates phospholipase C activity. In marked contrast, prostaglandin E_1 (PGE_1)-mediated increases in cAMP do not elicit an inhibition of stimulated PPI turnover, a result consistent with the possibility that the effects of cAMP on inositol lipid turnover are compartmentalized in SK-N-SH cells. A preliminary report of part of this work has appeared elsewhere (Akil and Fisher, 1988).

MATERIALS AND METHODS

myo-[2-^{3}H]Inositol (15 Ci/mmol) was obtained from American Radiolabeled Chemicals (St. Louis, MO, U.S.A.). *N*-[^{3}H]Methylscopolamine ([^{3}H]NMS) (80 Ci/mmol) was obtained from New England Nuclear (Boston, MA, U.S.A.). Forskolin (FSK), 3-isobutyl-1-methylxanthine (IBMX), 2-*O*-dibutyryl cyclic AMP (dBcAMP), theophylline, carbachol, and 8-bromo-cyclic GMP (8BcGMP) were obtained from Sigma Chemical (St. Louis, MO, U.S.A.). Guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) was purchased from Boehringer-Mannheim (Indianapolis, IN, U.S.A.). Cholera toxin (CT), 1,9-dideoxyforskolin, 7-*O*-hemisuccinyl-7-deacetyl forskolin, and 7β-deacetyl-7β-(γ-*N*-methylpiperazino)butyryl forskolin (L-85,8051) were obtained from Behring Diagnostics (La Jolla, CA, U.S.A.). Dowex-1 (100–200 mesh; ×8 in the formate form) was obtained from Bio-Rad Laboratories (Rockville Center, NY, U.S.A.). Tissue culture supplies were obtained from Corning Glass Works (Corning, NY, U.S.A.). Powdered Dulbecco's modified medium and fetal bovine serum were purchased from Grand Island Biological (Grand Island, NY, U.S.A.). [^{32}P]NAD (39 Ci/mmol) was obtained from New England Nuclear (Boston, MA, U.S.A.).

Cell culture conditions

Human SK-N-SH neuroblastoma cells were cultured in Dulbecco's modified Eagle's medium/10% fetal calf serum in an atmosphere of 90% air/10% CO_2 under conditions that have been described previously by Fisher and Snider (1987). Cells were first detached from tissue culture dishes by aspiration of the medium and the addition of 10 ml of Puck's D_1 solution (Honegger and Richelson, 1976). They were then collected by centrifugation at 300 *g* for 1 min and resuspended in buffer A (142 m*M* NaCl, 5.5 m*M* KCl, 2.2 m*M* $CaCl_2$, 3.6 m*M* $NaHCO_3$, 1 m*M* $MgCl_2$, 5.6 m*M* D-glucose, and 30 m*M* Na^+ HEPES buffer, pH 7.4). Cells utilized were 10–20 days old. Protein determinations were performed by the method of Geiger and Bessman (1972).

Determination of PPI turnover

Intact SK-N-SH cells were harvested as described above, then resuspended in buffer A containing [^{3}H]inositol (approximately 9 μCi/ml) at a protein concentration of 0.8–2 mg/ml protein. Cells were first allowed to prelabel for 45 min at 37°C, and then incubated (unless specified otherwise) for an additional 45 min in the presence or absence of carbachol (10 m*M*), the various agents, and 10 m*M* LiCl (final volume, 0.5 ml). Reactions were terminated by the addition of 1.5 ml of chloroform/methanol (1:2, vol/vol) and the total water-soluble inositol phosphate (IP) fraction was separated and quantitated by ion-exchange chromatography as previously described by Fisher and Snider (1987). Both basal and carbachol-stimulated release of ^{3}H-IP was linear with time over a 45-min incubation period. In other experiments, individual IP species were fractionated as previously described by Fisher and Bartus (1985) and Fisher et al. (1989). Approximately 75% of the label was recovered in inositol monophosphate (IP_1), with the remainder present in glycerophosphorylinositol, inositol bisphosphate, inositol trisphosphate, and inositol tetrakisphosphate. In some experiments, a 200-μl aliquot was removed from the organic phase to determine the incorporation of [^{3}H]inositol into the phospholipid fraction (Fisher and Bartus, 1985).

Determination of intracellular cAMP levels

SK-N-SH cells (0.8–2 mg protein/ml) were incubated with various agents for 45 min at 37°C (unless otherwise specified). Reactions were terminated by immersing the tubes in ice-cold water followed by centrifugation at 2,500 *g* for 3 min. The supernatant was discarded, and the pellet extracted with 1 ml of 7% perchloric acid. The extract was then neutralized with K_2CO_3 (1.77 *M*), dried down under N_2, and resuspended in 50 m*M* sodium acetate buffer (pH 6.2). cAMP quantitation was performed using a cAMP ^{125}I radioimmunoassay kit from New England Nuclear (Boston, MA, U.S.A.). In most experiments, cAMP determinations were carried out on the same incubations utilized for PPI turnover measurement.

CT treatment

Intact cells. SK-N-SH cells were cultured in Dulbecco's modified Eagle's medium/10% fetal calf serum containing 50 μg/ml CT for 5 or 24 h. Cells were then harvested, prelabeled with [^{3}H]inositol for 60 min, and incubated for 45 min at 37°C in the presence or absence of carbachol. The release of labeled IPs and/or cAMP concentrations determined were measured as described above.

Permeabilized cells. Cells were prelabeled for 3 days in medium containing 5–7.5 μCi/ml of [^{3}H]inositol. In one set of experiments CT was added to tissue culture dishes at a concentration of 50 μg/ml for an overnight incubation (approximately 16 h). Cells were then harvested and permeabilized with digitonin as previously described by Fisher et al. (1989), prior to incubation with carbachol, GTPγS, or both for 15 min at 37°C and measurement of IP release. In a second series of experiments, prelabeled cells were first permeabilized, and then incubated with activated CT (Tamir and Gill, 1988) at a concentration 50 μg/ml for 30–60 min. Release of labeled IPs was then measured following a 15-min incubation with carbachol, GTPγS, or a combination of the two agents.

ADP-ribosylation of SK-N-SH cells with [^{32}P]NAD and CT

Control or CT-pretreated SK-N-SH cells were homogenized in 50 m*M* sodium phosphate buffer (pH 7.4) containing protease inhibitors (10 μg/ml of leupeptin, aprotinin, and soybean trypsin inhibitor). Homogenates were then centrifuged at 500 *g*/5 min, pellets discarded, and supernatants centrifuged at 14,000 *g*/10 min. The pellets were then resuspended in 100 m*M* potassium phosphate buffer (pH 7.5) and membranes from control or CT-pretreated cells incubated in the absence or presence of activated CT (100 μg/ml; Tamir and Gill, 1988) with [^{32}P]NAD under the conditions described by Klinz et al. (1987). [^{32}P]ADP-ribosylated membrane proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli, 1970) and visualized autoradiographically as previously described by Fisher and Heacock (1988).

Radioligand binding

The binding of [^{3}H]NMS to mAChRs on intact SK-N-SH cells was determined as previously described by Fisher (1988).

Data analysis

Values quoted are means ± SEM for the number of separate experiments stated in parentheses. Student's two-tailed *t* tests were used to evaluate the statistical differences of the means of paired or unpaired sets of data. Percent inhibition of carbachol-stimulated ^{3}H-IP release was calculated from the equation:

$$\text{Percent inhibition} = \left[1 - \frac{B - D}{A - C}\right] \times 100$$

where *A* is ^{3}H-IP release observed in the presence of carbachol; *B* is ^{3}H-IP release observed in the presence of carbachol and inhibitory agent; *C* is basal ^{3}H-IP release observed in control incubations; and *D* is basal ^{3}H-IP release observed in control incubation containing the inhibitory agent. A negative value indicates that carbachol-stimulated ^{3}H-IP release was higher in the presence of the agent than in its absence.

RESULTS

Inhibition of carbachol-stimulated ^{3}H-IP release by FSK

Incubation of [^{3}H]inositol-prelabeled SK-N-SH cells with 50 μ*M* FSK, a diterpene that directly stimulates the catalytic subunit of adenylate cyclase (Seamon and Daly, 1986), resulted in a 41 ± 4% inhibition of carbachol-stimulated ^{3}H-IP release (n = 16). Addition of FSK also inhibited the ability of carbachol to stimulate the labeling of the inositol lipid fraction (33 ± 4% inhibition, n = 11), whereas no effect of FSK on basal lipid labeling was observed. The inhibitory effect of FSK was dose dependent with little effect detectable at 10 μ*M* and maximum inhibition observed at 100 μ*M* FSK, a concentration close to the solubility limit of FSK (Seamon and Daly, 1986) (Fig. 1). In all subsequent experiments, a concentration of 50 μ*M* FSK was used. The inhibitory effects of FSK on carbachol-stimulated ^{3}H-IP release were detectable within the first 5–10 min of incubation, and persisted for up to at least 40 min (Fig. 2). Although FSK reduced the extent of carbachol-stimulated ^{3}H-IP release, the concentration of agonist required to elicit a half-maximal increase in PPI turnover was not altered, indicating that FSK does not change the agonist's affinity for the mAChR (Fig. 3).

Because some of the effects of FSK have been previously ascribed to mechanisms that do not involve adenylate cyclase activation (Joost et al., 1988), additional experiments were conducted using either 7-*O*-

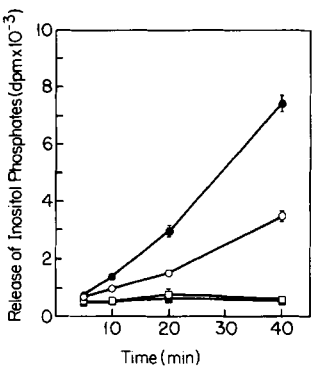

FIG. 2. Time course of FSK inhibition of carbachol-stimulated ^{3}H-IP release. Prelabeled SK-N-SH cells were incubated for the designated times at 37°C: Control (□); FSK (■); carbachol (●); carbachol plus FSK (○). Values shown are means ± SEM for triplicate samples from a single experiment. In four separate time-course experiments, values for the percentage inhibition of carbachol-stimulated ^{3}H-IP release obtained with 50 μ*M* FSK at 5, 10, 20, and 40 min of incubation were 24 ± 16, 54 ± 8, 34 ± 9, and 39 ± 12, respectively.

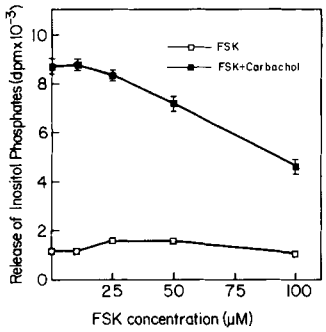

FIG. 1. Concentration dependence of FSK inhibition of stimulated ^{3}H-IP release. Prelabeled SK-N-SH cells were incubated with increasing concentrations of FSK for 45 min at 37°C in the presence (■) or absence (□) of carbachol (10 m*M*). Values shown are means ± SEM for triplicate samples from a single experiment. Where error bars are not shown, they fall inside the symbol. In three or four separate experiments, the addition of 10, 25, 50, and 100 μ*M* FSK resulted in 12 ± 7, 22 ± 8, 37 ± 7, and 66 ± 6% inhibition of carbachol-stimulated ^{3}H-IP release. Total inositol phosphate release was measured as described in Materials and Methods.

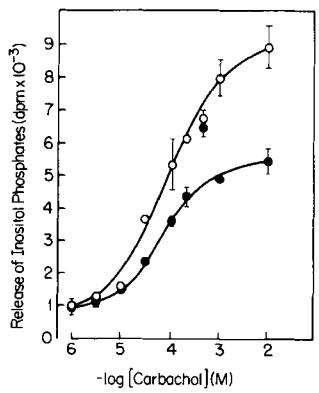

FIG. 3. Effect of FSK on dose dependence of carbachol-stimulated ^{3}H-IP release. Cells were incubated with carbachol at the indicated concentrations in the absence (○) or presence (●) of 50 μ*M* FSK. FSK did not alter basal ^{3}H-IP release. Values shown are means ± SEM for triplicate samples obtained from a single experiment. In the experiment shown, EC_{50} values for carbachol (calculated by log-probit analysis) were 80 μ*M* (presence of FSK) and 120 μ*M* (absence of FSK). In three separate experiments the mean values for the EC_{50} were 130 ± 21 μ*M* (absence of FSK) and 177 ± 73 μ*M* (presence of FSK).

TABLE 1. *Effects of FSK and analogs on carbachol-stimulated ^{3}H-IP release and inositol lipid labeling*

Agent	Inhibition (%) IP	Inositol lipids
FSK, 50 μM	41 ± 4 (16)	33 ± 4 (11)
7-*O*-Hemisuccinyl-7-deacetyl FSK, 50 μM	25 ± 4 (8)	11 ± 4 (6)
1,9-Dideoxy FSK, 50 μM	5 ± 8 (5)	2 ± 8 (5)
L-85,8051, 50 μM	27 ± 8 (3)	20 ± 8 (3)
200 μM	56 ± 6 (3)	25 ± 8 (3)

Cells were first prelabeled with [^{3}H]inositol for 45 min and then incubated for an additional 45 min at 37°C with carbachol (10 mM), in the presence or absence of the agent shown. LiCl (10 mM) was included in the assays during the incubation period to prevent the degradation of the primary IP formed, i.e., IP_1. Basal and stimulated ^{3}H-IP release and inositol lipid labeling were determined as described in Materials and Methods. Values shown are percent inhibition of carbachol-stimulated ^{3}H-IP release or inositol lipid labeling (means ± SEM) or range (n = 2) for the number of separate experiments indicated in parentheses. Basal ^{3}H-IP release and inositol lipid labeling were not affected by any of the agents listed.

hemisuccinyl-7-deacetyl FSK, an active structural analog of FSK (Pfeuffer and Metzger, 1982), L-85,8051, an active water-soluble FSK analog (Laurenza et al., 1987), or 1,9-dideoxy FSK, an inactive hydrophobic FSK analog (Laurenza et al., 1987). Addition of either 7-*O*-hemisuccinyl-7-deacetyl FSK or the water-soluble analog L-85,8051 significantly inhibited carbachol-stimulated ^{3}H-IP production (Table 1). In contrast, the addition of 1,9-dideoxy FSK had little or no effect on stimulated ^{3}H-IP release (Table 1). It appears, therefore, that the ability of FSK to inhibit carbachol-stimulated PPI hydrolysis is mediated directly through its activation of adenylate cyclase and is not a function of its hydrophobicity.

Inhibition of carbachol-stimulated ^{3}H-IP release by IBMX, theophylline, and dBcAMP

The addition of either IBMX or theophylline, both known inhibitors of cAMP phosphodiesterase, resulted in a 51–70% inhibition of carbachol-stimulated ^{3}H-IP release, whereas basal IP release was unaffected (Table 2, part A). Similarly, these agents also inhibited carbachol-stimulated inositol lipid labeling, whereas no effect on basal lipid labeling was observed. When IBMX was included in incubations containing FSK, the resulting inhibition (85 ± 8, n = 3) was less than additive ($p < 0.05$), a result indicative of a common site of action for these two agents. In contrast to the marked effects of inhibitors of cAMP phosphodiesterase on carbachol-stimulated ^{3}H-IP release, direct addition of the cell permeant analog of cAMP, dBcAMP, at a concentration of 1 mM had only a modest inhibitory effect (11%), the extent of which varied considerably between experiments (Table 2, part B). The direct addition of 8BcGMP was without effect.

Effects of FSK and inhibitors of cAMP phosphodiesterase on ligand binding to mAChRs on SK-N-SH cells

The inhibitory effects of FSK (and analogs), IBMX, or theophylline on mAChR-stimulated PPI turnover do not appear to be mediated through an inhibition of ligand binding to the mAChR, because none of the agents tested had a significant effect on the binding of [^{3}H]NMS to binding sites on intact cells. Thus, in three separate experiments, specific [^{3}H]NMS binding in the presence of 50 μM concentrations of FSK, 1,9-dideoxy FSK, 7-*O*-hemisuccinyl-7-deacetyl FSK, or L-85,8051 was 100 ± 0, 97 ± 2, 97 ± 3, and 102 ± 4% of control, respectively. [^{3}H]NMS binding obtained in the presence of 1 mM IBMX or 2 mM theophylline was 99 ± 6 and 94 ± 4% of control, respectively.

Intracellular cAMP concentrations

The basal concentration of cAMP in SK-N-SH cells, measured under the same conditions as those employed for PPI turnover, was 24 ± 2 pmol/mg protein (n = 12). To establish whether cAMP intracellular concentrations were increased in the presence of FSK, IBMX, and theophylline, changes in the concentration of cAMP were measured. FSK (50 μM) increased the concentration of cAMP to 281 ± 62% of control (n = 6), IBMX (1 mM) to 228 ± 22% of control (n = 10), and theophylline (2 mM) to 171 ± 40% of control (n = 3). cAMP could not be measured in the presence of dBcAMP or 8BcGMP because of cross-reactivity in the radioimmunoassay. Thus, the agents that inhibit stimulated PPI turnover also elicit a measurable increase in the concentration of cAMP in SK-N-SH cells.

Inhibition of carbachol-stimulated ^{3}H-IP release by CT

mAChR-stimulated PPI turnover was also inhibited by preincubation of SK-N-SH cells with CT, an agent known to activate adenylate cyclase through ADP-ribosylation of the α-subunit of a stimulatory guanine nucleotide binding protein (G_s; Gilman, 1987). Prein-

TABLE 2. *Effects of (A) inhibitors of cAMP phosphodiesterase, (B) cell-permeant cyclic nucleotide analogs, and (C) CT on carbachol-stimulated ^{3}H-IP release and inositol lipid labeling*

Agent	Inhibition (%) IP	Inositol lipids
(A)		
IBMX, 1 mM	51 ± 3 (15)	23 ± 5 (12)
Theophylline, 2 mM	70 ± 2 (6)	59 ± 8 (3)
(B)		
dBcAMP, 1 mM	11 ± 5 (11)	10 ± 5 (11)
8BcGMP, 1 mM	−12 ± 3 (4)	−2 ± 2 (3)
(C)		
CT, 50 μg/ml		
5 h	45 ± 10 (3)	42 ± 11 (2)
24 h	37 ± 4 (7)	33 ± 4 (3)

See footnote to Table 1 for experimental details.

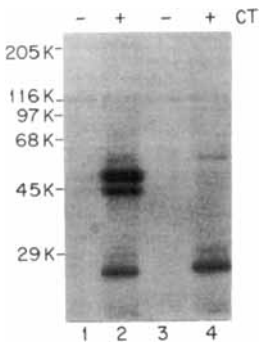

FIG. 4. [^{32}P]ADP-ribosylation of control or CT-pretreated cells by CT. Intact SK-N-SH cells were pretreated in the absence or presence of CT (50 μg/ml) for 24 h. Membrane preparations were then exposed to [^{32}P]NAD and activated CT for 30 min, as described in Materials and Methods. Samples were then electrophoresed on SDS-PAGE. Lanes 1 and 2, control cells; lanes 3 and 4, CT-pretreated cells. +/−CT, membranes incubated in presence or absence of CT. Note the absence of labeling of the 42- and 46-kilodalton bands in lane 4, indicating that previous ADP-ribosylation with endogenous NAD had occurred.

cubation of intact cells with CT (50 μg/ml) for either 5 h or 24 h inhibited carbachol-stimulated ^{3}H-IP release by 37–45%, whereas basal ^{3}H-IP release remained unaffected. Carbachol-stimulated, but not basal, phospholipid labeling was also inhibited by CT pretreatment (Table 2, part C). When IBMX (1 m*M*) was added to CT-pretreated cells, there was a further increase in the inhibition of carbachol-stimulated ^{3}H-IP release (61 ± 5%, n = 3). However, the sum of CT- and IBMX-mediated inhibitions was consistently found to be less than additive ($p < 0.05$). Preincubation of SK-N-SH cells with CT prevented the subsequent [^{32}P]ADP-ribosylation of two membrane proteins of 42 and 46 kilodaltons (Fig. 4), indicating that ADP-ribosylation with endogenous NAD had occurred. Furthermore, in three separate experiments either a 5-h or 24-h pretreatment of SK-N-SH cells with CT reduced (by 84–89%) the ability of PGE_1 (an agonist that couples to adenylate cyclase activation through G_s) to increase cAMP concentration. Whereas a 5-h CT pretreatment increased cAMP concentration to 145–187% of control, this increase was not observed following a 24-h pretreatment (112 ± 10% of control, n = 6). This may be attributable to an efflux of cAMP from cells that occurred during the prolonged incubation, as previously noted for bovine chromaffin cells (Chern et al., 1988).

Inhibition of stimulated PPI turnover by cAMP in permeabilized cells

To probe the site of action of cAMP on stimulated PPI turnover, digitonin-permeabilized SK-N-SH cells were employed. The addition of carbachol or GTPγS to permeabilized cells elicited a large increase in the release of ^{3}H-IP, as previously observed (Fisher et al., 1989). Overnight preincubation with CT (50 μg/ml) or dBcAMP (1 m*M*) prior to cell permeabilization reduced ^{3}H-IP release elicited by carbachol, GTPγS, or their combination, whereas basal ^{3}H-IP release was unaffected (Fig. 5). Although CT was markedly more effective than dBcAMP (28–73% versus 15–44%), the ability of both agents to inhibit significantly GTPγS-stimulated ^{3}H-IP release suggests that a primary site of action of cAMP occurs at or downstream from the G_p–phospholipase C interaction. Because basal ^{3}H-IP release was unaffected by pretreatment with CT or dBcAMP, a direct action of cAMP on phospholipase C activity appears unlikely. The ability of dBcAMP to inhibit PPI turnover in permeabilized cells following a 16-h incubation, in contrast to its relative ineffectiveness in short-term incubations (see Table 2, part B), suggests that it may not readily penetrate intact SK-N-SH cells.

In addition to its ability to increase the cAMP content of cells, CT is also known to inhibit stimulated PPI turnover through its interaction with a G_p that mediates phospholipase C activation (Lo and Hughes, 1987; Schnefel et al., 1988). To evaluate this possibility, labeled SK-N-SH cells were first permeabilized, pretreated for 30–60 min in the absence or presence of 50 μg/ml of activated CT, and then incubated with carbachol or GTPγS for another 15 min. Preincubation with CT did not inhibit either carbachol- or GTPγS-stimulated ^{3}H-IP release, even though ADP-ribosylation of membrane proteins was detected under these conditions. Thus, either G_p in SK-N-SH cells is not a substrate for CT, or alternatively, a more extensive incubation is necessary for ADP-ribosylation of G_p.

Effect of the agonist PGE_1 on carbachol-stimulated ^{3}H-IP release

The addition of agents that increase cAMP concentrations through mechanisms other than receptor activation results in an inhibition of mAChR-stimulated PPI hydrolysis in SK-N-SH cells. To determine whether an increase in cAMP concentration elicited by the ad-

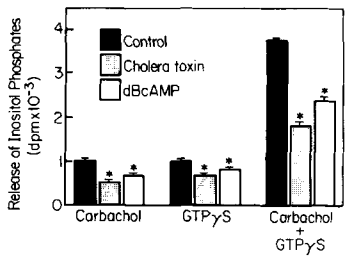

FIG. 5. Pretreatment with either CT or dBcAMP reduces the ability of both the agonist carbachol and GTPγS to stimulate ^{3}H-IP release from permeabilized SK-N-SH cells. Cells that had been prelabeled with [^{3}H]inositol for 3 days were treated with either CT (50 μg/ml), dBcAMP (1 m*M*), or buffer (control) for the last 16 h of labeling. Cells were then detached, permeabilized with digitonin, and incubated for 15 min at 37°C in the absence or presence of carbachol (10 m*M*), GTPγS (50 μ*M*), or both. *Significantly different from control, $p < 0.05$. Results shown are means ± SEM for triplicate samples from a single experiment. Values for basal ^{3}H-IP release in the experiment shown were 282 ± 25, 274 ± 15, and 276 ± 14 dpm for control, CT- and dBcAMP-pretreated cells, respectively. The concentration of Ca^{2+} was maintained at 60 n*M* as previously described by Fisher et al. (1989). In three separate experiments pretreatment of SK-N-SH cells with CT reduced the carbachol, GTPγS, or carbachol plus GTPγS-stimulated release of ^{3}H-IP by 51 ± 6, 59 ± 9, and 47 ± 9%, respectively. The corresponding values obtained following pretreatment with dBcAMP were 27 ± 9, 23 ± 2, and 26 ± 7%, respectively.

dition of an agonist can produce a similar inhibition, the effect of PGE_1 on carbachol-stimulated ^{3}H-IP release was studied. Addition of 30 μM PGE_1 increased cAMP concentrations to 208 ± 34% of control in the absence of 1 mM IBMX (n = 3), and to 2,768 ± 296% of control in the presence of IBMX (n = 9). These increases in cAMP concentration were not, however, accompanied by a significant inhibition of stimulated PPI turnover. Thus, the addition of PGE_1 alone did not inhibit carbachol-stimulated ^{3}H-IP release (−5 ± 9%, n = 5), whereas in the presence of IBMX, PGE_1 addition resulted in a 57 ± 6% inhibition (n = 5), a value similar to that obtained for IBMX alone (51 ± 3%, see Table 2, part A). PGE_1 had no effect on basal ^{3}H-IP release. Thus, PGE_1-mediated increases in cAMP do not appear to result in an inhibition of stimulated PPI turnover.

DISCUSSION

The principal conclusion to emerge from the present study is that an increased intracellular concentration of cAMP elicited by agents that act at either the level of G_s, adenylate cyclase, or cAMP phosphodiesterase results in an inhibition of stimulated PPI turnover in SK-N-SH neuroblastoma. The evidence in favor of this conclusion is threefold. First, the addition of CT, an agent known to ADP-ribosylate the α-subunit of G_s and cause its permanent activation (Gilman, 1987), resulted in a marked inhibition of carbachol-stimulated ^{3}H-IP release. Second, inclusion of either FSK, or its water-soluble analog, L-85,8051, both of which directly stimulate the catalytic unit of adenylate cyclase (Seamon and Daly, 1986; Laurenza et al., 1987), also resulted in an inhibition of stimulated inositol lipid hydrolysis. In contrast, the inactive analog of FSK, 1,9-dideoxy FSK (Joost et al., 1988), had little or no effect on stimulated PPI turnover. Third, although relatively low resting levels of cAMP are present in SK-N-SH cells, inhibition of cAMP phosphodiesterase by inclusion of agents such as IBMX or theophylline resulted in increased cAMP concentrations and also inhibition of stimulated PPI turnover.

When SK-N-SH neuroblastomata were extensively pretreated with either CT or dBcAMP, the ability of either carbachol or GTPγS to stimulate PPI turnover in digitonin-permeabilized cells was reduced to a similar extent (Fig. 5). This result indicates that the primary inhibitory site of action of cAMP resides either at or distal to the G_p–phospholipase C interaction, rather than at the level of the mAChR or mAChR–G_p coupling. cAMP has also recently been proposed to inhibit the activation of phospholipase C by G_p in platelets (Yada et al., 1989).

The ability of cAMP to inhibit stimulated PPI turnover has previously been observed for platelets (de Chaffoy de Courcelles et al., 1986), neutrophils (Takenawa et al., 1986; Della Bianca et al., 1986), kidney (Neylon and Summers, 1988), smooth muscle (Madison and Brown, 1988), lymphocytes (Lerner et al., 1988), and adrenal glomerulosa cells (Guillon et al., 1988). In contrast, increases in cAMP concentration do not appear to inhibit stimulated PPI turnover in either hepatocytes (Kaibuchi et al., 1982) or in cerebral cortex (Hollingsworth and Daly, 1985). Despite the latter observation, our results with SK-N-SH cells (and recent observations with NG 108-15 cells; Campbell et al., 1988) suggest that inositol lipid hydrolysis may be subject to regulation by the cyclic nucleotide in at least some neural tissues.

Although inhibition of stimulated PPI turnover has been observed following receptor-mediated activation of adenylate cyclase (Kaibuchi et al., 1982; Della Bianca et al., 1986; de Chaffoy de Courcelles et al., 1986), most of the evidence implicating a regulatory role for cAMP in inositol lipid hydrolysis has been obtained from the use of agents that increase cAMP concentrations independently of receptor activation. An unexpected finding from the present study was that the increases in cAMP mediated by PGE_1 (in contrast to those generated through receptor-independent mechanisms) were not accompanied by an inhibition of carbachol-stimulated ^{3}H-IP release in SK-N-SH cells, even though the intracellular concentrations of cAMP were increased by up to 28-fold in the presence of the agonist. Conceivably, two explanations could account for this paradoxical result. First, it is possible that the rise in cAMP concentration and inhibition of stimulated PPI turnover observed in the presence of CT, IBMX, and FSK are parallel but unrelated events. For example, in addition to its known ability to inhibit cAMP phosphodiesterase, IBMX can block the inhibitory guanine nucleotide binding protein, G_i (Parsons et al., 1988), and (like FSK) can directly inhibit the glucose transporter system in adipocytes (Kashiwagi et al., 1983). Although the possibility of such a *direct* inhibitory action of these agents on stimulated PPI turnover in SK-N-SH cells cannot be excluded, evidence in favor of an intermediary role for cAMP in the inhibition can be summarized as follows: (1) although CT, FSK, and IBMX have distinctly different proposed modes of action on the adenylate cyclase system, they affect cAMP content and stimulated PPI turnover in a similar manner; (2) when two of the agents are combined, e.g., CT and IBMX or FSK and IBMX, the resulting inhibition is less than additive, a result indicative of a common site of action for these agents; and (3) the specificity with which the FSK analogs inhibit PPI turnover mirrors their ability to activate adenylate cyclase. An alternative explanation for the inability of PGE_1 to inhibit PPI turnover may lie in the compartmentation of cAMP within SK-N-SH neuroblastoma, such that the cellular concentration of cAMP attained in the presence of the agonist does not necessarily reflect its concentration in the biologically relevant compartment. Evidence to suggest the compartmentalization of both cAMP (and cAMP-dependent protein kinase) and inositol trisphosphate has been reported for a number of

tissues (Spearman and Butcher, 1982; Merritt et al., 1986; Ambler et al., 1987). A further indication that separate metabolic compartments may exist for these two second messenger systems within the same cell was recently obtained from studies of tracheal smooth muscle. In these cells, agents that elevate cAMP result in an inhibition of histamine-stimulated PPI turnover, but not that mediated by mAChR activation (Madison and Brown, 1988). Although an explanation for the inability of PGE_1 to inhibit carbachol-stimulated PPI turnover in SK-N-SH cells is not yet apparent, our results nonetheless stress the need to evaluate the effects of *both* receptor-mediated and -independent increases in cAMP when assessing the regulatory role of this cyclic nucleotide on inositol lipid hydrolysis.

The observation that increases in cAMP can inhibit stimulated PPI turnover in some tissues has led to speculation over the possible physiological significance of such a "cross-talk" between the two signaling mechanisms (Nishizuka, 1986; Jakobs et al., 1986). Although this remains an attractive hypothesis, two considerations are pertinent when evaluating this possibility in SK-N-SH cells. First, as previously noted, there are differential effects of increased cAMP concentrations on stimulated PPI turnover dependent on the agent employed. Second, in SK-N-SH cells the relationship between inositol lipid hydrolysis and adenylate cyclase may be additionally complex, because activation of the M_3 mAChRs on these cells also results in a secondary *increase* in cAMP concentration (Baumgold and Fishman, 1988; Baron and Siegel, 1988). Thus, although it is evident that increases in cAMP mediated by the addition of certain agents can inhibit stimulated PPI turnover in SK-N-SH cells, the physiological significance of this inhibition remains to be determined.

In summary, the present results indicate that increases in cAMP concentration mediated by agents that act independently of receptor activation inhibit mAChR-stimulated PPI hydrolysis in human SK-N-SH neuroblastoma cells. These results point to the possibility that stimulated inositol lipid hydrolysis may be similarly affected by cAMP in other neural preparations.

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