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Differential Sensitivity of Phosphoinositide Metabolism to Sodium Fluoride and Carbachol Treatments in PC12 Cells[1]

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Exposure to sodium fluoride (NaF) resulted in an increased accumulation (up to 10-fold) of total [^{3}H]inositol phosphates (T-InsP) in rat PC12 cells. The magnitude of the NaF effect was comparable to that for muscarinic acetylcholine receptor-mediated stimulation of T-InsP accumulation in the presence of saturating concentrations of carbachol, but effects of NaF and muscarinic agonists were additive at subsaturating concentrations. The NaF effect was atropine insensitive; was not mimicked by effects of NaCl (10 m*M*), aluminum fluoride (1 to 100 μM), forskolin (up to 100 μM), or dibutyryl cyclic AMP (1 m*M*); and was not altered by treatment with pertussis or cholera toxins (1 μg/ml for 24 h). By contrast, the carbachol response was fully sensitive to atropine and partly sensitive to pertussis toxin. Chelation of extracellular calcium ion following 10 min of pretreatment with EDTA or EGTA (3 m*M*) inhibited carbachol-stimulated T-InsP accumulation by 50%, but resulted in an enhancement of NaF-stimulated T-InsP accumulation. By contrast, inhibition of the mobilization of intracellular calcium ion with 8-(*N,N*-diethylamino)octyl-3,4,5-trimethoxybenzoate inhibited NaF stimulation of T-InsP accumulation by more than 50% but inhibited carbachol-stimulated T-InsP accumulation to a much lower extent. Enhanced calcium influx and cell depolarization stimulated by high extracellular concentrations of KCl markedly potentiated carbachol, but not NaF, stimulation of T-InsP accumulation. This differential sensitivity to muscarinic antagonists, cell depolarization, and manipulation of intra- and extracellular calcium ion indicates that different mechanisms underly NaF and carbachol stimulation of T-InsP accumulation. However, stimulation of T-InsP accumulation in the presence of carbachol alone, NaF alone, or carbachol plus NaF was inhibited to a similar extent in the presence of the phorbol ester, phorbol 12-myristate-13-acetate. Taken together, these observations suggest that NaF and carbachol effects are mediated through distinct mechanisms but share a common target, perhaps a GTP-binding protein and/or phospholipase C, whose activity is known to be influenced by protein kinase C.

INTRODUCTION

There is substantial evidence that agonist-stimulated second messenger responses are mediated via specific interactions between liganded metabotropic receptors, associated guanine nucleotide binding proteins (G proteins), and target enzymes (1–3). For example, stimulation of polyphosphoinositide metabolism is mediated through G proteins (G_p) that activate phospholipase C to produce at least two second messengers: inositol triphosphate ($InsP_3$) and diacylglycerol. Among the tools commonly used to determine whether G proteins are involved in the mediation of second messenger signaling is sodium fluoride (NaF), which has been shown to modulate the activity of adenylate cyclase and the production of cyclic AMP through its interaction with the stimulatory and inhibitory G proteins, G_s and G_i. There is evidence that NaF can also modify agonist-stimulated formation of inositol phosphates in various preparations. These effects sometimes have been attributed to interaction of NaF with G proteins (4–6). However, other studies (7, 8) have reported that sodium fluoride activates polyphosphoinositide hydrolysis by a mechanism distinct from those involving G proteins.

In preliminary studies assessing roles of G proteins in the coupling of muscarinic acetylcholine receptors (mAChR) to phospholipase C, we have found that NaF enhances resting and carbachol-stimulated total tritiated inositol phosphate (T-InsP) accumulation in rat pheochromocytoma PC12 cells (9). The current report extends these observations. It suggests that the NaF effect is not directly mediated through receptor–G protein interactions, but does share with receptor–G protein-mediated phosphoinositide metabolism a common target that is sensitive to the activity of protein kinase C.

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MATERIALS AND METHODS

Cell Culture

The rat pheochromocytoma PC12 (obtained from Dr. D. Schubert, Salk Institute) is a continuous clonal cell line of neural crest origin derived from a tumor of the rat adrenal medulla (10). Cells were maintained as previously described (11). Briefly, cells were cultivated in proliferative growth in Dulbecco's modified Eagle's medium buffered with sodium bicarbonate and containing 4 m*M* glutamine, 4.5 g/liter glucose, 10% horse serum, 5% fetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml) and amphotericin B (2 μg/ml). For the assays conducted in this study, cells were seeded routinely at initial plating densities of 20 to 500 cells/mm^2 in 15.5-mm-diameter dishes.

Measurement of Inositol Phosphates

Quantitation of inositol phosphates was performed on cells grown for 2 days to a final density of 10^6 cells/15-mm well in 1 ml of standard medium supplemented with serum and antibiotics (see above). Metabolic labeling of inositol phosphates was initiated by removal of spent medium and replacement with otherwise identical fresh medium supplemented with ^{3}H-labeled *myo*-inositol to achieve a final specific activity of 1 μCi/ml (1 μCi per dish, 40 Ci/mmol). Dishes were returned to the incubator (37°C, 5% CO_2, humidified atmosphere) for 48 h. It is during the last 24 h of this ^{3}H-labeled *myo*-inositol loading period that drugs such as pertussis and cholera toxins (see figure legends) were exposed to cells at final concentrations of 1 μg/ml. Cells were then washed three times with incubation medium (118 m*M* NaCl, 4.7 m*M* KCl, 3 m*M* $CaCl_2$, 1.2 m*M* $MgSO_4$, 1.2 m*M* KH_2PO_4, 0.5 m*M* EDTA, 10 m*M* glucose, and 20 m*M* Hepes, pH 7.4) and thereafter incubated for 30 min with medium containing 10 m*M* LiCl to inhibit inositol monophosphatases. It is during the last 10 min of the LiCl preincubation period that drugs such as EGTA, EDTA, or 8-(*N,N*-diethylamino)octyl-3,4,5-trimethoxybenzoate (TMB-8) were added. Drugs such as aluminum fluoride, NaCl, forskolin, phorbol 12-myristate-13-acetate (PMA), or dibutyryl cyclic AMP (dbcAMP) were added to cell cultures in the last minute of LiCl preincubation to achieve the final concentrations indicated in the table and figure legends to test their effects on subsequent activation of phosphoinositide metabolism. Other drugs, generally carbachol and/or NaF at the indicated final concentrations, were then added for specified periods of time (from 15 s to 60 min) to initiate phosphoinositide turnover. Reactions were terminated by removal of medium and addition of 2 ml of 10% (wt/vol) trichloroacetic acid (TCA). Cells were harvested by scraping the dishes with a rubber policeman, and the samples were collected in a glass centrifuge tube. Samples were then sonicated for 10 s, and TCA was removed by four cycles of diethyl either extraction (2 vol). Samples were then neutralized with sodium tetraborate using phenol red as a pH indicator.

Water-soluble inositol phosphates were separated on small columns of Dowex-1X8 (200-mesh formate form) that were "preconditioned" by rinses with 2 *M* formic acid (FA)/1 *M* ammonium formate (AF). The sample containing phosphate esters was applied to the column, and [^{3}H]inositol derivatives were eluted by a modification of a method previously described (12–15). Following two rinses with H_2O and an additional column wash with 50 m*M* AF/5 m*M* sodium tetraborate, the major inositol phosphate metabolites, inositol monophosphates ($InsP_1$), inositol bisphosphates, and $InsP_3$ isomers, were eluted sequentially with 0.2 *M* AF/0.1 *M* FA, 0.4 *M* FA/0.1 *M* AF, and 1 *M* AF/0.1 *M* FA, respectively. Each of these steps was followed by an additional wash with an equal volume of the corresponding buffer to elute the last traces of inositol phosphate. Aliquots were taken and counted after addition to liquid scintillation cocktail. Our earliest studies indicated that accumulation of T-InsP under the conditions used in this study is dominated by accumulation of $InsP_1$ (>90% of T-InsP). Therefore, some experiments report data as accumulation of T-InsP and others as $InsP_1$ production only, but the results obtained using either measure of phosphoinositide metabolism were equivalent. Nevertheless, some studies were conducted to quantitate production of $InsP_3$ isomers.

RESULTS

Inositol Phosphates Accumulation in Response to Sodium Fluoride Treatment

PC12 cells exposed to sodium fluoride (10 m*M*) exhibit an accumulation of $InsP_1$ that increases linearly with time of exposure to NaF (Fig. 1). This effect is concentration dependent, with an EC_{50} for NaF of about 5 m*M*, and reaches a maximum at 20 m*M* (Fig. 2). For treatment of cells at higher concentrations of NaF, T-InsP levels fall below peak values (Fig. 2). The NaF effect is additive with carbachol stimulation of mAChR-mediated T-InsP accumulation for concentrations of NaF below 10 m*M* (Fig. 3), but at higher concentrations of NaF, the response saturates and there is no further increase in T-InsP accumulation in PC12 cells also exposed to increasing concentrations of carbachol (Fig. 3). Other studies indicate that treatments with NaF or carbachol produce early and quantitatively comparable increases in $InsP_3$ levels (2- to 3-fold increase over control levels; Table 1) as well as the more steady accumulation of $InsP_1$ (4- to 12-fold increase).

Pharmacological Distinction between NaF and Carbachol Responses

Atropine treatment has no effect on T-InsP accumulation alone or on NaF stimulation of T-InsP accumu-

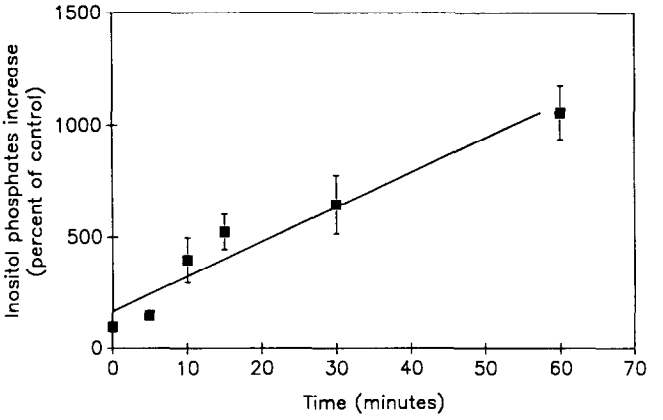

FIG. 1. Time course of inositol monophosphates accumulation in response to NaF treatment of PC12 cells. Cells labeled with ^{3}H-labeled *myo*-inositol as described under Materials and Methods were exposed for the indicated period of time (abscissa, minutes) to 10 m*M* NaF before the reaction was terminated with ice-cold TCA, and measurements of levels of $InsP_1$ were made following separation by anion exchange chromatography. Data are presented (ordinate) as the percentage of $InsP_1$ accumulation relative to control cells (identically seeded plates treated with vehicle alone). Control levels (100%) represent 347 ± 42 cpm. Data are means ± SEM (bars) for four experiments. The magnitude of inositol phosphates accumulation varied from 4- to 12-fold between experiments due to differences in "basal" levels of $InsP_1$ accumulation.

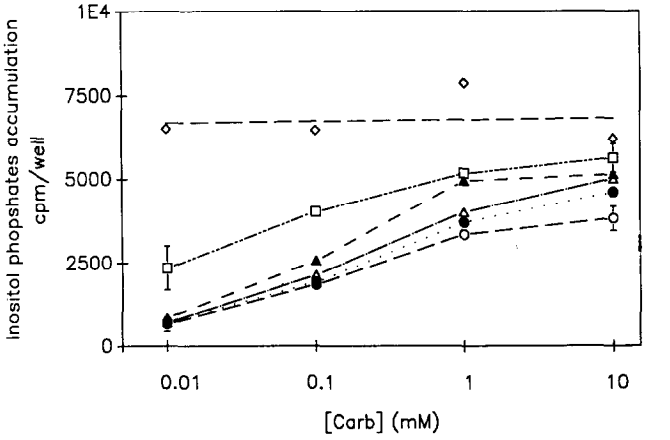

FIG. 3. Dose-dependence curves for carbachol- and NaF-stimulated accumulation of total tritiated inositol phosphates in PC12 cells. T-InsP accumulation (ordinate, cpm/well) is plotted as a function of carbachol concentration (abscissa, m*M*) for 60 min of treatment in medium also supplemented with NaF at 0.1 m*M* (○), 0.3 m*M* (●), 1 m*M* (△), 3 m*M* (▲), 10 m*M* (□), or 30 m*M* (◇). No NaF addition gave a control value of 689 cpm, which was not significantly different from that obtained for samples treated with 0.01 m*M* NaF alone. Although the results of a single experiment are shown, numerous replicates ($n > 3$) consistently demonstrated additivity of NaF and carbachol effects at concentrations of NaF below 20 m*M*, but achievement of a maximal response is invariably reached at NaF concentrations between 20 and 30 m*M*.

lation, but completely blocks carbachol-stimulated responses (Table 2). These results indicate that the NaF effect is not mediated indirectly by a secondary release of acetylcholine or directly at muscarinic receptors.

Fluoride Ion Specificity of NaF-Stimulated T-InsP Accumulation

Mechanisms by which sodium fluoride enhances T-InsP accumulation in PC12 cells were investigated. The results (Table 2) indicate that NaF-stimulated increases in T-InsP accumulation are not mimicked by NaCl or by lower concentrations of fluoride ion (from 10 to 100 μM) derived from aluminum fluoride. For example, AlF concentrations above 100 μM were cytotoxic, indicating that comparable concentrations of aluminum ion could not have been present as a significant contaminant in NaF, which is without effect on cell survival. These and other studies with NaF stocks of different purity (from 99 to 99.99%) indicate that NaF effects are not attributable to an aluminum impurity, but are most likely due to effects of fluoride ion.

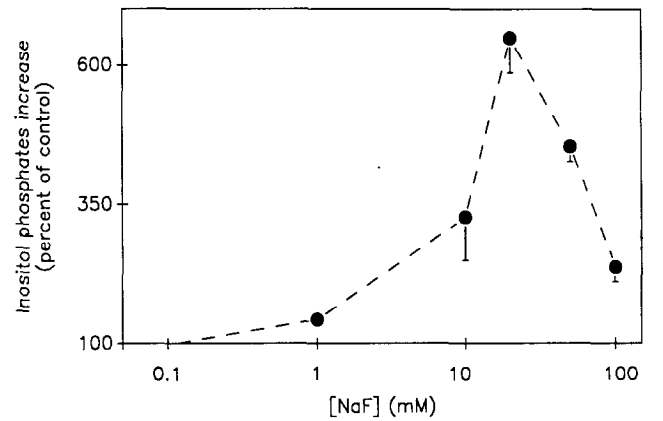

FIG. 2. NaF concentration dependence for total tritiated inositol phosphates accumulation. T-InsP accumulation (ordinate, percentage of control) was determined for PC12 cells (●) following 60 min of incubation in the presence of NaF at the indicated concentrations (abscissa, m*M*). Basal levels were 362 ± 11 cpm. Data are means ± SEM of triplicates. *$P < 0.005$ for [NaF] ⩾ 10 mM (paired comparisons test).

Is There Involvement of a G-Protein in the NaF Effect?

The possibility that NaF effects are mediated through a G_s-like G protein was examined in cells preincubated

TABLE 1

Inositol Triphosphates Levels Following NaF or Carbachol Treatments

Control	70 ± 07
Carbachol	274 ± 39*
NaF	180 ± 15*

Note. Inositol triphosphates levels were measured as described (see Materials and Methods) following exposure of PC12 cells for 60 min to carbachol (1 m*M*) or NaF (20 m*M*). Levels are expressed in cpm/10^6 cells and represent the mean ± SEM of triplicates or quadruplicates from three different experiments. *$P < 0.01$ when compared to control and not significantly different from each other.

TABLE 2

Effects of Drug Treatments on T-InsP Accumulation

Drug treatment	T-InsP accumulation (% of control)	Drug treatment	T-InsP accumulation (% of control)
Agonist responses		*Depolarization/Ca^{2+}*	
Control	100	KCl	99 ± 07
Carb	815 ± 123	KCl + Carb	1418 ± 166
NaF	831 ± 75	KCl + NaF	980 ± 104
NaF + Carb	1066 ± 14	PMA	87 ± 15
		Carb + PMA	450 ± 37
Muscarinic antagonists		NaF + PMA	589 ± 42
Atropine	101 ± 06		
Carb + atropine	110 ± 15	*cAMP and G protein targets*	
NaF + atropine	780 ± 75	FSK	114 ± 11
		dbcAMP	135 ± 28
Fluoride specificity		CTx	121 ± 23
		CTx + Carb	734 ± 106
NaCl	102 ± 08†	CTx + NaF	910 ± 64
AlF 10 μM	145 ± 47	PTx	70 ± 10
AlF 100 μM	91 ± 30	PTx + Carb	532 ± 178
		PTx + NaF	826 ± 139

Note. T-InsP accumulation (percentage of control; cells treated with vehicle alone) in response to treatment for 60 min with 1 mM carbachol (Carb), 10 mM sodium fluoride (NaF), 5 μM atropine, 10 mM sodium chloride (NaCl), 10–100 μM AlF, 100 mM potassium chloride (KCl), 1 μM phorbol 12-myristate-13-acetate (PMA), 100 μM forskolin (FSK), or 1 mM dibutyryl cyclic AMP (dbcAMP), alone or in different combinations, as indicated. Drugs were applied without or after 24 hr of pretreatment with 1 μg/ml of pertussis toxin (PTx) or cholera toxin (CTx). Results are presented as means ± SEM of 3 to 10 experiments except where the dagger indicates that the value provided reflects the range of two experiments performed in duplicate.

with cholera toxin, which is known to directly activate G_s. Cholera toxin treatment alone results in no change in T-InsP accumulation and does not modify carbachol- or NaF-stimulated responses (Table 2). We have previously shown that levels of endogenous cyclic AMP in some cells are greater in the presence of NaF than in the presence of cholera toxin (15), which raised the possibility that NaF effects on T-InsP accumulation might be mediated indirectly through increased production of cyclic AMP. However, treatment of cells with the diterpene, forskolin, which activates adenylate cyclase directly, produces no significant change (14%) in T-InsP accumulation in contrast to the marked increase (731%) in T-InsP accumulation observed on NaF treatment (Table 2). Other studies show that dbcAMP treatment is also without effect on T-InsP accumulation (Table 2). Therefore, increases in cyclic AMP production cannot account for the marked increase in T-InsP accumulation observed following exposure to NaF. Preincubation of cells with pertussis toxin does not mimic or prevent the NaF response but induces a moderate decrease in carbachol-stimulated T-InsP accumulation (Table 2).

Effects of Intra- and Extracellular Calcium Ion Modulation on T-InsP Accumulation Stimulated by Carbachol and NaF

The role of calcium ion in the mediation of carbachol and/or NaF-stimulated T-InsP accumulation was first tested by chelating extracellular calcium ion with EGTA or EDTA (3 mM). The carbachol response is inhibited from 400% of control values to 165% when EGTA is present in the culture medium. By contrast, the NaF response increases modestly but significantly from 358 to 441% (Fig. 4). Control levels of T-InsP in PC12 cells are unaffected by EGTA treatment, and similar results were observed with EDTA treatment (data not shown). Inhibition of the mobilization of intracellular calcium ion with TMB-8 fails to affect either resting or carbachol-stimulated accumulation of T-InsP (Fig. 5). By contrast, the NaF-stimulated response is inhibited in a concentration-dependent manner with 64% inhibition occurring at 50 μM TMB-8. At higher concentrations of TMB-8 (100 μM), the inhibitory effect is more pronounced, but interpretation of the results is more complicated since these concentrations of TMB-8 also produce decreases in "basal" T-InsP accumulation. NaF addition results in an enhancement of carbachol-stimulated T-InsP accumulation, and the extent of this enhancement is also inhibited by 68% in the presence of 50 μM TMB-8 (Fig. 5). Cell depolarization (50–100 mM KCl) alone fails to affect basal levels or NaF-stimulated accumulation of T-InsP (Table 2), but significantly potentiates (by 70%) carbachol-stimulated T-InsP accumulation (Table 2).

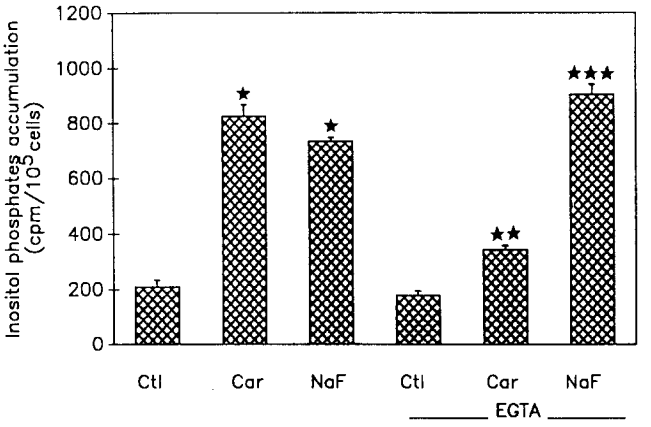

FIG. 4. Effects of chelation of extracellular calcium ion on total inositol phosphates accumulation. T-InsP accumulation (ordinate; cpm per 10^5 cells) is shown for PC12 cells exposed for 60 min to vehicle alone (Ctl), 1 mM carbachol (Car), or 10 mM sodium fluoride (NaF) or under the same conditions following 10 min of preincubation with 3 mM EGTA (histograms above horizontal, labeled bar). Similar results are observed with EDTA pretreatment. Values are means ± SEM from greater than three experiments each performed in duplicate or triplicate. *$P < 0.005$ vs control; **$P < 0.005$ vs control and $P < 0.025$ vs carbachol; ***$P < 0.01$ vs NaF. Statistical significance was established by paired comparisons test.

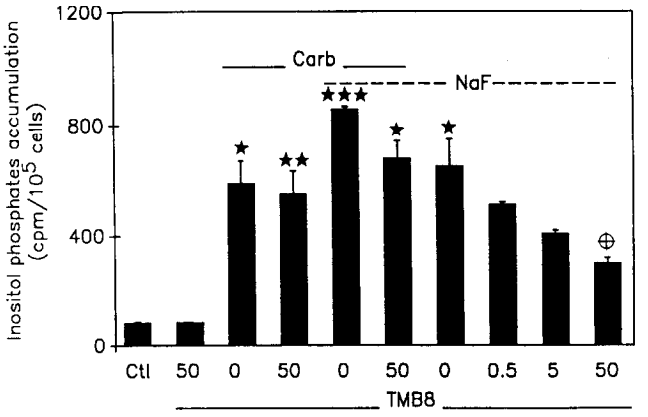

FIG. 5. Effect of inhibition of intracellular calcium ion mobilization on total inositol phosphates accumulation. T-InsP accumulation (ordinate; cpm per 10^6 cells) is shown for PC12 cells exposed (presence of drugs is indicated by horizontal lines and labels on the figure or below the abscissa) to 1 m*M* carbachol (Carb), 10 m*M* sodium fluoride (NaF), and/or TMB-8 (at concentrations in μM indicated on the abscissa). Values represent means ± SEM of two to six experiments. $^*P < 0.005$ vs control; $^{**}P < 0.05$ Carb + TMB-8 vs control and $P < 0.025$ Carb + TMB-8 vs Carb; $^{***}P < 0.005$ vs control and $P < 0.025$ vs Carb; $^{\oplus}P < 0.01$ NaF + TMB-8 vs NaF. Paired comparisons test was used to determine statistical significance.

Effects of C-Kinase Activation on Carbachol- and NaF-Stimulated T-InsP

Acute treatment of cells with PMA, which is presumed to cause stimulation of protein kinase C activity, inhibits carbachol- or NaF-stimulated T-InsP accumulation to approximately the same degree (Table 2).

DISCUSSION

The findings of this study are (1) that sodium fluoride treatment stimulates the accumulation of T-InsP in rat PC12 cells; (2) that the NaF effect is additive with stimulation of T-InsP accumulation via mAChR when NaF and carbachol are applied at subsaturating doses, but that the accumulation of T-InsP saturates at high concentrations of each agent; (3) that the NaF effect does not appear to be mediated through a G protein that couples stimulatory receptors to adenylate cyclase (see below) or accumulation of endogenous cyclic AMP; (4) that T-InsP accumulation stimulated by NaF or by carbachol exhibits opposite sensitivity to chelation of extracellular calcium ion, to inhibition of intracellular calcium ion mobilization, or to depolarization-induced calcium entry; and (5) that NaF and carbachol effects show the same sensitivity to modulation of protein kinase C activity. The major conclusions drawn from these observations are (i) that activation of phosphoinositide metabolism by treatment with NaF or carbachol occurs via different pathways and requires calcium ions originating from different pools, but (ii) both pathways intersect at a common target that is sensitive to activity of protein kinase C, possibly phospholipase C and/or a GTP-binding protein coupled to it.

Several other studies have shown that NaF treatment enhances inositol phosphate accumulation (4–6, 8). Our results support those findings. However, these earlier reports assumed that NaF effects on phosphoinositide metabolism are mediated through a G protein (5, 7) based on the finding that NaF stimulates adenylate cyclase activity by interaction with N_s (G_s) (16). Our findings are not fully compatible with such a process, since the described NaF effects are not mimicked by aluminum ion as aluminum fluoride and are neither mimicked nor blocked by cholera or pertussis toxins. By contrast, pertussis toxin inhibits carbachol T-InsP accumulation as has been found in previous reports using SH-SY5Y cells (17) and PC12 cells (18). Furthermore, an indirect effect via NaF-stimulated increases in endogenous cyclic AMP is not tenable because the NaF-stimulated increase in T-InsP accumulation is not mimicked by forskolin or dbcAMP treatments (Table 2). Thus, the NaF response does not appear to involve G_s or G_i.

Another recent study has shown that NaF-stimulated T-InsP accumulation in brain cortical membranes is sensitive to calcium ion, is not affected in the presence of guanosine 5′-*O*-thiodiphosphate, and occurs via a different mechanism than a carbachol-stimulated response that exhibits opposite ion/drug sensitivity (8). Our data using the PC12 cell line substantiates the conclusion that different mechanisms underlie accumulation of T-InsP stimulated by NaF or by carbachol, but we find that the carbachol-stimulated response is inhibited and the NaF response is slightly potentiated in the presence of EGTA (or EDTA, i.e., in the absence of extracellular calcium ion). We also show that exposure of cells to high extracellular KCl (which should promote external calcium ion entry) enhances the carbachol effect but has no effect on NaF-stimulated T-InsP accumulation and that the carbachol-stimulated response is unaffected by the inhibition of intracellular calcium ion mobilization (TMB-8 treatment), whereas the NaF effect is markedly inhibited. Thus, the current results suggest that NaF stimulation of T-InsP accumulation involves initially the mobilization of calcium ion from intracellular stores, which would be consistent with several reports indicating that levels of free, cytoplasmic calcium ion are enhanced in the presence of NaF (19–21).

Recent studies using different cell types (22) suggest that there are complex relationships between agonist-stimulated accumulation of inositol phosphates and temporal and spatial features of agonist-specific cytoplasmic calcium ion "signatures," which vary depending on whether cytoplasmic calcium ion activity is increased due to influxes from extracellular stores or mobilization of intracellular stores. Other studies demonstrate that NaF stimulation of phosphoinositide metabolism inhibits hormone release from secretory cells (21), suggesting that

the balance between effects of extracellular calcium ion entry into cells (which activates hormone release) and effects of mobilization of intracellular calcium ion (stimulated by NaF and acting as a negative feedback mechanism?) can have important influences on cell function. These relationships might be elucidated by an improved understanding of the precise temporal and causal relationships between the effects of NaF on intracellular calcium ion mobilization and on T-InsP accumulation. Clues to these relationships could also be provided by an improved understanding of the mechanism(s) by which chelation of extracellular calcium ion inhibits carbachol-stimulated T-InsP accumulation and potentiates the NaF effect.

The complexity of the phosphoinositide metabolic pathway allows one to envision how differential sensitivity to calcium ion of kinases and phosphatases acting on phosphatidylinositols (PtdIns) and inositol phosphates may not only contribute to feedback regulation of phosphoinositide metabolism, but may also afford means by which changes in calcium ion concentrations induced by non-receptor-mediated mechanisms, such as those that seem to be involved in the NaF effect, can affect accumulation of inositol phosphates (23). For example, NaF has been reported to stimulate phospholipase C and inhibit phosphatidylinositol kinase activity to promote direct formation of $InsP_1$ from PtdIns but to inhibit carbachol-stimulated phospholipase C activity under conditions of enhanced PtdIns kinase activity (24). However, such a mechanism does not appear to be involved in the effects observed in the present study, since NaF treatment alone produces increased accumulation of $InsP_3$ as well as increased $InsP_1$ production and augments, rather than inhibits, T-InsP accumulation in the presence of carbachol.

Several other hypotheses regarding the pathways and possible targets involved in the NaF effect are suggested by the present results. One hypothesis is that NaF treatment first stimulates mobilization of intracellular calcium ion, which then leads to G protein-independent stimulation of phospholipase C. This hypothesis is the simplest and is compatible with the striking calcium ion sensitivity of at least some phospholipase C subtypes (23), but does not discount the possibility that there is activation of some other calcium-sensitive step in the PtdIns turnover pathway. A second, alternative hypothesis is that NaF coordinately stimulates a G protein capable of initiating phospholipase C activity and mobilizes intracellular calcium to elevate cytoplasmic calcium ion activity required for full phospholipase C activation (or for activation of some other calcium-sensitive process). However, the hypothetical NaF-sensitive and phospholipase C-coupled G protein must be distinct from the pertussis toxin-sensitive G protein that is most immediately coupled to mAChR, as well as from G_s and G_i. A third possibility is that NaF stimulates mobilization of intracellular calcium, which then acts through an intervening, calcium-sensitive G protein to activate phospholipase C. However, evidence for the existence of such a calcium-sensitive G protein is presently lacking.

The current observation that carbachol-stimulated PtdIns turnover is inhibited by chelation of extracellular calcium ion but is enhanced during elevated extracellular potassium-induced cell depolarization not only distinguishes mechanisms involved in NaF and carbachol effects, but also provides insight into possible mechanisms of both carbachol and NaF responses. We favor a general hypothesis that carbachol simply activates a mAChR-coupled, pertussis toxin-sensitive G protein to initiate PtdIns hydrolysis, but that subsequent or concurrent elevation of cytoplasmic calcium ion levels is required to activate one or more calcium-sensitive steps in the metabolic pathway and to maximally stimulate PtdIns turnover. This hypothesis would explain how NaF treatment-induced mobilization of intracellular calcium augments PtdIns turnover at lower concentrations of carbachol, and it suggests that calcium ion originating from different pools can converge to "cross-modulate" PtdIns turnover. By its convergence at phospholipase C, this hypothesis also is compatible with the demonstrated equal sensitivity of NaF and carbachol effects to modulation of protein kinase C, which is known to have phospholipase C as a substrate (25).

In summary, our observations suggest that NaF activates phosphoinositide metabolism through a mechanism distinct from that involved in carbachol stimulation of T-InsP accumulation. The NaF and carbachol effects have divergent requirements for intra- or extracellular calcium ion but exhibit the same sensitivity to C-kinase activation. The rat pheochromocytoma cell line PC12 may be useful for studies involving mechanisms for activating phosphoinositide metabolism other than via direct, agonist–receptor interactions.

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