ACTIVATION OF PROTEIN KINASE C INHIBITS NaF-INDUCED INOSITOLPHOSPHOLIPID TURNOVER IN ISOLATED CANINE PARIETAL CELLS

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ABSTRACT

Effects of protein kinase C (PKC) activation by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) on NaF-induced inositolphospholipid metabolism were investigated in canine parietal cells. NaF dose-dependently stimulated the formation of [³H]inositolphosphates, which was characterized by a rapid generation of [³H]inositoltrisphosphate (IP₃) within 3 min. Furthermore, NaF elicited a rapid decrease in ³²P-labeling of phosphatidylinositol-4,5-bisphosphate (PIP₂) with a simultaneous increase in [³²P]phosphatidic acid (PA). Although TPA alone had no effect, it significantly inhibited the inositolphospholipid turnover enhanced by NaF. Since NaF is known to directly activate guanine nucleotide binding proteins (G proteins), PKC activation appears to inhibit inositolphospholipid turnover at a level between a G protein and phospholipase C-induced PIP₂ breakdown.

We have previously demonstrated that two acid secretagogues, carbachol and gastrin, stimulate inositolphospholipid turnover followed by translocation of protein kinase C (PKC) activity from the cytosol to membranes (6, 7). In addition to the stimulation of acid secretion by carbachol and gastrin, PKC activation is also involved in the mechanism that inhibits a signal transduction in canine parietal cells by attenuating inositolphospholipid turnover, indicating that this inhibition is at least partly due to down-regulation of muscarinic and gastrin receptors (6). However, accumulating evidence also suggests that PKC exerts an inhibitory action on receptor-stimulated inositolphospholipid turnover at more than one site (4, 5, 18).

On the other hand, recent studies have indicated that NaF can directly activate various G proteins including the one involved in phospholipase C activation with resultant phosphatidylinositol-4,5-bisphosphate (PIP₂) breakdown (3, 9, 13, 17). Thus, by using NaF, it is possible to activate the intracellular signal transduction system without involving the receptor. The present study was undertaken to examine whether the activation of PKC also exerts

an inhibitory action on canine parietal cells at a level distal to receptor activation upon agonist binding.

MATERIALS AND METHODS

Mucosal cells were dispersed from stripped canine fundus by sequential exposure to collagenase (0.35 mg/ml) and ethylene-diaminetetra-acetic acid (EDTA, 1 mM), and parietal cells were further enriched by centrifugal elutriation (7, 16).

To measure the formation of inositolphosphates, cells (4×10^6) were preincubated for 2 h at 37°C in 0.4 ml of HEPES-buffered Krebs-Ringer bicarbonate buffer, 1 mg/ml bovine serum albumin and 5.6 mM D-glucose, pH 7.4 (HKRBG), containing 5 μ Ci [³H]inositol (Amersham, Arlington Heights, IL, U.S.A.). At 110 min of incubation, lithium chloride was added to a final concentration of 10 mM. Further incubation was then initiated by adding various test agents (100 μ l), and after 30 min the incubation was terminated by adding 1.5 ml of chloroform:methanol (1:2). Water-soluble inositolphosphates were separated from [³H]insitol by

ion-exchange chromatography as described by Berridge *et al.* (2). In some experiments, cells were pre-labeled with [^3H]inositol for 4 h and incubated under test conditions for 3 min. The aqueous products were then separated on a Dowex-1 column into inositolmonophosphate (IP), inositolbisphosphate (IP₂), inositoltrisphosphate (IP₃), and inosoltetrakisphosphate (IP₄) (1, 7).

In the ^{32}P -labeling experiment, parietal cells (100×10^6) were preincubated for 2 h at 37°C in 10 ml of phosphate-free buffer (142 mM NaCl, 5.6 mM KCl, 2.2 mM CaCl_2 , 3.6 mM NaHCO_3 , 1 mM MgCl_2 , 5.6 mM D-glucose and 30 mM HEPES, pH 7.4) containing 1 mCi [^{32}P]orthophosphate. Cells were then washed three times and resuspended in phosphate-free buffer. Aliquots ($400 \mu\text{l}$) of the cell suspension (4×10^6 cells) were added to $100 \mu\text{l}$ of phosphate-free buffer with or without NaF (final concentration, 20 mM) and incubated for various periods at 37°C . The reaction was terminated by adding 1.5 ml of chloroform:methanol (1:2), and phospholipids were extracted (15), and separated by TLC using chloroform:methanol:acetone:acetic acid:water (40:14:13:12:8) as a solvent (10). To measure *de novo* synthesis of phospholipids, cells were incubated in phosphate-free buffer containing both test substances and [^{32}P]orthophosphate ($5 \mu\text{Ci}/2 \times 10^6$ cells) for 20 min, and phospholipids were then extracted and separated as described above.

For the measurement of cyclic AMP production, cells (10^6) were preincubated in 0.9 ml of HKRBG for 10 min at 37°C , various test materials ($100 \mu\text{l}$) were then added, and the incubation continued for another 10 min. Cyclic AMP produced was extracted and measured as described previously (8).

RESULTS AND DISCUSSION

NaF significantly and dose-dependently increased the formation of [^3H]inositolphosphates in parietal cells at concentrations ranging from 2 to 20 mM (Fig. 1). The maximum increase in [^3H]inositolphosphate formation induced by 20 mM NaF was $278 \pm 21\%$ of the basal value, which was higher than the increase produced by 10^{-4} M carbachol and 10^{-6} M pentagastrin (Table 1). Detailed analysis of the inositolphosphates by Dowex column chromatography showed a significant and rapid increase in labeled IP₃ and IP₂ within 3 min of incubation upon exposure to 20 mM NaF (Table 2). Moreover, NaF (10 mM) elicited a time-dependent increase in

Fig. 1 Effects of NaF on the production of [^3H]inositolphosphates in canine parietal cells. Cells (4×10^6) were incubated for 30 min in 0.5 ml HKRBG with or without NaF. Values are presented as means $\pm$ SEM from four separate experiments and are expressed as percentages of the control value. Each experiment consisted of triplicate samples. * $P < 0.01$ vs control

Table 1 Effects of TPA (10^{-7} M) on NaF-, Carbachol- and Pentagastrin-Induced Increase in [^3H]Inositolphosphate Production in Parietal Cells

Agents	TPA (10^{-7} M)		% Inhibition by TPA
	(-)	(+)	
NaF (20 mM)	$278 \pm 21^*$	$213 \pm 19^{**}$	23.4
Carbachol (10^{-4} M)	$236 \pm 20^*$	$136 \pm 12^{**}$	42.4
Pentagastrin (10^{-6} M)	$218 \pm 28^*$	$133 \pm 14^{**}$	39.0

After preincubation in the presence or absence of TPA (10^{-7} M) for 2 h, parietal cells were incubated for 30 min with respective test agents. Data represents means $\pm$ SEM from four separate cell preparations and are expressed as percentages of the control (no stimulant and without TPA pretreatment). Each experiment was done in triplicate. * $P < 0.01$ vs control; ** $P < 0.01$ vs respective values without TPA pretreatment.

^{32}P -labeling of [^{32}P]phosphatidic acid (PA) with a concomitant decrease in PIP₂-labeling that began as early as 10 sec of incubation (Fig. 2). In contrast, although histamine (10^{-4} M) significantly increased cyclic AMP production in parietal cells,

Table 2 Effects of NaF (20 mM) on the Production of IP, IP₂, IP₃ and IP₄ in Canine Parietal Cells

	IP	IP ₂	IP ₃	IP ₄
Control	965±102	288±43	168±28	102±18
NaF (20 mM)	1,125±156	422±46*	362±34*	130±21

After prelabeling with [³H]inositol for 2 h, the cells were incubated with or without NaF (20 mM) for 3 min. Values represent means±SEM from four separate experiments and are expressed as dpm/4×10⁶ cells. Each experiment was done in triplicate. *P<0.01 vs respective control values

NaF at concentrations between 2 and 20 mM did not influence cyclic AMP content (data not shown). These findings clearly indicate that NaF activates membrane phospholipase C in canine parietal cells, which results in a rapid breakdown of PIP₂ and production of IP₃. Since NaF has been reported to directly activate certain G proteins involved in phospholipase C activation in various cells (3, 9, 13, 17), it appears that NaF activates a G protein linked with phosphodiesteratic cleavage of polyphosphoinositide in canine parietal cells.

In a previous study, we found that activation of PKC by 12-O-tetradecanoylphorbol-13-acetate (TPA) attenuated not only carbachol- but also gastrin-induced polyphosphoinositide breakdown by down-regulating muscarinic and gastrin receptors in parietal cells (6). To examine whether the activation of PKC inhibits inositolphospholipid turnover via a mechanism other than receptor down-regulation, we investigated the effect of activation of PKC by TPA on NaF-induced polyphosphoinositide breakdown. Although TPA (10⁻⁹-10⁻⁷ M) alone had no effect on inositolphospholipid turnover (data not shown), it significantly and dose-dependently inhibited the NaF (20 mM)-induced increase in [³H]inositolphosphate formation (Fig. 3). Moreover, TPA (10⁻⁷ M) reduced the NaF (10 mM)-induced increase in *de novo* [³²P] PA synthesis, although 4β-phorbol (10⁻⁷ M), a phorbol compound with no tumor-promoting activity, had no effect (Fig. 4). These results strongly suggest that, in addition to receptor down-regulation, PKC activation inhibits in parietal cells one of the steps between G protein activation and polyphosphoinositide hydrolysis. In agreement with our study, Vicentini *et al.* (18) reported that PKC activation inhibits muscarinic-mediated polyphosphoinositide hydrolysis without altering the binding charac-

Fig. 2 Effects of NaF (10 mM) on phospholipid labeling in canine parietal cells. Cells were prelabelled with [³²P]orthophosphate (40 μCi/4×10⁶ cells) for 2 h in phosphate-free buffer. After preincubation, cells were further incubated in 0.5 ml phosphate-free buffer with (●—●) or without (○---○) 10 mM NaF for indicated periods. Each value represents the mean±SEM from three separate experiments, and each experiment was performed in duplicate.

teristics of the receptors in PC12 cells, suggesting that PKC activation interferes with the intracellular signal transduction event distal to receptor acti-

Fig. 3 Effects of TPA and 4 β -phorbol on NaF (20 mM)-induced increase in [3 H]inositolphosphate production in canine parietal cells. After preincubation in the presence or absence of various concentrations of TPA (●—●) or 4 β -phorbol (○---○), parietal cells were incubated with NaF (20 mM) for 30 min. Data are means $\pm$ SEM from four separate experiments, and are expressed as percentages of the control value obtained with NaF alone. * P <0.01 vs control (without TPA or 4 β -phorbol pretreatment)

vation (14). In this regard, it may be important to note that only a 23.4% reduction in NaF-induced [3 H]inositolphosphate accumulation was produced by TPA (10^{-7} M), whereas the same concentration of TPA inhibited carbachol- and gastrin-induced [3 H]inositolphosphate accumulation by 42.4% and 39.0%, respectively (Table 1). It is possible that the inhibition of NaF-induced polyphosphoinositide breakdown by TPA represents a part of the inhibition of carbachol- or gastrin-induced polyphosphoinositide hydrolysis by PKC. The post-receptor site where PKC exerts its inhibitory action in parietal cells is unknown at present. However, it has been reported that the α -subunit of G_i is phosphorylated by PKC in a cell-free system, suggesting that the G protein itself is a site of action (11). On the other hand, Kikuchi *et al.* (12) have indicated that an unknown factor is involved in PKC-induced inhibition of polyphosphoinositide hydrolysis at a step

Fig. 4 Effects of TPA (10^{-7} M) and 4 β -phorbol (10^{-7} M) on *de novo* synthesis of phosphatidic acid in canine parietal cells. After preincubation in the presence or absence of 10^{-7} M TPA or 4 β -phorbol for 2 h, cells were incubated in phosphate-free buffer supplemented with [32 P]orthophosphate ($5 \mu\text{Ci}/4 \times 10^6$ cells) in the presence or absence of NaF (10 mM) for 20 min. Values are means $\pm$ SEM from four separate experiments, and each experiment consisted of duplicate samples. * P <0.01 vs control (without NaF), ** P <0.01 vs NaF alone (without phorbol ester pretreatment)

between G protein and phospholipase C.

Our previous and present studies raise the possibility that PKC exerts its inhibitory action on muscarinic- or gastrin-stimulated polyphosphoinositide hydrolysis at least at two sites, i.e. at muscarinic or gastrin receptor, and at a post-receptor site between G protein and phospholipase C.

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