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Modulation of protein kinase C activity by NaF in bone marrow derived macrophages

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Stimulation of murine bone marrow derived macrophages with NaF, prelabeled with [1-^{14}C]oleate and [^{3}H]inositol, increased the production of inositol phosphates and the release of 1,2-[^{14}C]diacylglycerol (DAG). Moreover, NaF also induced activation of protein kinase C. These results indicate that bone marrow derived macrophages exhibit a phosphatidyl-4,5-bisphosphate phospholipase C activity, sensitive to NaF, which might be modulated by G-proteins. Activation of protein kinase C could have been mediated by NaF-induced release of DAG.

Protein kinase C; NaF; G-protein; Phosphoinositide phospholipase C; (Bone marrow derived macrophage)

1. INTRODUCTION

A novel guanine nucleotide binding protein, often termed G_p, may be involved in coupling receptor activation to the breakdown of phosphatidylinositol 4,5-bisphosphate (PtdIns-(4,5)P_2) catalyzed by the polyphosphoinositide phospholipase C [1–5]. This generates two second messengers, inositol 1,4,5-trisphosphate ($InsP_3$) and diacylglycerol (DAG). DAG stimulates protein kinase C [6], while $InsP_3$ is postulated to activate the release of intracellular calcium from the endoplasmic reticulum [7,8]. This transmembrane signalling system bears striking resemblance to the familiar adenylate cyclase system, which is also regulated by guanine nucleotide-binding proteins (G-proteins). The G-proteins of both systems seem to have functional homology since treatment with fluoride not only stimulates adenylate cyclase [9,10] but also polyphosphoinositide phospholipase C (reviewed in [11]). Fluoride has been shown to induce the dissociation of the α-subunit of G-proteins by mimicking the effect of the γ-phosphate of GTP on the α-subunit [12]. The released α-subunit then activates the corresponding enzyme system. Here we report that NaF stimulates phosphatidylinositol turnover in bone marrow derived macrophages, and that concomitantly activation of protein kinase C is observed.

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2. EXPERIMENTAL

2.1. *Bone marrow macrophage culture*

Cells obtained by flushing the femur of male Balb/c mice (age 8–12 weeks) were suspended at a density of 1×10^5/ml in DMEM supplemented with 10% heat-inactivated fetal calf serum (FCS), 30% L 929 conditioned medium, 5% horse serum, 1 mM sodium pyruvate, 2 mM glutamine, 60 μM mercaptoethanol, penicillin (100 units/ml) and streptomycin (100 μg/ml). Cells were cultured in 87 cm^2 Teflon bags (Heraeus, Hanau, FRG) for 7 days in 10% CO_2 at 37°C. L 929 conditioned medium containing colony-stimulation factor, was collected 7 days after starting cultures of the fibroblast line L 929 with 1×10^5 cells/ml.

2.2. *Measurement of [^{3}H]inositol phosphates*

Bone marrow derived macrophages were suspended at a concentration of 3×10^6/ml in inositol-free basal medium Eagle (BME) diploid (Flow), supplemented with 5% dialyzed heat inactivated FCS, 2 mM glutamine, 60 μM mercaptoethanol and 18 μCi/ml [2-^{3}H]inositol. After 20 h of incubation, the cells were washed and aliquots were preincubated for 30 min before

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adding NaF (25 mM) and LiCl (10 mM). After 60 min, the reaction was terminated by the addition of 1 ml of 1 M $HClO_4$. The neutralized supernatants were fractionated by anion-exchange chromatography (Dowex 1 × 8) and the levels of [^{3}H]inositol phosphates were determined as described by Berridge [13].

2.3. *Assay of 1,2-DAG*

Bone marrow derived macrophages at a cell density of 1 × 10^6/ml in RPMI 1640 containing 0.5% bovine serum albumin (essentially fat acid free), 2 mM glutamine and 60 μM mercaptoethanol were labeled with [^{14}C]oleate (0.1 μCi/ml) for 20 h at 37°C in 5% CO_2 atmosphere. The cells were then washed and aliquots were preincubated for 60 min at 37°C. After adding chloroform/ethanol (1:2, v/v) the lipids were extracted according to Schacht [14]. Separation of lipids was carried out on silica gel G (Merck) plates using the solvent system, light petroleum, diethyl ether, acetic acid (50:50:2, by vol.) [15].

2.4. *Protein kinase C assay*

Macrophages (2 × 10^7/ml) were incubated in balanced salt solution in the presence or absence of NaF (25 mM) for various times. Cells were then washed and suspended in ice-cold extraction buffer (20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 0.5 mM EGTA, 2 mM dithiothreitol, 0.33 mM sucrose, 0.01% leupeptin). Cells were disrupted by sonication for 1 min, and the homogenates were ultracentrifuged at 100000 × *g* for 1 h at 4°C. The supernatant cytosolic fractions were harvested. The corresponding membrane pellets were homogenized with a glass rod in extraction buffer containing 0.1% Triton X-100, incubated for 60 min at 4°C, and centrifuged again for 1 h at 100000 × *g* to obtain the detergent soluble particulate fraction.

PKC activity was quantified by incubating 2–4 μg cellular protein for 10 min at 37°C in a volume of 250 μl Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 0.4 mg/ml histone III-S, 40 μg/ml PtdSer, 0.8 μg/ml 1,2-diolein, 500 μM $CaCl_2$ and 10 μM ^{32}P-ATP (spec. act. 0.5 μCi/mmol). The reaction was terminated by the addition of 2 ml trichloroacetic acid (25%) and 250 μl of cold bovine serum albumin (1 mg/ml). After 20 min at 4°C, the resulting precipitate was recovered by centrifugation (10 min, 3000 rpm). The pellet was dissolved in 1 N KOH, precipitated again and collected on a Whatman GF/C glass fiber filter. The filters were washed with trichloroacetic acid (25%) followed by acetone. Transfer of ^{32}P to histone was quantified by liquid scintillation counting. The assay was linear with respect to time and protein concentration. Protein content was determined by the Biorad protein assay (Biorad, München, FRG).

3. RESULTS AND DISCUSSION

Bone marrow derived macrophages were prelabeled for 20 h with [^{3}H]inositol in order to label their inositol-containing lipids. Addition of fluoride to these cells triggered the breakdown of inositol phospholipids, which is mirrored in an increase of inositol phosphates and diacylglycerol. Generation of both compounds implies the activation of phosphoinositide phospholipase C (PLC).

The treatment with fluoride led to a 4–5-fold increase in Ins1P, no change in Ins(1,4)P_2, and a slight decrease in Ins(1,4,5)P_3 levels (table 1). Since the incubations were carried out in the presence of LiCl, which has been shown to inhibit Ins1P-phosphatase [16], Ins1P accumulated. However, recent reports indicate that LiCl might also affect accumulation of other inositol phosphate isomers as detected by the use of HPLC [17].

In response to treatment with fluoride not only the formation of inositol phosphates but also the DAG levels rose (table 2). These results suggest that increased inositol phosphate levels are not only due to an inhibition of inositol phosphate phosphatases by fluoride, an inhibitor of phosphoprotein phosphatases [18], but also reflects an activation of PLC. In accordance with these data, fluoride induced activation of PLC has also been demonstrated in other cell systems [19–22]. Data from Cockcroft and Tayler [19] support the idea that activation occurs via the G-protein and not by a direct effect on the enzyme, since GDP-β-S inhibits GTP-γ-S as well as fluoride-induced activation.

Accumulating evidence indicates that hormone, neurotransmitter or growth factor induced formation of $InsP_3$ and DAG is linked to activation of protein kinase C (PKC). Here we show that this signal transduction cascade might also be triggered by fluoride. Measuring PKC activity in the cytosolic and membrane fractions from macrophages that had been stimulated with 25 mM NaF for 8 min, showed a 30% decrease of the cytosolic enzyme activity and a 25% increase in the membrane fraction (fig.1). In the absence of

Table 1

Effect of NaF on inositol phosphate levels in bone marrow derived macrophages

	Inositol phosphates (cpm/3 × 10^6 cells)		
	Ins1P	Ins(1,4)P_2	Ins(1,4,5)P_3
Control	4948 ± 259	1234 ± 183	781 ± 166
NaF (25 mM)	20116 ± 2100	1153 ± 253	303 ± 70

Prelabeled bone marrow derived macrophages were incubated at 37°C for 60 min with or without NaF (25 mM) in the presence of LiCl (10 mM). [^{3}H]Ins1P, [^{3}H]Ins(1,4)P_2 and [^{3}H]Ins(1,4,5)P_3 were determined as described in section 2. Values are means ± SD of three experiments

Table 2

Effect of NaF on the release of 1,2-diacylglycerol from prelabeled bone marrow derived macrophages

	cpm/10^6 cells	
	1 min	2 min
Control	1230 ± 60	1270 ± 45
NaF	1771 ± 135	1900 ± 150

Bone marrow derived macrophages, prelabeled with [^{14}C]oleate for 20 h, were washed three times and incubated with or without NaF (25 mM) for the indicated times. Radioactivity incorporated into 1,2-diacylglycerol was determined. Each value represents the mean ± SD of triplicate determinations

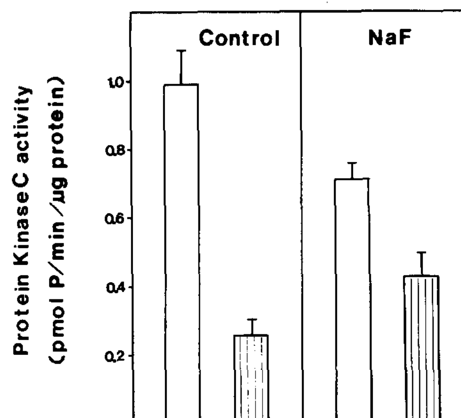

Fig.1. NaF induced modulation of PKC in bone marrow derived macrophages. Macrophages (2 × 10^7/ml) were stimulated with NaF (25 mM) in a balanced salt solution for 8 min. PKC activity was determined in the cytosolic (open bars) and particulate fractions (hatched bars) of the cell homogenate obtained by ultracentrifugation as described in section 2. Values are means ± SD of five experiments.

NaF no decrease in enzyme activity was observed during the preincubation time of 8 min (not shown). To ensure that the protein kinase C assay was carried out in a fluoride free medium, the cytosol and membrane preparations were desalted by use of Sephadex G-25 column chromatography before being tested. Thus treated samples showed no change in PKC activity when NaF was added at time 0 of preincubation (not shown).

Membrane associated PKC exhibited different characteristics to the cytosolic enzyme. In the absence of Ca^{2+}, diolein and phosphatidylserine, enzyme activity measured in the cytosol decreased from 0.98 ± 0.12 to 0.3 ± 0.07 pmol/min per µg protein (n = 4), whereas in the membrane fraction activities in the presence and absence of Ca^{2+}, diolein and phosphatidylserine amounted to 0.32 ± 0.06 (n = 4) and 0.34 ± 0.05 (n = 4), respectively. One could speculate that the membrane associated enzyme is already fully active or that the enzyme is proteolytically cleaved, hereby becoming independent of the co-substrates. Further work is in progress to identify the characteristics of this enzyme. The inhibitory effect of NaF was not only observed when preincubation was performed in the presence of NaF, but also when NaF was added to the assay mixture. As shown in table 3 addition of NaF to the assay mixture led to a decrease in enzyme activity by about 45%, suggesting that NaF might act by inhibiting phosphatases, or by inhibiting the enzyme directly. PKC extracted from prestimulated washed cells was also inhibited by NaF, although less efficiently (15%) than the enzyme extracted from non-prestimulated cells, indicating that the less active enzyme is still subject to modulation.

In summary our data show that treatment of intact bone marrow derived macrophages with NaF resulted in a raise of $InsP_3$ and DAG, associated with modulation of PKC. Whether modulation of PKC is due to the raise of $InsP_3$ and DAG or due to the action of NaF as phosphatase inhibitor, remains to be established. Assuming that PKC might be activated by dephosphorylation, then NaF, by inhibiting phosphatases would prevent dephosphorylation, thus rendering PKC inactive. Despite

Table 3

Addition of NaF to the assay mixture inhibits PKC activity

Addition during preincubation	PKC activity (pmol/µg protein per min)	
	Without NaF	With NaF
Non	1.10 ± 0.14	0.62 ± 0.09
NaF	0.73 ± 0.06	0.60 ± 0.05

Bone marrow derived macrophages (2 × 10^7/ml) were preincubated at 37°C in the presence or absence of NaF (25 mM). After 8 min, PKC activity was determined in the cytosolic fractions. The assay was carried out in the presence or absence of NaF (25 mM). Values are means ± SD of three determinations

these reservations we can conclude that fluoride provides an effective pharmacologic tool to stimulate $PtdInsP_2$ hydrolysis and to modulate PKC in intact bone marrow derived macrophages.

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