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SODIUM FLUORIDE UNMASKS THE ACCUMULATION OF LYSOPHOSPHATIDYLCHOLINE IN INTACT PANCREATIC ISLET CELLS

Stewart A. Metz*[1] and Marjorie Dunlop

*Medicine and Research Services, Denver VA Medical Center (111H)
1055 Clermont Street, Denver, CO 80220
and the University of Colorado Health Sciences Center

Department of Medicine
Royal Melbourne Hospital/University of Melbourne
Parkville, Victoria 3050 AUSTRALIA

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SUMMARY: When intact but dispersed neonatal islet cells, prelabelled with [^{14}C-Me]choline, were stimulated with a calcium ionophore, ionomycin alone elicited only small rises in lysophosphatidylcholine; in contrast, pretreatment for 20 min with sodium fluoride (20mM) unmasked a consistent accumulation of lysophospholipid (to 155% of control at 1 min, 162% at 5 min and 212% at 10 min). Fluoride was shown to inhibit (by 40-50%) the reacylation of exogenous acyl- or alkyl-linked lysophosphatidylcholines by a delayed and indirect effect, whereas, in contrast, 12-O-tetradecanoylphorbol-13-acetate or dioctanoylglycerol actually augmented acylation. Thus, increased production of lysophosphatidylcholine in intact islets is obscured by rapid removal mechanisms, one of which might involve protein kinase C (or diglycerides directly). The use of sodium fluoride partially obviates this clearance, but this finding may necessitate a re-interpretation of claims that G protein agonists such as fluoride directly activate phospholipase A_2 in some cells.

A role for the activation of phospholipase A_2 (PLA_2; E.C.# 3.1.1.4) in the regulation of insulin release has been proposed. Both of the byproducts of PLA_2 action--arachidonic acid and lysophospholipids--promote insulin secretion, as does exogenous PLA_2 (1-3). Conversely, inhibitors of PLA_2 reduce physiologic insulin release (2). However, while the presence of PLA_2-like activity has been demonstrated in islet homogenates (4,5), no study has unambiguously identified PLA_2 directed towards *endogenous* substrate in *intact* islet cells. Such a demonstration is important, since the effects of nutrient stimuli such as glucose require intact islets to be studied in a physiologic fashion. Therefore, we sought to determine if lysophosphatidylcholine (lysoPtdCho) accumulation could be induced in intact, dispersed neonatal islet cells, as a marker of PLA_2 activation. The Ca^{++}-selective ionophore ionomycin induced unequivocal lysoPtdCho formation only when cells were pretreated with sodium fluoride (NaF), as a consequence of the partial inhibition of the reacylation of lysophospholipid. This finding is in accord with our previous studies showing that another pharmacologic probe, p-hydroxy-

[1]To whom correspondence should be addressed.

Abbreviations Used: PtdCho, phosphatidylcholine; LysoPtdCho, lysophosphatidylcholine; GroPCho, glycerophosphorylcholine; PLA_2, phospholipase A_2; NaF, sodium fluoride; TPA, 12-O-tetradecanoylphorbol-13-acetate.

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mercuribenzoic acid, inhibits lysoPtdCho acylation (and hydrolysis), and leads to lysoPtdCho accumulation (2,6) and insulin release. This approach should facilitate future studies of the regulation of PLA_2 in intact islet cells. However, at the same time, this effect of NaF suggests alternative interpretations of studies suggesting that GTP-binding proteins directly activate PLA_2 (7,8).

MATERIALS AND METHODS

Materials: Sources for materials have been described (9). Radionuclides were from Amersham International (Amersham, UK). Staurosporine was from Calbiochem (La Jolla, CA).

Cell Incubations: Dispersed monolayer-cultured neonatal rat islet cells were prepared and overnight-prelabelled (x18 hrs) with [^{14}C-Me]choline (1μCi/ml; 55mCi/mmol), [^{14}C]stearic acid (0.2μCi/ml; 55.3mCi/mmol) or [1-^{14}C] hexadecanol (1μCi/ml; 24mCi/mmol) as previously described (9). The next day, islet cells were incubated in flasks containing Krebs-Ringer bicarbonate buffer with 25μM unlabelled choline but no albumin (glucose = 5.6mM), and gassed in 95% O_2, 5% CO_2 (pH 7.4; 37°C). Incubations were generally for 5 min except where indicated otherwise. Twenty minute preincubation periods in the same medium (in the presence or absence of NaF) preceded all incubation periods. For studies of the uptake and metabolism of exogenous arachidonate or lysophosphatidylcholines, [^{14}C]arachidonate (55mCi/-mmol), 1-[1-^{14}C-palmitoyl]lysoPtdCho (59mCi/mmol), or 1-O-hexadecyllysoPtd [^{14}C-Me]Cho (58mCi/mmol) (0.33, 0.35, 0.35 μCi/ml, respectively) were provided in the absence of BSA for the indicated times, and were washed three times in 0.1% delipidated bovine serum albumin after removal of media.

Extraction and Lipid Analysis: Cells were extracted and analyzed by thin layer chromatography as previously described (9). Recoveries of relevant metabolites were >98%, 93-96%, 94% and >98% for PtdCho, acyl-lysoPtdCho, alkyl-lysoPtdCho and GroPCho, respectively.

Data Analysis: To correct for variations in cell number or phospholipid mass between flasks, data for the formation of endogenous lysoPtdCho are expressed as dpm in [lyso-PtdCho/(lysoPtdCho + PtdCho)]. Results for the acylation of exogenous lysoPtdCho are expressed as %: 100 x [PtdCho/(lysoPtdCho + PtdCho)]. Data are x±SEM for (n) individual determinations or experiments, as indicated, and were analyzed, as appropriate, by non-paired or paired t test, respectively.

RESULTS

Production of lysophosphatidylcholine (LysoPtdCho)

In incubations of 15 sec-10 min, ionomyin (1μM) induced only a small increase in choline-labelled lysoPtdCho. For example, at 3.5-5 min of incubation, ionomycin-treated samples were 110±2% of control samples (n=17 determinations) in seven experiments ($p<.05$). The modest degree of rise in lysoPtdCho was not due to its hydrolysis to GroPCho by islet lysophospholipase (6), since ionomycin also did not increase GroPCho accumulation (101±2% of control; n=23 determinations; see also Table 1). Since sodium fluoride (NaF) has a number of properties which might potentiate phospholipase A_2 (see Discussion), islet cells were pretreated for 20 min with NaF (20mM), followed by a further 1, 5 or 10 min experimental incubation. In four experiments, NaF alone failed to elevate lysoPtdCho levels (95±7%; n=11 determinations) and increased GroPCho only insignificantly (108±3% of control; 11 determinations) (see also Table 1). In contrast, in each of six experiments, the combination of ionomycin plus NaF increased lysoPtdCho (cf. Table 1) at 5 mins to 162±11% of control (df 32; $p<.001$). The combination of agonists (but not either alone) also increased lysoPtdCho at 1 min of incuba-

Table 1

Representative Experiment Depicting the Effects of Ionomycin and Sodium Fluoride (NaF) on Lysophosphatidylcholine (LysoPtdCho) and Glycerophosphorylcholine (GroPCho) Accumulation in [^{14}C-Me]choline-Prelabelled Islet Cells

	LysoPtdCho, % dpm^{+}	GroPCho, dpm/flask^{+}
A. Control	1.42±.04 (3)	87226±3943 (3)
B. Ionomycin, 1μM (x5 min)	1.49±.06 (3)	88768±2437 (3)
C. NaF, 20mM°	1.40±.02 (3)	103482±4508 (3)*
D. NaF + Ionomycin (x1 min)°	2.16±.04 (3)*	96148±3559 (3)
E. NaF + Ionomycin (x5 min)°	1.87±.08 (5)*	118115±4060 (5)*

$^{+}$ % dpm = dpm in [LysoPtdCho/(LysoPtdCho + PtdCho)]; GroPCho = total GroPCho generated (sum of medium plus the aqueous phase of the cellular extracts). Values are mean ± SEM for (n) determinations.

*Difference vs. a (control) is significant (p<.05 or greater)

°In C, NaF alone was present for a 20 min preincubation and a 5 min incubation
In D, NaF was present for a 20 min preincubation and a 1 min incubation, the latter also in the presence of ionomycin
In E, NaF was present for a 20 min preincubation and a 5 min incubation, the latter also in the presence of ionomycin

tion (to 155±4% of control; df 8; p<.001) and, most impressively, at 10 mins of incubation (to 212±36%; df 7; p<.01). GroPCho also increased (116±4% of control; 16 determinations) at 5 mins (p<.05).

These findings were verified using islets labelled in the sn-1 position with [^{14}C] stearic acid. NaF (in the preincubation and incubation periods) plus ionomycin increased stearate-labelled lysoPtdCho to 153±7% of control values at 5 min (df9, p<.01), indicating that a phospholipase of the A_2 type, directed towards diacyl-linked PtdCho, was probably involved. Indeed, in preliminary studies in which islets were labelled with [1-^{14}C] hexadecanol to form 1-O-hexadecyl-linked PtdCho species, ionomycin plus NaF was less effective (125±8% of control; df4; p<.05), suggesting that alkyl-linked PtdCho is not the preferred substrate for this islet PLA_2. In fact, preliminary studies using HPLC suggested that acyl-linked lysoPtdCho, rather than alkyl-linked lysoPtdCho (i.e., lyso-platelet activating factor), was the predominant species formed (data not shown). Interestingly, NaF plus ionomycin was ineffective if NaF was present only during the incubation period (111±7% of basal; n=6 determinations; p=n.s.) in choline-prelabelled cells, as well as in stearate-prelabelled cells (data not shown), implying that the effect of NaF is a delayed and indirect one. Carrying out the incubation in medium derived from a NaF-containing preincubation period was also ineffective (not shown), suggesting that diffusible factors could not reproduce the effect of a preincubation period in NaF.

NaF Inhibits the Incorporation of Fatty Acids into Lysophospholipids

Since our previous studies suggested that lysoPtdCho is rapidly metabolized by islet tissue (6), we questioned whether NaF could inhibit the esterification of free fatty acids into

lysophospholipids. In preliminary studies, when islet cells were incubated with [^{14}C] arachidonate (0.33μCi/ml) or [^{14}C]palmitate (1μCi/ml), the presence of NaF dramatically reduced the uptake of the former (but not the latter) into phospholipids, while unesterified arachidonate and neutral lipids reciprocally accumulated (data not shown). However, since this effect did not require a preincubation with NaF, it may be irrelevant to the accumulation of endogenous lysoPtdCho (see above); furthermore, it could conceivably represent mere isotope dilution since NaF increases arachidonate release from these cells (9). To circumvent this trivial possibility, unlabelled islets were incubated with 1-[1-^{14}C-palmitoyl]lysoPtdCho or 1-O-alkyl-lysoPtd [^{14}C-Me]Cho in the presence or absence of NaF. NaF did not reduce the total uptake of label into cells. However, the acylation of both of these lysophosphatidylcholines was inhibited about 50% by NaF at all points from 15-120 min of incubation (Fig. 1; $p<.001$, ANOVA); this effect also required a pre-exposure to NaF (Fig. 1, inset).

NaF can augment the production of diglycerides in islet cells (9). Since diglycerides or their analogues can potentiate the phospholipase A_2-induced hydrolysis of PtdCho (10) or inhibit the reacylation of lysoPtdCho (11,12), either directly (13) or due to activation of protein kinase C (10,11), we examined the effect of dioctanoylglycerol (50μg/ml) or 12-O-tetradecanoylphorbol-13-acetate (TPA; 0.5μM). However, these agents actually stimulated acylation of acyl- or alkyl-linked lysoPtdCho (to 140±3% and 268±17% of controls for dioctanoylglycerol and TPA, respectively; n=6 each; $p<.01$ and $<.001$, respectively). In further contradistinction to the effects of NaF, TPA also failed to synergize with ionomycin, since the two agents together did not increase endogenous lysoPtdCho levels. Furthermore, the effect of NaF was resistant to inhibition by the protein kinase C inhibitor staurosporine (500nM).

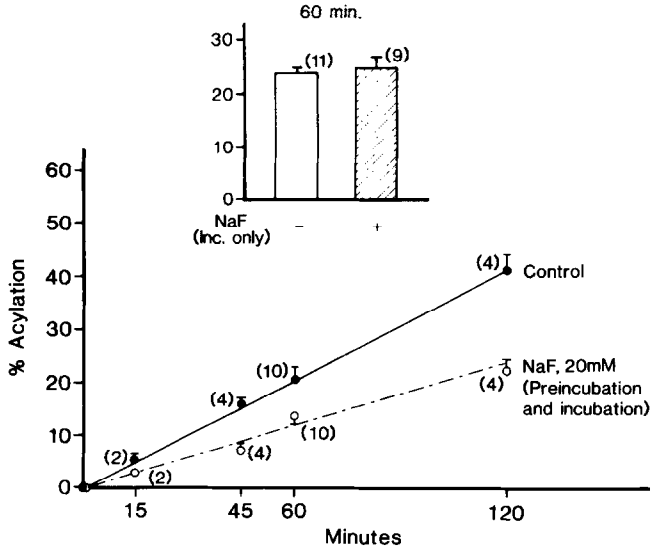

Fig. 1. Effect of NaF on the acylation of exogenous 1-O-alkyllysoPtd-[^{14}C-Me]choline or 1-[1-^{14}C-palmitoyl] lysoPtdCho. Data for the two substrates (from five separate experiments) were identical and so are combined. Main figure shows the effect of NaF when present during the incubation periods (of 15-120 min, as indicated) as well as during a preincubation period. Inset shows lack of effect of NaF when present during a 60 min incubation (inc.) period only.

DISCUSSION

These studies demonstrate that the synergistic combination of ionomycin (to activate PLA_2) and NaF (working, at least in part, by inhibiting the reacylation of lysoPtdCho) unmasks the presence of phospholipase A_2 directed against *endogenous* substrates in *intact* pancreatic islet cells. Based on previous estimates (6), the increment in lysoPtdCho achieved by these agents is probably sufficient to augment insulin release even though NaF only inhibited lysoPtdCho clearance partially. Much more complete inhibition of clearance is needed to fully appreciate the amount of lysophospholipids generated (6). The failure of NaF to elevate basal levels of lysoPtdCho implies that basal lysoPtdCho may derive from mechanism(s) other than PLA_2 (perhaps including PLA_1, transacylases, and/or artefactual lipid hydrolysis during sample work-up); evidence for two such pools of lysoPtdCho in islets has been presented (14).

The exact mechanism of NaF is not totally resolved by these studies, but does not appear to be direct or immediate. In fact, the direct effect of NaF appears to be a slight *stimulation* of acylation (15). NaF can activate G proteins which, in turn may activate phospholipase A_2 (7,8), or, conceivably, inhibit the reacylation process (directly, or via a rise in cellular cyclic AMP or Ca^{++} concentrations; ref. 15). As indicated above, diglycerides produced by NaF (9) might also modulate either process; however, neither dioctanoylglycerol nor a phorbol ester mimicked the effect of NaF and a protein kinase C inhibitor did not block it. In fact, both TPA and dioctanoylglycerol actually promoted the reacylation of lysophosphatidylcholines. NaF might also promote phospholipase A_2 activation by potentiation of the rise in cytosolic free Ca^{++} concentration (16) via stimulation of Ca^{++} mobilization or influx (16), or by inhibiting Ca^{++} extrusion from the cell (17). By elevating cytosolic pH (18), fluoride might also potentiate PLA_2, which usually is favored at alkaline pH (19). Alternatively, by inhibiting islet glycolysis (20), NaF might reduce ATP levels and thereby inhibit the acyl CoA synthetase/transferase cascade which reacylates lysoPtdCho. Further studies will be needed to answer this question definitively. However, no matter what the exact molecular mechanism of action of NaF, the finding that it blunts the reacylation of lysophospholipids and the reincorporation of arachidonate suggests a role of fluoride (possibly acting via a GTP-binding protein) to inhibit the reacylation process, rather than necessarily to stimulate deacylation, as had been concluded by others (7).

Thus, these data re-emphasize the potential regulatory role of fatty acid reincorporation mechanisms in the availability of free arachidonate and lysophospholipids (2,6). Since both a phorbol ester and a diglyceride potentiated the reacylation of lysophospholipids, it may be that protein kinase C (or diglyceride directly) modulates that process physiologically. Although a delayed stimulation of phospholipid acylation by activators of protein kinase C has also been reported in cultured smooth muscle cells (21), in most studies only *inhibitory* effects have been reported (11,12). Thus, our findings are novel and suggest a heretofore unsuspected effect of protein kinase C activation (or of diglyceride directly) on phospholipid turnover in pancreatic islets. Glucose, the major physiologic islet secretagogue, increases diglyceride levels (22) and may activate protein kinase C (23,24) in islet cells; these effects may now help

to explain the stimulation of arachidonate esterification (25) and the apparently paradoxical reduction in lysoPtdCho levels (26) observed after glucose stimulation.

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