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RAPID COMMUNICATION

INHIBITION OF THE METABOLISM OF PHOSPHATIDYLETHANOL AND PHOSPHATIDIC ACID, AND STIMULATION OF INSULIN RELEASE, BY PROPRANOLOL IN INTACT PANCREATIC ISLETS

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Recent studies have demonstrated the presence of a phospholipase of the D type (PLD) in several mammalian tissues. In the absence of alcohols, PLD hydrolyzes membrane phospholipids to yield phosphatidic acid (PtdOH); in the presence of ethanol, the phosphatidyl group is transferred to the alcohol, yielding a unique phospholipid, phosphatidylethanol (PtdEtOH) [1]. Despite the recent proliferation of articles describing the use of PtdEtOH as a sensitive and pathognomonic marker for PLD activation, the metabolic clearance of this phospholipid has been overlooked, and has been assumed to be negligible [2]. Recently, we reported [3] that intact adult pancreatic islets or dispersed neonatal islet cells [4] contain a PLD which is activated by the phorbol ester 12-0-tetradecanoylphorbol-13-acetate (TPA) or by sodium fluoride (NaF) in the presence of $AlCl_3$. Surprisingly, we found [3] that preformed PtdEtOH is metabolized at a rate of 30-50% per hour after removal of the agonist and the ethanol; however, the mechanisms leading to this degradation were not identified. In the current studies, we report that the *in situ* degradation of endogenous PtdEtOH (or PtdOH) was blocked by propranolol, a classic inhibitor of phosphatidic acid phosphohydrolase (PAPH) [5-7]. Furthermore, we report that propranolol (in the absence of ethanol) led to the accumulation of endogenous PtdOH, accompanied by the stimulation of insulin release. These findings provide further, unique evidence to support our earlier studies [8,9] suggesting that PtdOH may be an intracellular signal for insulin release.

MATERIALS AND METHODS

[1-^{14}C] Arachidonic acid (53 mCi/mmol) was from New England Nuclear (Boston, MA). dl-Propranolol was from Sigma (St. Louis, MO) and was dissolved in water. All preincubation and incubation periods were carried out in Krebs-Ringer Bicarbonate buffers as described [3] at 37°, pH 7.4, at a glucose concentration of 3.3 mM. Islets were isolated from fed, male Sprague-Dawley rats and insulin secretion was assessed in static incubations, as previously described in detail [10,11]. Insulin secretion was assessed using duplicate determinations in the radioimmunoassay (RIA). To assess phospholipid metabolism, islets were labeled overnight (x 18-20 hr) with [^{14}C] arachidonic acid (6 μCi/mL) in RPMI 1640 medium containing 5% fetal calf serum, 11.1 mM glucose and 20 mM Hepes. The next day, islets were washed three times in KRB containing 0.5% dilipidated bovine serum albumin, distributed into groups of 200 islets/tube, and treated as described below. Incubations were terminated using cold MeOH and lipids were extracted overnight in chloroform/methanol/conc. HCl (200:100:1), followed by phase separation induced by 0.75 mL of 500 mM KCl/50 mM EDTA and centrifugation, as previously described [3,4,9]. The organic extracts were taken to dryness under argon and were brought up in 50 μL chloroform/methanol (2:1) containing 250 ng each of PtdOH and PtdEtOH (Avanti; Pelham,

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AL); 20 µL was streaked (in duplicate) onto pre-activated LK6D silica gel 60a TLC plates (Whatman; Clifton, NJ), which were developed using the upper phase of iso-octane/ethyl acetate/acetic acid/water (5:9:2:10). Relevant areas of the TLC plates were scraped according to the superimposed autoradiograph. The identities of PtdOH and PtdEtOH were confirmed using two additional TLC systems [3,4,9]. Data for PtdOH or PtdEtOH are expressed as percent of total phospholipids (to normalize for variations in islet phospholipid applied to each individual TLC lane), using duplicate determinations. Statistical analyses were by non-paired or paired *t*-testing, as appropriate.

RESULTS

Effects of propranolol on the metabolism of phosphatidic acid and phosphatidylethanol

We have reported [3] that either TPA (2 µM) or fluoroaluminate (20 mM NaF plus 10 µM $AlCl_3$), when provided in the presence of a saturating ethanol concentration (2.5%, v/v), increases PtdEtOH in islets up to 700% of basal levels. To assess the disappearance of endogenous PtdEtOH *in situ*, islets were pretreated with either agonist plus EtOH for 60 min; some of the islets were then immediately extracted (= control or 100% levels) whereas other islets were incubated for variable times in the absence of agonist or ethanol (in order to preclude ongoing PtdEtOH synthesis). The provision of EtOH "ablates" PLD-derived PtdOH formation in favor of PtdEtOH [3,12]; however, AlF_4^- also stimulates phospholipase C and thus generates diglyceride [3,4,13] which is phosphorylated to yield increments in PtdOH [3]. Therefore, the pre-incubation with AlF_4^- permitted us to assess the subsequent removal of both PtdOH and PtdEtOH simultaneously.

After removal of AlF_4^- and EtOH, PtdOH declined precipitously, with an apparent "half-life" of under 15 min, and reached a new steady-state level by about 30 min (Fig. 1). In contrast, PtdEtOH disappeared more slowly, declining by 33% after 1 hr and only by 47% after 2 hr of additional incubation (Fig. 1). Inclusion of dl-propranolol (250 µM) during this additional 60-min incubation period (i.e. after removal of AlF_4^- and EtOH) not only prevented the decline in PtdOH but dramatically increased PtdOH from 0.77±0.05% of phospholipids at 60 min (N=12) to 2.89±0.53% (mean±SEM, N=9 determinations; P<0.001), levels actually higher than those (1.22±0.09%; N=7; P<0.01) in islets that had not been incubated further (=0 min). Propranolol (250 µM) also vitiated the metabolism of PtdEtOH (no additional incubation: 1.26±0.04% of phospholipids, N=7; 60-min incubation = 1.01±0.03%; N=12, P<0.001 vs no incubation; 60-min incubation plus propranolol = 1.33±0.07%; N=9; P=NS *vs* no further incubation). Likewise, propranolol prevented the degradation of the PtdEtOH formed as a result of pretreatment with TPA plus EtOH. In the absence of the drug, PtdEtOH fell 41% from 1.28±0.05 (N=4) to 0.75±0.01% (N=6; P<0.001); no decrement was seen in the presence of propranolol (1.23±0.04%, N=9; P=NS *vs* no further incubation).

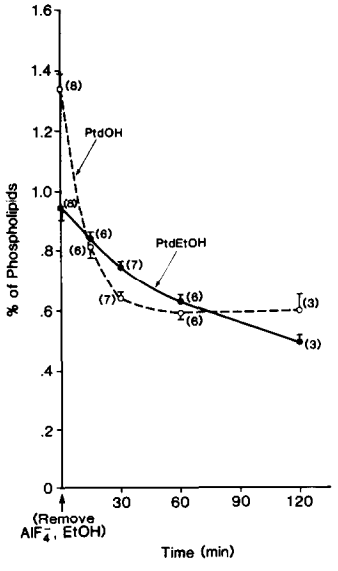

Fig. 1. Disappearance of endogenous PtdOH and PtdEtOH in intact islets. Compounds (labeled with [^{14}C]-arachidonate) were generated during a 60-min preincubation in the presence of AlF_4^- and ethanol (see text for details). AlF_4^- and ethanol were removed at time 0, at which time absolute DPMs in total phospholipids were 156685±1612. Values are means (±SEM) for (N) determinations.

Effects of propranolol on phosphatidate levels and on insulin release

The effect of propranolol to augment PtdOH levels was also evident in islets that had not been pretreated with agonists or EtOH. PtdOH levels were increased by propranolol concentrations of ≤ 50 µM through 500 µM, accompanied by significant, but much smaller percentage increases in diglycerides, monoglycerides and unesterified arachidonic acid

(Fig. 2). These effects of propranolol were accompanied by the stimulation of insulin release at identical concentrations. In each of nine separate experiments, 250 μM propranolol increased insulin release ($P<0.01$). The threshold for this effect was about 50 μM propranolol ($P<0.05$; see Fig. 2, inset); the maximal response was seen at 250-500 μM. Likewise, when islets were pretreated with AlF_4^- for 60 min in the absence of EtOH (to prevent the concurrent accumulation of PtdEtOH), and then were incubated for a further 60 min in the absence of agonist, the inclusion of propranolol in the second incubation period markedly increased insulin release from 96±11 to 244±38 μUnits/60 min (df 14; $P<0.001$). Propranolol (250 μM) did not interfere with the RIA for insulin.

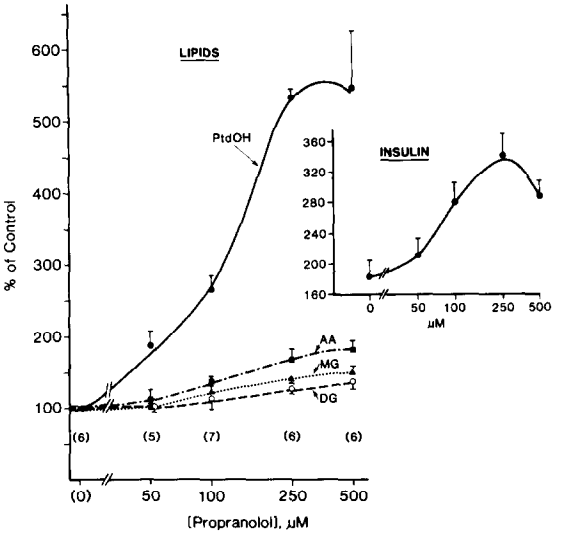

Fig. 2. Effects of various concentrations of propranolol on levels of PtdOH, diglycerides (DG), monoglycerides (MG), and unesterified arachidonate (AA) during 60 min incubations of islets studied at a non-stimulatory glucose concentration (3.3 mM) and in the absence of agonist. Data are means ± SEM for (N) determinations. Control values (as % of total phospholipids) were: PtdOH, 0.72±0.08; MG, 0.24±0.02; DG, 5.75±1.37; AA, 1.83±0.09; absolute DPMs in total phospholipids (in the absence of propranolol) were 196795±4572. Inset shows effects on insulin release (μUnits/60 min) in four separate experiments.

DISCUSSION

These data permit two novel conclusions. First, they directly demonstrate that, contrary to the expectations of some [2], PtdEtOH is metabolized *in situ* by cells (albeit more slowly than PtdOH). Our earlier studies [3] suggested that the activation of phospholipases A_2, C, or D does not play a quantitatively important role in PtdEtOH removal. The inhibition of the catabolism of PtdEtOH (and PtdOH) by propranolol suggests instead that PtdEtOH is degraded (at least in part) via phosphatidate phosphohydrolase [5-7]; direct studies of that enzyme, which is present in the islet [14], will be required to validate this conclusion. Thus, the rate of removal of PtdEtOH should be considered in future studies of PtdEtOH accumulation when used to assess PLD activation. In fact, while the physiologic secretagogue glyceraldehyde increased PtdEtOH levels modestly in dispersed neonatal islet cells in the absence of propranolol, a much greater formation of PtdEtOH was revealed in the presence of the drug.[§]

Second, these data support our earlier findings [8,9] which suggested that PtdOH is a secretagogue in the islet, either directly or possibly via the accumulation of other lipid mediators as well. Our earlier studies employed exogenous PtdOH [8] or the provision of exogenous PLD to generate PtdOH endogenously [9]. The current studies suggest that a pool of endogenous PtdOH which accumulates due to propranolol (presumably as a result of the inhibition of PAPH since propranolol did not directly activate PLD[§]) is also insulinotropic. Others have also observed insulinotropic effects of propranolol [15,16] at similar concentrations (which are well above those required to block beta adrenergic receptors). Together these three approaches (provision of exogenous lipid mediator, generation of lipid via provision of the appropriate exogenous phospholipase, and inhibition of the metabolic clearance of endogenous lipid) closely parallel the experimental paradigms which we used to support a physiologic role for lysophospholipids in insulin release [10,11,17].

In the current studies, propranolol increased not only PtdOH levels but also levels of diglyceride, monoglyceride and unesterified arachidonic acid, probably as a result of the secondary activation of phospholipase C by PtdOH [8]. It is possible that these other lipids contributed to the observed stimulation of insulin release; however, we were able to dissociate insulin release from the accumulation of these other lipid moieties in our previous studies using exogenous PLD

[§]Dunlop, M., Metz, S., manuscript in preparation.

[9]. It should be noted that while propranolol and exogenous PLD both increase secretion pari passu with increments in PtdOH, the insulinotropic effects of propranolol were less pronounced than those of PLD at comparable levels of PtdOH (cf. current studies to Ref. 9). Although the PtdOH was also generated endogenously in the latter study, it was formed in the outer plasma membrane leaflet (since exogenous PLD presumably does not enter cells). This finding suggests that PtdOH in that location (or in the medium) is more effective than PtdOH at intracellular locations and is compatible with an extracellular, receptor-mediated effect of PtdOH [8] or possibly of lysoPtdOH (cf. Ref. 18). It is possible, then, that intracellular PtdOH mimics receptor stimulation (albeit relatively weakly) by inhibiting GTPase [19]. Alternatively, the enzymatic source or the rate of rise of PtdOH may be critical. Further studies will be needed in this regard. We also recognize that it is possible that propranolol has effects other than those on PtdOH accumulation. For example, as a cationic amphiphilic drug, it may displace membrane-bound Ca^{2+} [20]. However, in view of reported effects of PtdOH on Ca^{2+} fluxes [8,21], such an effect may well be mediated via local increments in PtdOH, especially since many cationic amphiphilic drugs inhibit PAPH [5].

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