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EFFECTS OF PHOSPHATIDYLETHANOL ON INOSITOL 1,4,5-TRIPHOSPHATE LEVEL OF CULTURED NG108-15 CELLS

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Abstract – Tempting to further understand the molecular mechanism of pharmacological action of ethanol, we evaluated effects of phosphatidylethanol (PET) on inositol 1,4,5-triphosphate (IP_3) level and protein kinase C (PKC) activity in cultured NG108-15 cells. PET increased intracellular concentration of IP_3. PET incorporation into membranes of NG108-15 cells had no effect on the phosphorylation of the PKC-specific substrate MBP_{4-14}, thus indicates that PET does not affect PKC activity in this system.

INTRODUCTION

The use of ethanol goes back to the dawn of civilization, but in spite of intense efforts its mode of action at the molecular level has remained elusive. Alling *et al.*[1] demonstrated that phosphatidylethanol (PET) was formed in different organs of ethanol-treated rats. PET is an unique and anionic phospholipid formed in cell membranes only in the presence of ethanol. This phospholipid is formed through a transphosphatidylation reaction mediated by phospholipase D[2,3]. The ability to activate phospholipase D and thus stimulation of PET formation if ethanol is present has been ascribed to several receptor agonists and protein kinase C (PKC) activators[4-6]. Thus considerable quantities of PET may be formed in ethanol-exposed tissues, including brain. Some recent studies have, in addition, suggested that PET be degraded very slowly[7,8]. Therefore, significant amounts of PET may be accumulated during a period of ethanol exposure.

PET has been shown to affect Na^+/K^+-ATPase, 5′-nucleotidase, membrane fluidity and fusion of membrane vesicles[9,10]. Lundqvist *et al.*[11] have recently reported that PET affects the intracellular levels of inositol 1,4,5-triphosphate (IP_3).

PKC is a key enzyme in signal transduction that has shown to be activated by 1,2-diacylglycerol formed by phospholipase C. PKC itself has also been shown to mediate a regulatory influence on phospholipase C. Therefore, it is possible that the increase in IP_3[11] could be related to an effect of PET on PKC. Asaoka *et al.*[12] have indeed demonstrated that PET can act as an activator of PKC in an *in-vitro*, cell-free system. An effect of PET on PKC-activity would be interesting especially since PKC has been suggested to be a common target for a large variety of cytotoxins including ethanol[13-15].

The aim of the present study was therefore to examine whether PKC down-regulation or inhibition would abolish the IP_3 response to exogenous PET and whether exogenous PET influences PKC phosphorylation activity.

MATERIALS AND METHODS

Chemicals Dulbecco's modified Eagle's medium and cell medium supplements were purchased from GIBCO (Grand Island, NY, USA). [3H]-Inositol 1,4,5-triphosphate (specific activity, 15-20 Ci/mmol) and [γ-32P]-ATP were obtained from New England Nuclear (USA). IP3, phosphatidylcholine (PC) from egg yolk, phospholipase D from peanut, 12-O-tetradecanoyl phorbol 13-acetate (TPA), myelin basic protein (MBP), 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H7), staurosporine (SS), phenylmethylsulphonylfluoride (PMSF), MBP_{4-14} and other reagents were purchased from Sigma Chemical Co. (St, Louis, MO, USA).

Synthesis of 1,2-Dimyristoylphosphatidylethanol (PET) For the transphosphatidylation reaction, phospholipid was dis-

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solved in diethyl ether at a concentration of 5 mg/ml for 1,2-DMPC. A 1.5 vol. of 100 mM acetate buffer (pH 5.6) containing 100 mM $CaCl_2$ was added to the 1,2-DPMC solution in ether, followed by ethanol and phospholipase D to a final concentration of 5% and 0.125%, respectively (approximately 2 units enzyme/ 2.5 ml solution). The incubation flask was tightly closed and incubation was carried out at 28-30°C under stirring to ensure complete mixing of both phases. After 30 min an equal amount of phospholipase D was added and incubation continued for 60 min. Ether was then evaporated under a stream of nitrogen and the reaction stopped by the addition of 4.3 vol. of chloroform/methanol (5:8, v/v), followed by stirring for 30 min. Extraction and partitioning were performed according to Omedeo Salé *et al.*[16] with the omission of the filtration step. PET was easily separated from undigested 1,2-DMPC and traces of phosphatidic acid (PA) by DEAE Sephadex A25 ion-exchange column in the acetate form[16]. Before applying it to the column, the lipid mixture was washed in the chloroform/methanol/water system of Folch *et al.*[17] in the presence of HCl (0.02 N) to convert the Ca^{2+} salt form of PA to the acid form. The lipid solution was applied in chloroform/methanol (7:3, v/v) (up to 8 mg of lipid per ml of bed volume); the column was washed with 10 bed volumes of the same solvent for the recovery of undigested 1,2-DMPC and with 10 bed volumes of chloroform/glacial acetic acid (3:1, v/v) for the recovery of free fatty acids, PET was eluted with 20 bed volumes of 10 mM sodium acetate in methanol, purified by phase partitioning into the chloroform/ methanol/ water system[17] and checked by high-performance thin-layer chromatography (HPTLC, Whatman Chemical Separation Inc.) in three different solvent systems: (A) chloroform/acetone/methanol/acetic acid/water (50:15:15:10:5); (B) chloroform/methanol/water (110:40:6); (C) chloroform/methanol/acetic acid/water (60:40:4:2). Homogeneity of the sample was also checked by reverse phase thin-layer chromatography (RP18, HPTLC, Merk) developed twice at 34°C in the solvent system methanol/acetonitrile/water (19:5:1, by vol.)(the chromatographic tank was pre-equilibrated at 34°C before the first run) PET was quantified by phosphorus determination[18]. Fast atom bombardment-mass spectrometry (FAB-MS) was recorded on a VG 7070 EQ mass spectrometer and samples applied to a matrix of glycerol.

Cell Culture NG108-15 cells were cultured in 35 mm diameter plastic dishes containing 1 ml of Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum, 2 mM L-Glutamate, 2% 50 x HAT (final concentrations, 0.1 mM hypoxanthine, 4 μM aminopterin, 16 μM thymidine), 100 μg/ml of streptomycin and 100 IU/ml of penicillin. The culture dishes were maintained for 5 days after passage at 37°C in an incubator under a humidified atmosphere will 10% CO_2. Medium was changed daily. At the time of the experiments, the cells had reached confluence.

Incubation with Exogenous PET PET in chloroform/ methanol (2:1 by volume) was evaporated under nitrogen gas, 1 ml of the cell culture medium without fetal calf serum was added and the suspension was mixed and sonicated in a water bath. The concentration was adjusted to the required PET concentration-using medium without fetal calf serum. The cells were then incubated with this PET-containing medium for various times with or without the PKC inhibitors SS or H7. Control cells were incubated for the same times with the same medium but without PET. After the incubation, cells were harvested as described and processed further for lipid analyses or IP_3 assays.

IP_3 Assays A receptor-binding assay developed by Bredt *et al.*[19] was used with slight modifications. Harvested cells in trichloroacetic acid (TCA, 0.5 M) were centrifuged at 2000 x g for 15 min at 4°C. The pellet was mixed, resuspended in 250 μl 1 M NaOH and stored for protein analysis[20]. The supernatant was transferred to glasstubes and extracted four times with 1.25 ml water-saturated diethylether. Adding 20 μl 500 mM Tris buffer, pH 8.4, neutralized the extract. Rat or bovine cerebella were homogenized in cold buffer A (50 mM Tris, 1 mM EDTA, 1 mM 2-mercaptoethanol, pH 7.7) and washed by repeated centrifugations at 15000 x g for 15 min. Cerebella membranes were stored in aliquots at -70°C. 0.2 pmol [^{3}H]- IP_3 (20 cpm/fmol) was added to Eppendorf Microfuge tubes together with aliquots of the cerebella membranes diluted with buffer B (same as buffer A but with pH 8.6) to yield 60 μg protein/tube. Standard solutions of IP_3 or neutralized cell extracts were added to a final volume of 275 μl/tube. After mixing, tubes were left on ice for 10 min. The displacement of [^{3}H]- IP_3 bound to cerebella membranes was stopped by centrifugation at 12000 x g in a Beckman Microfuge. The supernatant was carefully aspirated and the pellet was resuspended in 500 μl water and taken to scintillation vials for scintillation counting. A standard curve of 1-100 nM IP_3 was used. 50% Displacement of bound [^{3}H]- IP_3 was obtained at 10-15 nM IP_3. The displacement curves from rat and bovine cerebella had similar profiles. Identical concentrations of IP_3 were detected using membranes from the two sources. Coefficient of variation was 6.6% (within assay variation) and 6.8% (between assay variation).

Phorbol Ester Pretreatment In order to down-regulate the PKC activity in NG108-15 cells, the cells were incubated with 100 nM TPA for 16 h in the cell culture medium. The TPA-containing medium was removed, cells were washed with fresh medium and the PET containing medium was added as described above. After varying time periods, incubations were terminated through removal of the medium and addition of ice-cold TCA (0.5 M) after which the cells were harvested and IP_3 was assayed.

PKC Activity PKC activity was assayed in isolated membranes from NG108-15 cells, as described by Chakravarthy *et al.*[21] using the specific PKC substrate MBP_{4-14}[22] or the non-specific substrate MBP and [^{32}P]-ATP. Cells which had been incubated for 5 h in the presence or absence of PET, were scraped in ice cold lysation buffer (50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 5 mM EGTA, 100 μM PMSF) and homogenized with

a Polytron^R homogenizer. The suspension was centrifuged at 48400 x g for 25 min to obtain a crude membrane pellet. Membranes were subsequently incubated in 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 2 μM $CaCl_2$, 200 μM sodium pyrophosphate, 2 mM NaF, 200 μM PMSF, 40 μM MBP_{4-14} and 100 μM [^{32}P]-ATP (100-200 cpm/pmol). After 10 min at 20°C, stimulations were interrupted by addition of 40 μl 1 M TCA and 80 μl of the solution was spotted on Whatman P81 filters. These were washed in 75 mM phosphoric acid and taken for scintillation counting.

Statistical Analysis Statistical analyses were performed using Student's two-tailed *t*-test. Differences were considered significant if $P < 0.05$. Data are presented as mean ± SEM.

RESULTS

PET Effects on Cellular IP_3 PET was incorporated into membranes of NG108-15 cells. The incorporation caused an increase in the basal cellular levels of IP_3. This increase was time- and dose-dependent and similar to the increase caused by exposure of the cells to ethanol with a concomitant endogenous PET synthesis[11]. The effect was significant and was not due to a general effect of exogenous phospholipid since phosphatidylcholine at equimolar concentrations did not elicit the same change (Figure 1).

Effects of PKC Inhibition and Down-regulation When cells were preincubated with the PKC inhibitors SS or H7, the same PET-induced increase in IP_3 was observed relative controls as when the inhibitors were not present (Figure 2). Thus the inhibition of PKC activity did not affect the ability of PET to induce changes in cellular inositol phosphate metabolism. Down-regulation of PKC activity through phorbol ester treatment over night did not abolish the PET response either (Figure 3). On the contrary, there was a slight enhancement of the PET-induced increase in IP_3 through this treatment.

PET Effects on PKC Activity PET incorporation into membranes from NG108-15 cells had no effect on the phosphorylation of the PKC-specific substrate MBP_{4-14}, thus indicating that PET does not affect PKC activity in this system (Figure 4). When the non-specific phosphorylation substrate MBP was used instead of the PKC-specific peptide, a (non-significant) tendency towards an increase in phosphorylation activity was seen in PET-treated membranes compared to controls (Figure 4).

DISCUSSION

In the present paper we further characterize the previously described stimulatory effect of PET on levels of IP_3 in NG108-15 cells[11]. We demonstrate that PKC does not seem to be involved in the observed PET-specific changes since no change in PKC activity was observed in cells treated with PET. Moreover, neither PKC inhibition nor down-regulation abolished the effects of exogenous PET.

The described negative results of the effects of PET on PKC are in contrast to those of Asaoka *et al.*[12] who demonstrated that PET was able to partially mimic the effects of phosphatidylserine in a cell-free system. PET was thus by these authors shown to have a stimulatory effect on PKC in this system. NG108-15 cells normally contain phosphatidylserine at levels equivalent to approximately 5-10%

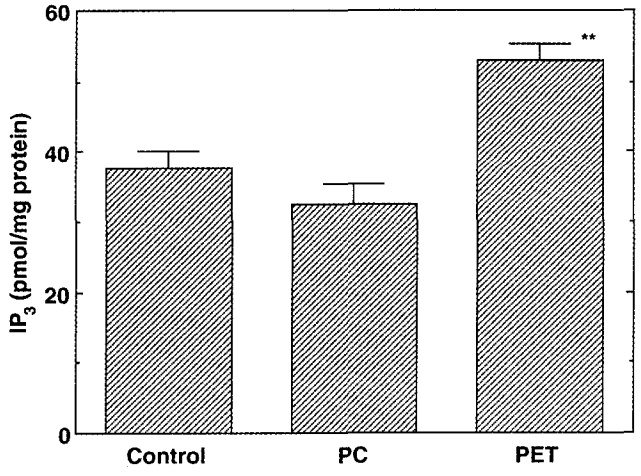

Figure 1. Effects of PET and phosphatidylcholine (PC) on IP_3 levels in NG108-15 cells. NG108-15 cells were incubated for 3 h in the presence of PET or PC (30 μM). Data are represented as the mean ± SEM of five determinations. Double asterisk signifies $P < 0.01$ compared to the control according to Student's *t*-test.

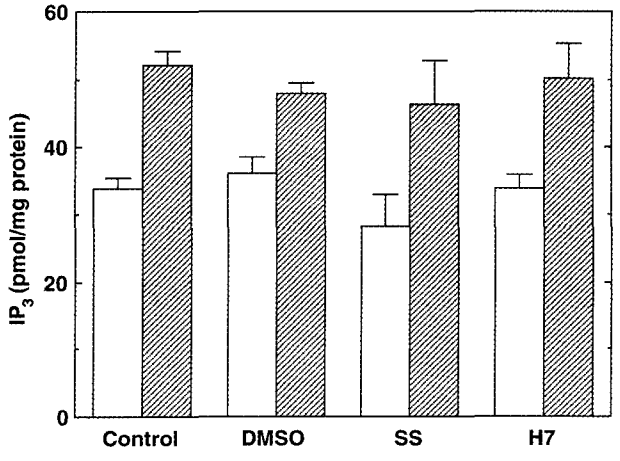

Figure 2. PKC inhibition in controls (open bars) and PET-treated (hatched bars) NG108-15 cells. NG108-15 cells were incubated for 1 h in 30 μM PET in the presence or absence of staurosporine (SS) or 1-(5-isoquinolinysulfonyl)-2-methylpiperazine (H7). Data are represented as the mean ± SEM of five determinations. Control, water; DMSO, dimethylsulfoxide; SS, SS in DMSO; and H7, H7 in water.

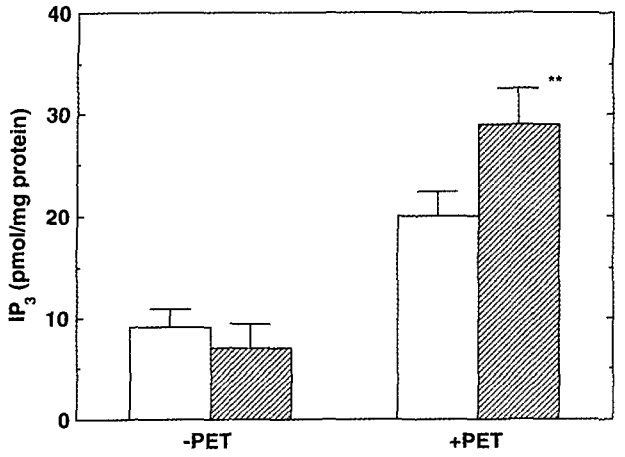

Figure 3. PKC down-regulation and PET-induced IP_3 increase in DMSO (open bars) and 12-O-tetradecanoyl phorbol-13-acetate (TPA)-treated (hatched bars) NG108-15 cells. NG108-15 cells were incubated with DMSO or TPA overnight (15 h, 100 nM) before being exposed to 30 μM PET for 3 h. Data are represented as the mean ± SEM of five determinations. Double asterisk signifies $P < 0.01$ compared to the control according to Student's *t*-test.

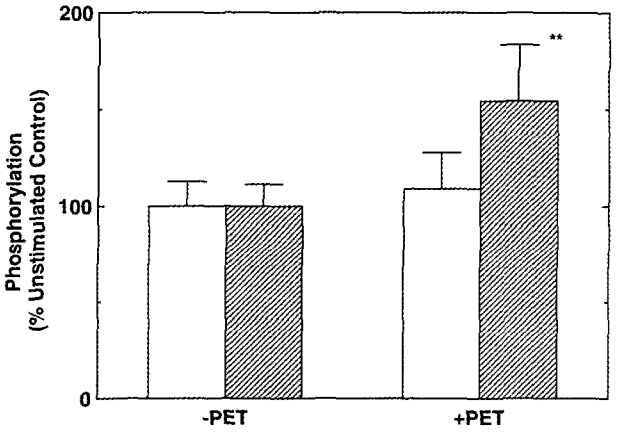

Figure 4. The phosphorylation of the PKC-specific phosphorylation substrate myelin basic protein 4-14 (MBP_{4-14}) (open bars) and non-specific phosphorylation substrate MBP (hatched bars) by controls and PET-treated membranes from NG108-15 cells. Data are represented as the mean ± SEM of five determinations. Double asterisk signifies $P < 0.01$ compared to the control according to Student's *t*-test.

of total phospholipids in their cell membranes and it seems reasonable to suppose that the endogenous levels of this activator are in great excess of that which is required to activate the PKC present in the cells. This would then conceal the effect that PET might have on its own if present in membranes without or with a small amount of phosphatidylserine. In addition, Asaoka[23] has also demonstrated a PKC subclass specificity in the activation by PET, with PKC_γ being the isozyme most activated at physiological Ca^{2+} concentrations similar in size to the concentrations used here (2-3 μM). In the present study, we have not specifically examined the effects of PET on the different PKC isozymes and theoretically therefore, a small isozyme-specific effect might remain undetected in our assay of total PKC phosphorylation activity, especially since the total PKC activity in NG108-15 cells is rather small compared to many other cell lines. In any case, since non-specific PKC inhibition and down-regulation did not remove the PET effect on IP_3 levels, it seems unlikely that PKC activity changes play a major role in mediating this effect.

An interesting side effect of PET became evident when we used the non-specific phosphorylation substrate MBP. In these assays it seemed that PET stimulated the phosphorylation or alternatively inhibited the dephosphorylation of this substrate. The specificity and functional role of such an effect remain to be elucidated.

There are several possibilities for other mechanisms through which the PET effect on IP_3 may be mediated. In other systems PET has been shown to have specific effects on certain membrane-bound enzymes including Na^+/K^+-ATPase and 5′-nucleotidase[9]. Therefore, a direct effect on the enzymes involved in the metabolism of IP_3 may also be a possibility but other more indirect effects can of course not yet be excluded. PET has, for example, also been shown to have various effects on the physical properties of membranes and an indirect effect on membrane-associated enzymes through changes in membrane parameters is also a possibility that merits further study[9,10].

In conclusion, we have presented evidence suggesting that PET increases IP_3 levels in NG108-15 cells through a mechanism which does not involve a change in the activity of PKC. The exact mechanism behind the increase in basal IP_3 levels remains to be elucidated.

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