Volume 314, number 3, 489–492 FEBS 11923 December 1992

Evidence for receptor-linked activation of phospholipase D in rat parotid glands

Stimulation by carbamylcholine, PMA and calcium

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Received 22 October 1992

In order to test if phospholipase D (PLD) activity exists in the rat parotid gland, we took advantage of the fact that, in the presence of ethanol, PLD generates phosphatidylethanol (PEth) via a transphosphatidylation reaction. Lipid extracts of parotid acini prelabelled with [^{3}H]myristic acid were analyzed by thin layer chromatography to determine [^{3}H]phosphatidylethanol ([^{3}H]PEth) formation. Carbamylcholine (1 mM) stimulated [^{3}H]PEth formation in the presence of 2% ethanol, this effect was completely inhibited by atropine (10 μM). PMA (0.1–1 μM) and ionomycine (10 μM) also caused [^{3}H]PEth generation. We conclude that a phospholipase D activity is present in the rat parotid gland and is regulated by muscarinic cholinergic receptors. Protein kinase C and calcium could also modulate this activity. This report provides the first evidence for the existence and receptor-linked regulation of phospholipase D in an exocrine gland, the rat parotid gland.

Phospholipase D: Muscarinic receptor; Phorbol ester; Calcium; Parotid acini

1. INTRODUCTION

Many hormone and neurotransmitter receptors transduce signals in a wide variety of cells through the activation of phospholipases and the production of second messenger molecules derived from phospholipids. Evidence is rapidly accumulating to indicate that phospholipase D (PLD) is involved in signal transduction processes [1–11].

Phospholipase D cleaves the terminal phosphodiester bond of phospholipids to form phosphatidic acid (PA) and appropriate bases. In addition, PLD possesses the unique ability to catalyse a transphosphatidylation reaction in which the phosphatidyl moiety of the phospholipid substrate is transferred to a primary alcohol such as ethanol, thereby producing phosphatidylalcohol [12]. This ability is currently used to evidence PLD activity in intact cells.

Mammalian PLD prefers phosphatidylcholine (PC) as a substrate, although phosphatidylinositol (PI) and phosphatidylethanolamine (PE) may also be degraded [1–3,6,13,14]. Receptor-linked activation of PLD may occur via several distinct mechanisms and may involve multiple factors including calcium [9–11,13,15,16], protein kinase C (PKC) [5,9,11,15–19] and G-proteins [1,13,15,16,20]. It has been proposed that PLD may be regulated by direct receptor coupling via G-proteins [16]. However, some studies demonstrated that PLD regulation may involve G-protein- as well as PKC-dependent mechanisms [5,11,16,18]. Since PKC activation is downstream to phospholipase C stimulation, it has been suggested that phospholipase D regulation may occur as a secondary effect of phospholipase C activation [5,8,15,16].

In the rat parotid gland, two main signal transduction processes have actually been described: the cAMP pathway involving adenylate cyclase and the calcium/phospholipid pathway involving a phospholipase C. Muscarinic cholinergic receptors have been shown to be coupled to PLC acting on phosphatidylinositol bisphosphate and leading to diacylglycerol (DAG) and inositol trisphosphate (IP_3) production [21]. DAG activates PKC and IP_3 mobilizes intracellular calcium.

In the present study, we report for the first time that a phospholipase D activity is also present in the acinar cells of parotid gland and may constitute a novel signal transduction mechanism. By measuring phosphatidylethanol formation, we show that this phospholipase D activity is modulated by muscarinic cholinergic receptors. We also show that a PKC activator (4β-phorbol 12-acetate 13-myristate) and a calcium ionophore stimulate this phospholipase D activity.

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Abbreviations: PLD, phospholipase D; PEth, phosphatidylethanol; PKC, protein kinase C; PMA, 4β-phorbol 12-myristate 13-acetate; PA, phosphatidic acid.

2. MATERIALS AND METHODS

2.1. *Materials*

2.1.1. Animals

Male albino Sprague–Dawley rats, 5–6 weeks old were used in this study.

2.1.2. Chemicals

[9,10(*n*)-^{3}H]myristic acid (2 TBq/mmol; 54 Ci/mmol) was purchased from Amersham, England. Collagenase type CLS II (173 U/mg) came from Seromed, Germany. Hyaluronidase type I-S (290 U/mg), bovine serum albumine (fatty acid-free BSA), 4β-phorbol 12-myristate 13-acetate (PMA), ionomycine and standard lipids were obtained from Sigma, St. Louis, MO, USA. Carbamylcholine came from Merck, Germany. Silica gel G TLC plates were purchased from Cera-Labo, France, and solvents were from Prolabo. All reagents were of the highest purity available.

2.2. *Methods*

2.2.1. *Preparation of parotid acini, radiolabelling and treatments*

Rats were sacrified by cervical dislocation. Parotid glands were rapidly removed, trimmed of their fatty and connective tissues, fragmented in lobules and washed with Krebs–Ringer bicarbonate buffer supplemented with 0.55 mM glucose, 2 mM β-hydroxybutyrate, 10 mM HEPES and 0.1% BSA at 37°C. Parotid acini were preparated by incubation of parotid lobules in collagenase (1 mg/ml) and hyaluronidase (0.25 mg/ml) for 45 min at 37°C under a stream of O_2/CO_2 (95:5%), dissociation by pipetting and filtration (120 μm pore size).

Parotid acini were labelled for 3 h with [^{3}H]myristic acid (10 μCi/ml) at 37°C, under O_2/CO_2. Parotid acini were washed with buffer and incubated 20 min in buffer containing or not 2% ethanol. Parotid acini were separated in several incubations and drugs were added. The stimulation was continued for 20 min and stopped by adding chloroform/methanol/HCl 12 N (100:200:1, v/v).

When incorporation of [^{3}H]myristic acid was studied, parotid acini were incubated for different times with [^{3}H]myristic acid and incubations were stopped by adding chloroform/methanol/HCl 12 N.

2.2.2. Lipid analysis

Total cellular lipids were extracted according to the method of Bligh and Dyer [15] and were dissolved in chloroform. Neutral lipids and phospholipids were resolved by thin-layer chromatography (TLC) using two solvent systems. Chloroform/methanol/methylamine (94.5:45:15, v/v) allowed to separate PI: phosphatidylinositol (R_f = 0.31), PA: phosphatidic acid and PS: phosphatidylserine (R_f = 0.48), PC: phosphatidylcholine (R_f = 0.73), PE: phosphatidylethanolamine (R_f = 0.81) and neutral lipids (R_f = 1) (system 1). Chloroform/methanol/acetic acid/water (50:30:3.3:2, v/v) allowed to separate PC (R_f = 0.27), PI (R_f = 0.43), PS (R_f = 0.54), PA (R_f = 0.64), PE (R_f = 0.81) and neutral lipids (R_f = 1) (system 2). PA (R_f = 0.31) and PEth (R_f = 0.41) were resolved by TLC using the upper phase of a mixture ethylacetate/trimethylpentane/acetic acid/water (130:20:30:100, v/v) (system 3). The silica gels were analysed with a Berthold linear analyser. The plates were scraped and the radioactivity of each lipid species was determined by liquid scintillation to obtain an exact quantification. The lipids were identified by comparison with the standard lipid migration. Phosphatidylethanol was prepared according to a previously described method with small modifications [23].

[^{3}H]Phosphatidylethanol formation was expressed either in cpm or in percentage: radioactivity of PEth × 100/total radioactivity of the lipid extract. The results represented the means ± S.E.M. of *n* determinations as indicated in the figures.

3. RESULTS AND DISCUSSION

Because a unique feature of phospholipase D (PLD) is its ability to catalyse the transfer of the phosphatidyl moiety of phospholipids to alcohol (transphosphatidylation), the formation of phosphatidylalcohol is a useful

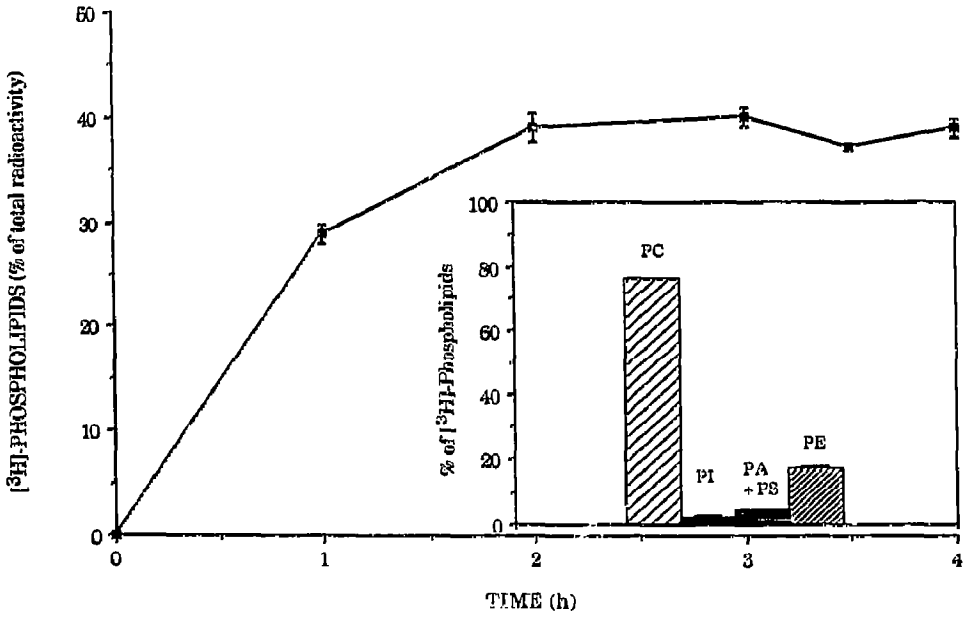

Fig. 1. Incorporation of [^{3}H]myristic acid in phospholipids. Parotid acini were incubated with [^{3}H]myristic acid for different times. [^{3}H]Phospholipids were separated by thin-layer chromatography with the solvent systems 1 and 2. Radioactivity in phospholipids was determined. Results are expressed as the percentage of total incubation radioactivity. Values are means ± S.E.M. from duplicate determinations. The insert represents radioactivity distribution in the different phospholipid classes after a 3 h incorporation. Results are expressed as the percentage of [^{3}H]phospholipids (706,730 ± 67,028 cpm).

indicator of PLD activity [2,3,9,12]. In order to test if PLD activity exists in parotid acini, we studied the formation of [^{3}H]phosphatidylethanol (PEth) from [^{3}H]phospholipids in the presence of ethanol.

The parotid acini were incubated with [^{3}H]myristic acid to label cellular lipids. We observed that a maximal incorporation of [^{3}H]myristic acid in the phospholipids was obtained after 3 h of incubation with the fatty acid (Fig. 1). Then, lipid extracts consisted of [^{3}H]phospholipids (706,730 ± 67,028 cpm) and [^{3}H]neutral lipids (273,075 ± 25,908 cpm) which represented respectively *40.16 ± 0.93% and 15.52 ± 0.36% of the total incubation* radioactivity. After 3 h of incubation, free [^{3}H]myristic acid disappeared from the lipid extracts while radioactive products appeared in the methanol/water fraction (777,008 ± 81,695 cpm, i.e. 44.32 ± 1.29% of total incubation radioactivity). These radioactive products would result from myristic acid metabolism in parotid acini. As shown in the insert of Fig. 1, phosphatidylcholine, the principal phospholipid class in mammalian tissues, was the phospholipid in which radioactivity was mainly localized (76.60 ± 0.01% of [^{3}H]phospholipids).

When the labelled parotid acini were incubated with 2% ethanol, we observed that a novel [^{3}H]phospholipid appeared in cellular lipid extracts (Fig. 2A,B,C). This phospholipid was identified as phosphatidylethanol (PEth) by comparing its migration on TLC plates (R_f) with the one of standard PEth and the one reported in the literature. Radioactivity localized in phosphatidylethanol (599 ± 36 and 861 ± 57 cpm) represented 0.16 ± 0.03 and 0.24 ± 0.04% of [^{3}H]lipid extract after respectively 20 and 40 min of incubation in the presence of ethanol. PEth generation indicated that a low basal phospholipase D activity existed in the parotid glands. When the parotid acini were incubated with 1 mM carbamylcholine in the presence of ethanol, the formation of [^{3}H]PEth was stimulated (1,965 ± 65 cpm), amounting to a 2.3-fold increase over the control level (Fig. 3). This stimulation was completely abolished by a 10 min preincubation of the acini with a low concentration of atropine (10 μM), a muscarinic cholinergic antagonist (Fig. 3), indicating that phospholipase D was activated by the muscarinic cholinergic receptors. We observed that phospholipase D activity was also modulated by a protein kinase C activator, 4β-phorbol 12-myristate 13-acetate (PMA) and by a calcium ionophore, ionomycin. Indeed, PMA at 1 μM increased [^{3}H]PEth formation threefold (2,526 ± 95 cpm) (Fig. 3). PMA at 0.1 μM also stimulated [^{3}H]PEth formation with an almost similar stimulation factor (2.6) (data not shown). Ionomycine, without any important effect at 1 μM, at 10 μM caused a 2.7-fold stimulation of [^{3}H]PEth formation (Fig. 3). We can note that PMA (1 μM) and ionomycine (10 μM) seemed more efficient to stimulate PLD than carbamylcholine used at a high concentration (1 mM).

The mechanisms of PLD activation by muscarinic receptors in parotid acini remain to be determined. Roles of guanine nucleotide binding protein, protein kinase C and calcium must be precised. The involvement of G-proteins in receptor-linked PLD regulation has been reported in some cells like hepatocytes [1,20], platelets [15], endothelial cells [24] and particularly in HL60 granulocytes in which a direct receptor coupling of PLD via G-protein has been evidenced [13,16]. Moreover, based on the observations that phorbol esters and calcium ionophores activated PLD, protein kinase C and calcium are largely described as activators of PLD [5,9,11,13,15–19]. Independent mechanisms of PLD activation by phorbol esters and receptor agonists have been reported in cells such as ovarian granulosa cells, cerebral cortex slices [5,11], while a synergy between

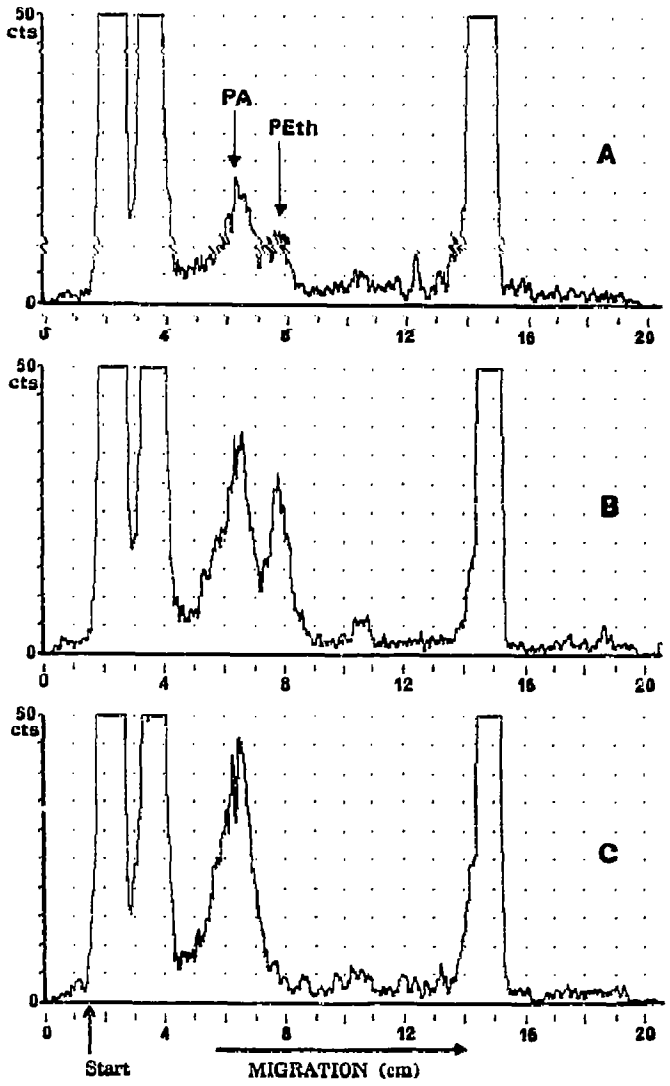

Fig. 2. Separation of [^{3}H]phosphatidylethanol by thin-layer chromatography. Labelled parotid acini were incubated with (A,B) or without ethanol 2% (C) for 20 min and stimulated (B,C) or not (A) with carbamylcholine (1 mM). Cellular lipids were separated by thin layer chromatography with the solvent system 3. Chromatograms obtained after analysis with linear scanner are represented.

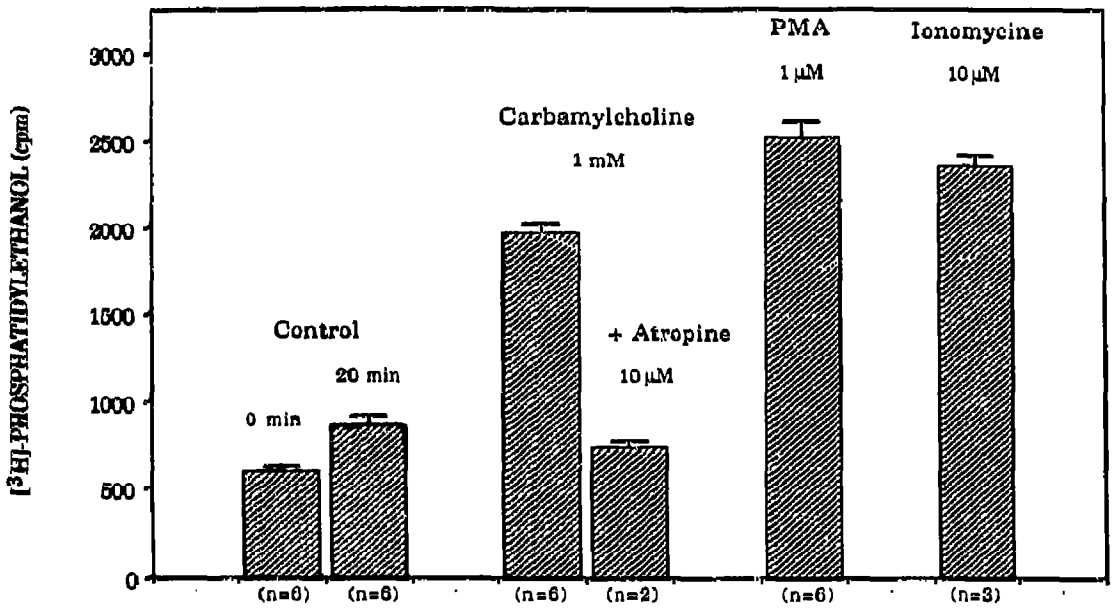

Fig. 3. Stimulation of [^{3}H]phosphatidylethanol formation. Labelled parotid acini were preincubated with ethanol 2% for 20 min. Parotid acini were stimulated or not for 20 min with carbamylcholine (1 mM), PMA (1 μM) or ionomycine (10 μM). When atropine was tested, it was added in the incubation medium for 10 min before stimulation. Phosphatidylethanol was separated by thin-layer chromatography with the solvent system 3. Atropine alone does not induce any significant stimulation (736 ± 36 cpm) (n = 2).

these two pathways was observed in HL60 cells and in promonocytic cell line U 937 [16,18]. Alternatively, a large number of agonists stimulating PLD, also stimulate phosphoinositide-specific phospholipase C and hence generation of IP_3 and DAG. Since DAG activates PKC and IP_3 mobilizes intracellular calcium, it has been concluded that PLD stimulation may be consequence of PLC activation [5,15,16]. This interrelationship between phospholipases C and D has also been suggested in astrocytoma cells stimulated by muscarinic receptors [8]. In rat parotid glands, carbamylcholine activated phospholipase C [21], but our results do not allow us to conclude if PLD activation is a consequence of PLC activation by muscarinic receptors (through DAG production and calcium mobilization) or if these receptors are directly coupled to PLD.

This report provides the first evidence for PLD existence in an exocrine gland and particularly the first evidence for receptor-linked activation of PLD in the rat parotid glands.

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