Protein kinase C and phospholipase D activation in rat parotid glands

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Abstract **We have previously demonstrated that muscarinic and α-adrenergic receptors regulated a phospholipase D (PLD) activity in parotid glands. Since phorbol 12-myristate, 13-acetate (PMA) induced production of phosphatidylethanol (PEt), a stable metabolite widely accepted as marker of PLD activation, we have investigated the role of protein kinase C (PKC) in PLD stimulation in parotid acini. We tested PKC inhibitors on PEt formation elicited by PMA, by muscarinic and adrenergic agents. Staurosporine and chelerythrine, which act on the catalytic domain of PKC, did not allow the attribution of a role for PKC in PLD activation. Indeed, staurosporine did not affect PMA-mediated PLD activity and chelerythrine showed an important non-specific effect, independent of PKC inhibition. On the other hand, calphostin C, which acts on the regulatory domain of PKC, affected PMA- and receptor-mediated PLD stimulation. We attributed this effect to PKC inhibition and we suggested PKC involvement in PLD regulation in parotid gland. Since only PKC inhibitor acting on the regulatory part of the enzyme affected PLD activity, we also suggested that PKC could be involved in PLD activation through a pathway independent of the phosphorylation mechanism.**

Key words: Phospholipase D; Phorbol ester; Carbamylcholine; Epinephrine; Protein kinase C

1. Introduction

Phospholipase D (PLD) catalyzes the hydrolysis of the terminal phosphodiester bond of phospholipids to yield the appropriate free polar head group and phosphatidic acid (PA), a potential lipid second messenger [1–3]. PA is also formed by either de novo synthesis or by the combined actions of phospholipase C (PLC) and diacylglycerol kinase. Phosphatidylcholine, mainly, but also phosphatidylinositol and phosphatidylethanolamine have been reported as substrates for PLD in different mammalian tissues [4–7]. In addition, PLD possesses the unique ability to catalyze a transphosphatidylation reaction in which the phosphatidyl moiety of phospholipids is transferred to a primary alcohol such as ethanol, thereby resulting in the formation of a phosphatidylalcohol such as phosphatidylethanol (PEt) [8]. Although PLD activation has been described in many cells, the mechanisms involved in the regulation of PLD activity still remain unclear.

Taking advantage of the unique ability of PLD to catalyze the transphosphatidylation reaction, we have recently demonstrated the presence of PLD and its regulation by muscarinic and α-adrenergic receptors in the rat parotid gland, an exocrine gland [9,10]. Indeed, we have reported that stimulation of these receptors induced labelled PEt production in [^{3}H]myristic acid- or [^{14}C]stearic acid-labelled parotid acini. Phorbol esters, activators of protein kinase C (PKC), and a calcium ionophore also caused PEt formation [10]. Furthermore, the production of labelled PA in the parotid glands during stimulation of muscarinic and α-adrenergic receptors was attributed to the combined actions of PLC and diacylglycerol kinase [10]. A relationship between the activation of phospholipases C and D may be possible in parotid acini.

The present work was undertaken in order to elucidate the role of PKC in the regulation of PLD activity in the rat parotid gland. For this, we tested PKC inhibitors on the formation of labelled PEt elicited in response to phorbol ester, to muscarinic and α adrenergic agonists. Three inhibitors were used: staurosporine and chelerythrine which act on the catalytic part of PKC, affecting the binding of either ATP or proteinic substrates on PKC and calphostin C which acts on the regulatory part of PKC. We found evidence for PKC involvement in PLD regulation, since calphostin C prevented labelled PEt production. However, staurosporine and chelerythrine were without significant inhibitory effect. Since only the PKC inhibitor acting on the regulatory part of the enzyme affected PLD activity, we suggested that PKC could be involved in PLD regulation in the parotid gland, through a pathway independent of the phosphorylation mechanism.

2. Materials and methods

2.1. Materials

Male albino Sprague–Dawley rats, 6–7 weeks old were used in this study.

[1-^{14}C]Stearic acid (2.1 GBq/mmol; 58 mCi/mmol) was purchased from Du Pont de Nemours/New England Nuclear division, France. Collagenase type CLS III Worthington (116 U/mg) was from Seromed, Germany. Bovine serum albumin (fatty acid-free BSA), 4β-phorbol 12-myristate 13-acetate (PMA) and epinephrine were obtained from Sigma Chemical Co., St. Louis, MO. Phosphatidylethanol standard was purchased from Avanti Polar Lipids Inc., Birmingham, AL. Carbamylcholine was from Merck, Darmstadt, Germany. Precoated silica gel plates were purchased from J.T. Baker Inc., 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 killed by CO_2 inhalation. Parotid glands were rapidly removed, trimmed of their fatty and connective tissues and fragmented into small pieces. Parotid gland pieces were washed with buffer (Krebs–Ringer bicarbonate buffer supplemented with 0.55 mM glucose, 10 mM HEPES and 0.1% BSA, pH 7.4) at 37°C. Tissue was incubated in this buffer plus collagenase (116 U/ml) for 30 min at 37°C under a stream of O_2/CO_2 (95%/5%). Parotid acini were dissociated by successive pipetting through pipette tips of decreasing diameters. At the end of this dissociation period, the preparation was filtered through nylon mesh (120 μm pore size). Filtrate was centrifuged for 5 min at 50 × *g* to eliminate medium and collagenase. The pellet was resuspended in buffer and washed by centrifugation through a solution of 4% BSA in the same buffer (at 50 × *g*, for 5 min). The resulting pellet,

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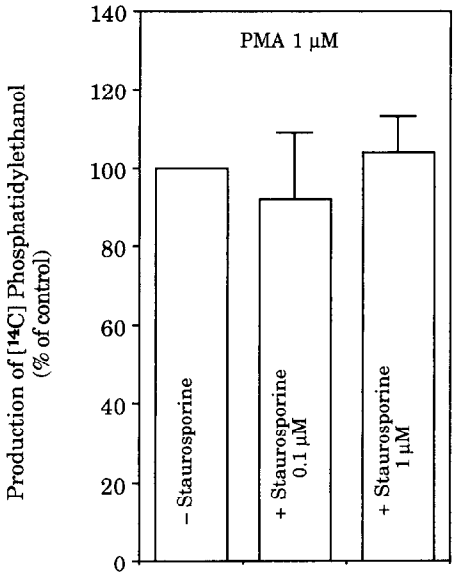

Fig. 1. Effect of staurosporine on the formation of [^{14}C]PEt. [^{14}C]Stearic acid-labelled-parotid acini were preincubated for 10 min in the presence or in the absence of staurosporine and then stimulated or not for 20 min with PMA (1 μM). [^{14}C]PEt production was determined as described in section 2. Data were expressed as the percentage of the control, i.e. the net [^{14}C]PEt production induced in parotid acini stimulated with PMA in the absence of staurosporine (0.026% ± 0.001).

which was the final parotid acinar preparation, was resuspended in buffer.

[^{14}C]Stearic acid was dissolved in buffer by vigorous vortexing and sonification. Parotid acini were incubated for 3 h in 5 ml of buffer supplemented with [^{14}C]stearic acid (10 μCi/ml) at 37°C, under a stream of O_2/CO_2. Radioactivity in the incubation medium was then eliminated by centrifugation at 50 × g, for 5 min. Parotid acini were resuspended in buffer and aliquoted in 1 ml of incubation medium.

Ethanol was added to the incubation medium to obtain a final concentration of 2% (340 mM). Acini were preincubated for 5 min and then reaction was started by addition of stimuli. The final concentration of dimethyl sulfoxide in which PMA was initially dissolved did not exceed 0.1% in the assay, a concentration that had no discernible effect on the parameters measured. The reaction was stopped by adding chloroform/methanol/HCl 12 N (100:200:1 by volume).

2.2.2. Lipid analysis. Total cellular lipids were extracted according to the method of Bligh and Dyer [11]. The lower chloroform phase was dried and spotted on silica gel plates. The plates were developed using the upper phase of a mixture consisting of ethylacetate/trimethylpentane/acetic acid/water (130:20:30:100 by volume), as described previously [10]. The silica gels were analysed with a TLC linear analyser (Berthold) for at least 1 h, to have a good estimation of the radioactivity. The background of the thin layer chromatography plate was determined and subtracted from each experimental sample. Lipids were located by staining with iodine vapor and identified by comparison with standard lipids.

The amount of [^{14}C]phosphatidylethanol and [^{14}C]phosphatidic acid produced was determined as a percentage of the total radioactivity (about 30,000 cpm). The basal value was substracted from the stimulated value to determine a net production of PEt or PA. Assays were done in duplicate. Each experiment was performed 2 times. Data obtained from a representative experiment were presented as the mean with the range for the duplicate samples.

3. Results and discussion

Staurosporine is an inhibitor of PKC which interferes with the binding of ATP on the catalytic domain of the enzyme [12,13]. This feature of staurosporine appears to be responsible for its lack of selectivity for PKC over several other kinases. We tested staurosporine at 0.1 μM and 1 μM on the [^{14}C]PEt production induced by the phorbol ester, PMA, in the parotid acini. We found that this response was not significantly affected

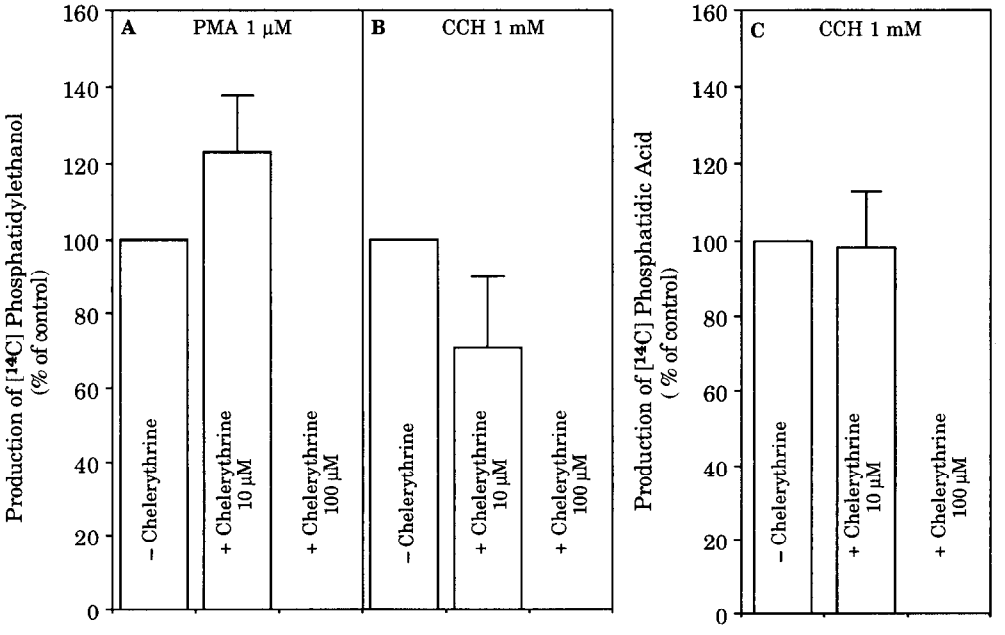

Fig. 2. Effect of chelerythrine on the formation of [^{14}C]PEt and of [^{14}C]PA. [^{14}C]Stearic acid-labelled-parotid acini were preincubated for 10 min in the presence or in the absence of chelerythrine and then stimulated or not for 20 min with PMA (1 μM) (A) or carbamylcholine (1 mM) (B,C). [^{14}C]PEt (A,B) and [^{14}C]PA (C) productions were determined as described in section 2. Data were expressed as the percentage of the control, i.e. the net [^{14}C]PEt or [^{14}C]PA production induced in parotid acini stimulated in the absence of chelerythrine (1 μM PMA: [^{14}C]PEt = 0.060% ± 0.001; 1 mM CCH: [^{14}C]PEt = 0.058% ± 0.008, [^{14}C]PA = 0.376% ± 0.059).

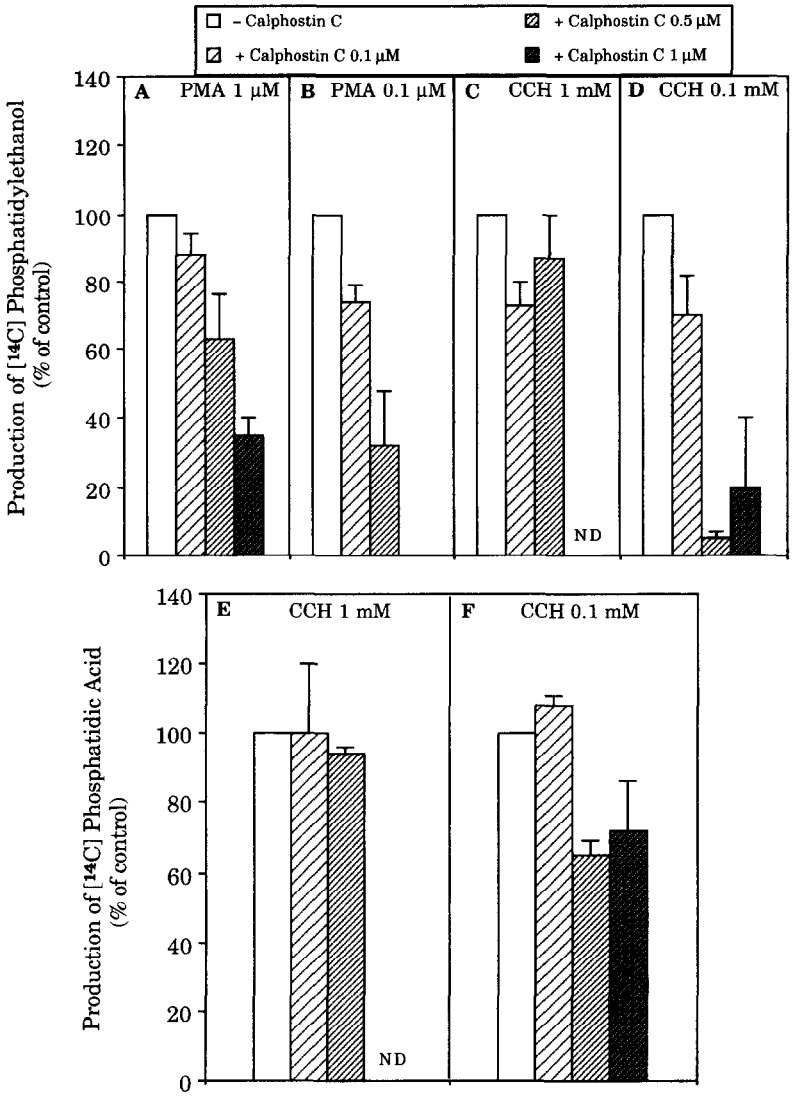

Fig. 3. Effect of calphostin C on the formation of [^{14}C]PEt and of [^{14}C]PA. [^{14}C]stearic acid-labelled-parotid acini were preincubated for 10 min in the presence or in the absence of calphostin C and then stimulated or not for 20 min with PMA (1 μM or 0.1 μM) (A,B) or carbamylcholine (1 mM or 0.1 mM) (C,D,E,F). [^{14}C]PEt (A,B,C,D) and [^{14}C]PA (E,F) productions were determined as described in section 2. Data were expressed as the percentage of the control, i.e. the net [^{14}C]PEt or [^{14}C]PA production induced in parotid acini stimulated in the absence of calphostin C (1 μM PMA: [^{14}C]PEt = 0.023% ± 0.003; 0.1 μM PMA: [^{14}C]PEt = 0.019% ± 0.001; 1 mM CCH: [^{14}C]PEt = 0.025% ± 0.003, [^{14}C]PA = 0.106% ± 0.010; 0.1 mM CCH: [^{14}C]PEt = 0.020% ± 0.001, [^{14}C]PA = 0.079% ± 0.005). N.D.: not determined.

by staurosporine (0.1 and 1 μM) treatment (Fig. 1). However, it should be noted that 1 μM staurosporine alone induced a weak [^{14}C]PEt formation (0.028% ± 0.002 vs. 0.021% ± 0.001 in non-treated samples). This result seems in agreement with other previous data indicating that staurosporine had different non-specific effects, independent of PKC inhibition [13]. In particular, it has been reported that staurosporine blocked PKC activation and independently induced PLD stimulation in human PMNs [14]. Thus, whereas 0.1 μM staurosporine did not affect PLD activity, used at 1 μM, it could itself weakly enhance this activity in parotid gland.

Chelerythrine interacts with the catalytic domain of PKC and is a competitive inhibitor with respect to the proteinic substrates of the kinase [15]. At 10 μM, chelerythrine did not affect significantly the [^{14}C]PEt production induced by PMA or by carbamylcholine (CCH), the muscarinic cholinergic agonist (Fig. 2A,B). On the other hand, preincubation of the parotid acini with chelerythrine at 100 μM completely inhibited these responses (Fig. 2A,B). However, the inhibition of these responses obtained with 100 μM chelerythrine could result from a non-specific effect of the PKC inhibitor rather than PKC inhibition itself. Indeed, 100 μM chelerythrine also completely inhibited the production of [^{14}C]PA stimulated by CCH (Fig. 2C). It is therefore likely that chelerythrine at 100 μM affected both phospholipase C and D activities, whereas when used at 10 μM it failed to affect all of them in the parotid gland. Considering our results, we have previously suggested that PLC activation was required for PLD activation by carbamylcholine [10]. In this case, non-specific inhibition of PLC would result in PLD inhibition and thus, chelerythrine did not provide evidence for a role of PKC in PLD regulation in the parotid gland.

Calphostin C is a potent and specific inhibitor of PKC which interacts with the regulatory domain of the kinase [16]. This compound showed inhibitory effects by competing at the bind-

ing site for diacylglycerol and phorbol esters [16]. In a first time, we tested calphostin C at different concentrations on the [^{14}C]PEt production elicited by PMA at maximal (1 μM) and submaximal (0.1 μM) concentrations. Fig. 3A,B showed that preincubation of parotid acini in the presence of calphostin C affected PLD activation by PMA. Calphostin C induced a concentration-dependent decrease of the control value (PMA stimulation without calphostin C treatment). However, a more important calphostin C effect was observed on the PLD activation obtained with PMA at a submaximal concentration (Fig. 3B). Indeed, a complete inhibition of the [^{14}C]PEt production was observed with 1 μM calphostin C, when parotid acini were stimulated with 0.1 μM PMA (Fig. 3B), while inhibition reached only 75%, when acini were stimulated with 1 μM PMA (Fig. 3A). These results could be interpreted as being a competitive inhibition by calphostin C of the PMA-induced PLD activation. Fig. 3C,D represents the effect of calphostin C at different concentrations on the [^{14}C]PEt production in parotid acini stimulated by CCH (1 mM or 0.1 mM). While we did not find any important effect on this response induced by 1 mM CCH (Fig. 3C), we observed a significant inhibition of calphostin C on the one induced by 0.1 mM CCH (Fig. 3D). This inhibition was observed at all the calphostin C concentrations tested and reached 95%. It should be observed that calphostin C at 0.5 and 1 μM affected also the [^{14}C]PA production induced by CCH (0.1 mM) (Fig. 3F). However, this effect on the PA level was very lower (35%) than the one related to the PEt level. Taking together these data obtained with calphostin C, it appears that PLD activation by phorbol ester and muscarinic receptor agonist would be mediated through PKC dependent processes. A similar conclusion was found for PLD activation by an α adrenergic agonist, epinephrine. Indeed, [^{14}C]PEt production induced by 10 μM epinephrine in parotid acini preincubated in the presence of 1 μM calphostin C represented only 7% of the response observed in acini non-treated with the PKC inhibitor (data not shown).

While the effects observed with calphostin C on PLD activation in parotid gland indicated a PKC involvement, the ones observed with chelerythrine and staurosporine led to conflicting conclusion. These contrasting data could be explained by the different targets of the different PKC inhibitors used. Indeed, PLD inhibition was obtained in parotid acini treated only with PKC inhibitor acting on the regulatory domain of the enzyme. It is possible that phosphorylation by PKC was not required to PLD activation even if PKC dependent processes mediated PLD activation. A similar conclusion has been previously reported by Conricode et al. [17]. These authors demonstrated that PMA stimulated PLD in fibroblast membranes supplemented with PKC, but did not need exogenous ATP. They concluded the involvement of PKC in the activation of PLD and suggested that ATP-dependent phosphorylation was not required [17]. Our data suggest that such a pathway of PLD activation could be involved in a cellular system.

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References

[1] Motasim Billah, M. (1993) Curr. Opinion Immunol. 5, 114–123.
[2] Motasim Billah, M., Pai, J.-K., Mullmann, T.J., Egan, R.W. and Siegel, M.I. (1989) J. Biol. Chem. 264, 9069–9076.
[3] Thompson, N.T., Bonser, R.W. and Garland, L.G. (1991) Trends Pharmacol. Sci. 12, 404–408.
[4] Huang, C., Wykle, R.L., Daniel, L.W. and Cabot, M.C. (1992) J. Biol. Chem. 267, 16859–16865.
[5] Balsinde, J., Diez, E., Fernandez, B. and Mollinedo, F. (1989) Eur. J. Biochem. 186, 717–724.
[6] Motasim Billah, M., Pai, J.-K., Mullmann, T.J., Egan, R.W. and Siegel, M.I. (1989) J. Biol. Chem. 264, 9069–9076.
[7] Taki, T. and Kanfer, J.N. (1979) J. Biol. Chem. 254, 9761–9765.
[8] Dawson, R.M.C. (1967) Biochem. J. 102, 205–210.
[9] Guillemain, I. and Rossignol, B. (1992) FEBS Lett. 314, 489–492.
[10] Guillemain, I. and Rossignol, B. (1994) Am. J. Physiol. 266, C692–C699.
[11] Bligh, E.G. and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911–917.
[12] Tamaoki, T., Nomoto, H., Takahashi, I., Kato, Y., Morimoto, M. and Tomita, F. (1986) Biochem. Biophys. Res. Commun. 135, 397–402.
[13] Rüegg, U.T. and Burgess, G.M. (1989) Trends Pharmacol. Sci. 10, 218–220.
[14] Périanin, A., Combadière, C., Pedruzzi, E., Djerdjouri, B. and Hakim, J. (1993) FEBS Lett. 315, 33–37.
[15] Herbert, J.M., Augereau, J.M., Gleye, J. and Maffrand, J.P. (1990) Biochem. Biophys. Res. Commun. 172, 993–999.
[16] Kobayashi, E., Nakano, H., Morimoto, M. and Tamaoki, T. (1989) Biochem. Biophys. Res. Commun. 159, 548–553.
[17] Conricode, K.M., Brewer, K.A. and Exton, J.H. (1992) J. Biol. Chem. 267, 7199–7202.