Absence of a direct role of phospholipid methylation in stimulus–secretion coupling and control of adenylate cyclase in guinea-pig and rat parotid gland

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The present study was undertaken to investigate a possible involvement of phospholipid methyltransferases in the coupling of receptor-mediated stimulation to secretion. Phospholipid methyltransferases were assayed in isolated parotid acini in the presence of carbamoylcholine or isoprenaline. Carbamoylcholine reduced the incorporation of methyl groups into phospholipids, whereas isoprenaline showed no effect. Amylase secretion stimulated either by carbamoylcholine or by isoprenaline could not be affected by inhibitors of methyltransferases (3-deaza-adenosine alone or plus homocysteine thiolactone) under conditions where phospholipid methylation was strongly inhibited. The activity of adenylate cyclase in isolated parotid microsomal membranes was not inhibited or stimulated by *S*-adenosyl-homocysteine or -methionine respectively. These results indicate that phospholipid methylation does not play an essential role in stimulus–secretion coupling in the parotid gland.

The stepwise methylation of PtdEtn to PtdCho has been shown to play an important role in receptor-mediated events in various cell types, such as histamine secretion in rat mast cells (Ishizaka *et al.*, 1980, 1981), chemotaxis in neutrophils (Pike *et al.*, 1979), histamine release in rat basophilic leukaemic cells (Crews *et al.*, 1980; McGivney *et al.*, 1981) and the β -adrenergic stimulated rise of cyclic AMP in rat reticulocytes (Hirata *et al.*, 1979), HeLa cells (Hirata *et al.*, 1980) and C⁶-astrocytoma cells (Strittmatter *et al.*, 1979) (for review, see Hirata & Axelrod, 1980). In these systems inhibition of methylation blocked the ability of the cell to respond to the external signal.

It has been claimed that the coupling of the β -receptor to the adenylate cyclase is facilitated by an enhanced phospholipid methylation by increasing the fluidity of the membrane (Hirata *et al.*, 1980). The methylation itself is stimulated by β -agonists (Hirata *et al.*, 1980).

Abbreviations used: PtdEtn, phosphatidylethanolamine; PtdMeEtn, phosphatidyl-*N*-methylethanolamine; Ptd(Me)₂Etn, phosphatidyl-*NN*-dimethylethanolamine; PtdCho, phosphatidylcholine; SAM, *S*-adenosylmethionine; SAH, *S*-adenosylhomocysteine; BSA, bovine serum albumin.

In the present work we have investigated whether phospholipid methylation is involved in receptor-mediated events in the parotid gland. The model has the advantage of having at least two very sensitive and easily measurable responses, namely (1) the amylase secretion as an overall response, (2) changes in the adenylate cyclase activity in response to β -adrenergic agents.

Amylase secretion can be stimulated by carbamoylcholine with Ca²⁺ as second messenger and by β -adrenergic agonists with cyclic AMP as second messenger (Butcher & Putney, 1980). According to the results obtained, phospholipid methylation does not seem to play an essential role in stimulus–secretion coupling in the parotid gland.

Materials and methods

Materials

[*Me*-³H]Methionine (sp. radioactivity 200 Ci/mol) was obtained from NEN, *S*-adenosyl-L-[*Me*-³H]-methionine (sp. radioactivity 15 Ci/mmol) and [³²P]-ATP from Amersham-Buchler (Braunschweig, Germany). Collagenase type IV (Charge 40 E 128) and soya-bean trypsin inhibitor (1 mg inhibits 1.4 mg of trypsin) were purchased from Worthington, Eagle's MEM with Earle's salts including NaHCO₃ (2.2 g/

litre) from the Boehringer-Mannheim Corp. (Mannheim, Germany), and BSA from the Behringwerke (Marburg, Germany). 3-Deaza-adenosine was obtained from the Southern Research Institute, Birmingham, AL, U.S.A., isoprenaline and carbamoylcholine from Sigma. All other chemicals were analytical grade from E. Merck (Darmstadt, Germany).

Preparation and incubation of guinea-pig parotid acini

Male Dunkin–Hartley guinea-pigs (body wt. 220–280 g) from the Lippische Versuchsanstalt, Extertal, Germany, were used. The acini were prepared by a modification of the method of Schmidt *et al.* (1979). Parotid lobules were incubated in Eagle's minimum essential medium (MEM) with Earle's salts gassed with O₂/CO₂ (19:1) and cut into small pieces. They were then incubated in a Ca²⁺-free medium (KH₂PO₄, 15.6 mM, pH 7.4; EGTA, 2 mM; NaCl, 120 mM; KCl, 4.8 mM; glucose, 5 mM; 3-hydroxybutyrate, 5 mM) at 37°C for 10 min in a shaking water bath. After centrifugation at 70 *g*_{max} for 30 s and washing twice with Ca²⁺-free Krebs–Ringer bicarbonate buffer to remove EGTA, they were incubated in a digestion medium (Eagle's MEM with Earle's salts; 1 mg of collagenase type IV/ml; 0.2 mg of soya-bean trypsin inhibitor/ml) for 45 min. They were then sucked in five steps through siliconized glass pipettes with inner diameters decreasing from 800 µm to 200 µm. This suspension was filtered through a nylon filter (pore size 100 µm) and washed twice (MEM; 0.2 mg of soya-bean trypsin inhibitor/ml; 1 mg of bovine serum albumin/ml). The acini were allowed to recover for 90 min. They were then incubated in Krebs–Henseleit bicarbonate buffer and gassed with O₂/CO₂ (19:1). After 30 min of pre-incubation they were stimulated with (–)-isoprenaline (20 µM) or carbamoylcholine (10 µM) and the incubation was continued for another 30 min. At the indicated times 0.5 ml was removed, centrifuged at 12000 *g*_{max} for 30 s, and the amylase content in the supernatant was measured. For determination of total amylase content the acini were broken up with Lubrol WX (0.2 mg/mg of protein), centrifuged and the amylase content was measured in the supernatant by the method of Bernfeld (1955).

Incorporation of methyl groups into phospholipids of parotid gland acini

Methylation of phospholipids in acini (50 mg wet wt./ml) was determined by carrying out the whole incubation (5.0 ml) in the presence of 25 µM-L-[Me-³H]methionine (sp. radioactivity 0.2 Ci/mmol). At the times indicated 1 ml was pipetted into 3 ml of chloroform/methanol/conc. HCl (100:50:1, by vol.) and the mixture was vigorously shaken. The phospholipids were extracted by the method of Bligh

& Dyer (1959) as described by Hirata *et al.* (1978). The methylated lipids were identified by t.l.c. on silica-gel plates with chloroform/methanol/aq. NH₃ (6:13:1, by vol.) as a solvent. The spots corresponding to co-chromatographed PtdMeEtn, Ptd(Me)₂Etn and PtdCho were scraped off, eluted by the method of Raheja *et al.* (1973), dried under N₂ and counted for radioactivity in a liquid-scintillation spectrometer in the presence of a toluene-based scintillator. The results were normalized by relating them to total PtdCho, which was determined by the method of Stewart (1979).

Determination of phospholipid methyltransferase and adenylate cyclase activities in rat parotid gland microsomes

Male Wistar rats (120–180 g body wt.) from MUS-Rattus, Brunnthal, Germany, were used. Parotid-gland lobules homogenized at 4°C in 10 vol. of 0.3 M-sucrose/5 mM-triethanolamine/HCl/0.1 mM-EDTA (pH 7.0). The homogenate was centrifuged at 12000 *g*_{max} for 10 min. The pellet was discarded and the supernatant spun at 100000 *g*_{max} for 60 min. The resulting pellet, corresponding to a mixture of endoplasmic reticulum and plasma membranes, was resuspended in a small volume of the homogenizing buffer and used for the assay of enzyme activities. The assay mixture for the total phospholipid methyltransferases contained (final concentrations): 40 mM-Tris/HCl, pH 7.4; 4 mM-MgCl₂; 100 µM-[Me-³H]SAM (sp. radioactivity 500 Ci/mol) and 500 µg of microsomal protein in a final volume of 50 µl. The mixture was incubated at 37°C for 10 min or 30 min. The reaction was stopped and the phospholipids were extracted and analysed as given above.

Before the determination of adenylate cyclase activity the microsomes were pre-incubated at 37°C for 15 min in a medium containing 40 mM-Tris/HCl, pH 7.4, and 4 mM-MgCl₂, plus or minus the indicated concentrations of SAM or SAH. The assay of adenylate cyclase itself was carried out by a modification of the procedure of Butcher *et al.* (1975). The mixture contained (final concentrations): 40 mM-Tris/HCl, pH 7.5 (37°C); 2.0 mM-EDTA; 0.1 mM-theophylline; 0.1 mM-GTP; 1 mM-cyclic AMP; 10 mM-phosphoenolpyruvate; 2 units of pyruvate kinase; 50 µg of bovine serum albumin and 20–30 µg of microsomal protein in a final volume of 50 µl. The incubation was performed at 37°C for 10 min and stopped by heating at 100°C for 4 min after addition of [³H]cyclic AMP as internal standard. The [³²P]cyclic AMP formed during the incubation was isolated by chromatography on Dowex-AG 50-W (X4; Bio-Rad) and Al₂O₃ columns (Sigma) and quantified by liquid-scintillation spectrometry as described by Salomon (1979). Protein was measured by the dye-binding assay of Bradford (1976).

Results

Phospholipid methylation and adenylate cyclase activity in isolated rat parotid-gland membranes

Plasma-membrane-containing parotid-gland microsomes respond to isoprenaline as well as to NaF with an increased activity of adenylate cyclase (Table 1). Neither the addition of SAM as a possible stimulator of phospholipid methylation nor the addition of SAH at concentrations sufficiently high to inhibit phospholipid methylation by more than 80% (Table 2) significantly affected the β -agonist-induced stimulation of adenylate cyclase. Why SAH did enhance fluoride activation of the enzyme remains unknown at present.

Characterization of methyl-group incorporation into phospholipids of guinea-pig parotid-gland acini

The incorporation of methyl groups from exogenous [$Me-^3H$]methionine into the different phospholipids is shown in Fig. 1. The incorporation was linear for at least 50 min. The total amount of PtdCho was not altered during the incubation (0.14 ± 0.02 mg of PtdCho/mg of protein), which justifies the use of total PtdCho as a reference parameter. In order to prove that the methyl groups were indeed transferred to the polar head groups of the phospholipid molecules, the labelled spots were eluted and digested with phospholipase C. As expected, the label could be completely removed from the phospholipids by this treatment (results not shown).

The incorporation of methyl groups into phospholipids could be inhibited by adding exogenous 3-deaza-adenosine (2–100 μ M) alone or in combination with homocysteine thiolactone to the incubation medium during the whole incubation time. After addition of 20 μ M-3-deaza-adenosine for 30 min phospholipid methylation was inhibited by

about 80% (see Table 3). This inhibition could only be slightly enhanced by additional homocysteine thiolactone (100 μ M) (Table 3). In the presence of the inhibitors, the incorporation of methionine into trichloroacetic acid-precipitable material was reduced by 20–30%.

Incorporation of methyl groups into phospholipids in guinea-pig parotid-gland acini during stimulation by muscarinic or β -adrenergic agonists

Isolated guinea-pig acini were preloaded for 30 min with [$Me-^3H$]methionine followed by stimulation with carbamoylcholine (10 μ M) or isoprenaline (20 μ M). As shown in Fig. 2(a), carbamoylcholine rapidly inhibited the incorporation of methyl groups into phospholipids. Although incorporation into PtdMeEtn was hardly affected, and the incor-

Table 2. *Inhibition of phospholipid methyltransferases in rat parotid-gland microsomes in the presence of SAH*

The activity of phospholipid methyltransferases in rat parotid-gland microsomes was determined as described in the text. Incubations were carried out for 10 min with SAM and SAH being present from the beginning. The activities without SAH were 6.6 ± 1.3 and 1.0 ± 0.2 pmol of methyl groups/mg of protein per 10 min with 100 and 10 μ M-SAM respectively. The values are means from four different experiments $\pm$ S.D. The degree of inhibition did not significantly differ for the three phospholipid classes.

Effector concentration (μ M)		
SAM	SAH	Inhibition (% of control)
100	20	65.3 ± 6.5
100	100	81.3 ± 8.0
100	200	77.0 ± 7.4
10	200	88.0 ± 6.0

Table 1. *Effects of SAH and SAM on β -agonist- or fluoride-mediated stimulation of adenylate cyclase from rat parotid-gland microsomes*

The activity of adenylate cyclase was determined as described in the text. The activity without any addition was 271 ± 62 pmol of cyclic AMP/mg of protein per 10 min, 1722 ± 22 pmol of cyclic AMP/mg of protein per 10 min after stimulation with isoprenaline and 6450 ± 385 pmol of cyclic AMP/mg of protein per 10 min after stimulation with NaF. The stimulation factors given in the Table were calculated by dividing the stimulated activity by the basal activity (means $\pm$ S.D. from four experiments). Abbreviation: n.d., not determined.

Effectors (μ M)		Stimulation of adenylate cyclase activity (stimulated activity/unstimulated activity)		
		Control	20 μ M-Isoprenaline	NaF
SAH	—	1.0	6.4 ± 0.1	23.8 ± 1.4
	20	1.2 ± 0.1	6.6 ± 0.3	32.9 ± 3.8
	100	n.d.	6.6 ± 0.3	29.6 ± 0.2
	200	1.1 ± 0.1	6.0 ± 0.3	33.2 ± 2.2
SAM	20	0.5 ± 0.3	7.0 ± 0.1	25.3 ± 2.3
	100	n.d.	6.4 ± 0.5	23.5 ± 4.4
	200	1.3 ± 1.0	6.0 ± 0.3	23.8 ± 0.9

Fig. 1. Incorporation of methyl groups into phospholipids by isolated acini from guinea-pig parotid glands. The acini were incubated at 37°C in Krebs–Henseleit buffer with [*Me*-³H]methionine for 40 min as described in the Materials and methods section. The values are means from two experiments. The bars indicate the range covered by the two values. □, PtdMeEtn; Δ, Ptd(Me)₂Etn; ○, PtdCho.

Table 3. Inhibition of the incorporation of methyl groups into phospholipids of guinea-pig parotid-gland acini from exogenous L-[*Me*-³H]methionine by 3-deaza-adenosine and homocysteine thiolactone

Acini were incubated in the presence of inhibitors for 30 min, the reaction was stopped and the phospholipids were extracted and analysed as described in the text. In control experiments without inhibitors the incorporation was 51000 ± 3500 d.p.m./mg of PtdCho per 30 min. The values given are means from three different experiments $\pm$ s.d. The degree of inhibition of *Me*-³H group transfer onto the three different phospholipid classes was similar.

Additions	Inhibition of phospholipid methylation (% of control)
3-Deaza-adenosine	
2 μ M	24 $\pm$ 3
20 μ M	75 $\pm$ 5
100 μ M	82 $\pm$ 1
3-Deaza-adenosine (20 μ M) + homocysteine thiolactone (100 μ M)	85 $\pm$ 7

poration into Ptd(Me)₂Etn only slightly lowered, the inhibition was most pronounced with respect to the accumulation of label in PtdCho.

In contrast, the incorporation of methyl groups was not altered by stimulation of the acini with isoprenaline (Fig. 2*b*).

Influence of methyltransferase inhibitors on stimulated amylase secretion

To further clarify whether phospholipid methylation plays an essential role during stimulated secretion in the parotid gland, receptor-mediated stimulation of amylase secretion was determined during inhibition of transmethylation processes. The concentrations of 3-deaza-adenosine used in these experiments (20 μ M) is sufficient to inhibit phospholipid methylation by about 80% (see Table 1); the inhibitors were present throughout the incubation. As shown in Table 4, carbamoylcholine-induced amylase secretion was not significantly affected by 3-deaza-adenosine alone or in combination with 100 μ M-homocysteine thiolactone. Even when submaximal concentrations of carbamoylcholine (1 μ M) were used, inhibition of methyl-group transfer did not significantly affect amylase secretion (results not shown).

When secretion of amylase was stimulated by isoprenaline again, methyltransferase inhibitors failed to significantly affect the release of amylase irrespective of whether maximally effective concentrations of isoprenaline (1 μ M) (see Table 3) or submaximal concentrations (10 nM–10 μ M) were used (results not shown).

Discussion

A direct involvement of phospholipid methylation in the process of stimulus–secretion coupling would require that an inhibition of phospholipid methylation should affect receptor-mediated secretion.

This was clearly not the case, irrespective of whether secretion was elicited by carbamoylcholine, which uses Ca²⁺ as second messenger, or by isoprenaline, which involves the formation of cyclic AMP. Under the conditions tested the degree of inhibition of phospholipid methylation was about 80%. This should be sufficient to detect an involvement of phospholipid methylation in stimulus–secretion coupling, unless one argues that only the remaining 20% is essential for receptor-mediated secretion to occur. This would require that specifically phospholipid methylation necessary for stimulus–secretion coupling should be resistant to inhibition, which seems highly unlikely. In the experiments of Ishizaka *et al.* (1980), an inhibition of phospholipid methylation by 60% was already associated with a 40% inhibition of secretion in stimulated mast cells.

The finding that isoprenaline stimulated secretion of amylase without an effect on the incorporation of methyl groups into phospholipids and that carbamoylcholine even inhibited methyl-group incorporation into phospholipids is also in line with the

Fig. 2. *Effect of amylase secretagogues on phospholipid methylation in isolated guinea-pig parotid-gland acini* The acini were preloaded with [Me - 3H]methionine for 30 min (see Fig. 1) and then stimulated with $10\mu M$ -carbamoylcholine (a) or $20\mu M$ -isoprenaline (b). The amount of methyl groups incorporated over 30 min was taken as 100%. ○, Control values without added agonists; ●, after stimulation with agonist. The values of (a) were taken from four separate experiments, and those in (b) from two separate experiments. Two further experiments with isoprenaline (results not shown) showed lower rates of phospholipid methylation in the control period as well as after stimulation, but again the rates of phospholipid methylation after isoprenaline did not differ from those of the control period.

Table 4. *Effects of inhibitors of phospholipid methylation on stimulated amylase release*

Guinea-pig parotid-gland acini were incubated at $37^\circ C$ in Krebs–Henseleit buffer and stimulated after 30 min with $10\mu M$ -carbamoylcholine or $20\mu M$ -isoprenaline. Portions were taken at the beginning, after 30 min (before stimulation) and, after 60 min, centrifuged, and amylase activity was determined in the supernatant. Total amylase activity was measured as described in the text. Where indicated, 3-deaza-adenosine ($20\mu M$) and homocysteine thiolactone ($100\mu M$) was added from the beginning. The numbers of determinations are shown in parentheses.

Effector + inhibitors	Amylase release (% of total)	
	0–30 min	30–60 min
Control	4.6 ± 1.1	4.5 ± 1.7 (6)
Carbamoylcholine	4.8 ± 1.3	15.2 ± 3.4 (8)
Carbamoylcholine + 3-deaza-adenosine	4.4 ± 1.7	14.8 ± 2.4 (9)
Carbamoylcholine + 3-deaza-adenosine + homocysteine thiolactone	4.5 ± 1.9	13.1 ± 2.6 (4)
Control	3.8 ± 2.2	5.4 ± 4.9 (8)
Control + 3-deaza-adenosine + homocysteine thiolactone	2.3 ± 0.5	2.7 ± 0.8 (4)
Isoprenaline	3.4 ± 2.0	26.5 ± 4.8 (7)
Isoprenaline + 3-deaza-adenosine	4.2 ± 0.8	26.3 ± 5.1 (4)
Isoprenaline + 3-deaza-adenosine + homocysteine thiolactone	2.5 ± 1.6	23.7 ± 2.7 (4)

idea that increased membrane phospholipid methylation is not directly involved in stimulus–secretion coupling. An inhibition of phospholipid methylation by carbamoylcholine is obviously a side effect, which seems to take place also in the rat pancreas (Gunther, 1980).

One might speculate that inhibitory effects of deaza-adenosine plus or minus homocysteine thiolactone could not be detected because maximally stimulating concentrations of effectors had been used. However, since inhibition of phospholipid

methylation failed also to affect amylase secretion elicited by submaximal concentrations of effectors, this argument does not seem to be valid.

Neither stimulation nor inhibition of phospholipid methylation affected the stimulation of adenylate cyclase activity in isolated parotid-gland membranes, which is in agreement with similar findings obtained by Colard & Breton (1981) in isolated liver cell plasma membranes. Although this finding by itself would not exclude the possibility of a direct involvement of phospholipid methylation in β -

agonist-mediated exocytosis, it is in line with the other findings. According to the results obtained, changes in phospholipid methylation seem not to be directly involved in stimulus-secretion coupling in the parotid gland.

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