Phosphoinositide breakdown in isolated rat parotid membranes

Stimulation by cholinergic and α-adrenergic agonists

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Parotid gland membranes labelled with [^{3}H]inositol were challenged with the cholinergic agonist, carbamylcholine, or with epinephrine in the presence of propranolol. Both agonists caused a significant breakdown of labelled phosphoinositides (17.5%) in membranes suspended in Krebs-Ringer bicarbonate buffer. This effect was abolished by the respective antagonists, atropine or phentolamine. The carbamylcholine-induced breakdown of labelled phosphoinositides did not require cytosol. The addition of cytosol alone, or the exposure of membranes to a medium of low ionic strength caused a significant breakdown of phosphoinositides (10–40%). No further breakdown due to the addition of carbamylcholine was observed under these conditions. It is suggested that neurotransmitter-induced breakdown of phosphoinositides is effected by membrane-associated enzyme(s) and can be observed only in a medium of high ionic strength.

Phosphoinositide breakdown *Parotid membrane* *Cholinergic receptor*

1. INTRODUCTION

Breakdown of phosphoinositides (PIs) induced by hormones or neurotransmitters is a common biochemical event that accompanies calcium mobilization in many tissues [1,2]. Michell [1] was the first to propose a link between PI breakdown and calcium mobilization. This hypothesis was based on the fact that PI breakdown occurs in the absence of extracellular calcium [3], suggesting that this is the primary event following the activation of hormone and neurotransmitter receptors on the cell membrane. Further support for this hypothesis can be found in [4–6]. A significant increase in breakdown products of PIs within the first seconds of hormonal stimulation was reported [4]. There is also new evidence that one of the breakdown products, inositol 1,4,5-trisphosphate, may serve as a messenger for the mobilization of intracellular calcium [7]. All these studies were performed in intact tissues or isolated cells. Hormone and neurotransmitter-induced changes in PI metabolism in cell-free preparations from several tissues have been observed. Thus, α-adrenergic and vasopressin-induced breakdown of PIs in liver membranes [8–11], serotonin-induced breakdown of phospatidylinositol in cell-free preparations of the blowfly salivary glands [12] and an increased phosphorylation of PIs and of diacylglycerol induced by ACTH in rabbit iris membranes have been reported [13]. Here, we provide evidence that cholinergic or α-adrenergic stimulation results in a loss of PI label in rat parotid membranes and describe some of the requirements for this response.

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2. MATERIALS AND METHODS

2.1. *Membrane and cytosol preparation*

Rat parotid gland fragments were prepared and

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incubated as in [14], and labelled with 10 μCi/ml [^{3}H]inositol (New England Nuclear or Amersham) in Krebs-Ringer bicarbonate buffer (KRB) for 1 h at 37°C under an oxygen:CO_2 mixture (95:5). At the end of the incorporation period the gland fragments were thoroughly washed and homogenized with a loose glass-Teflon homogenizer in 5 vols of 0.27 M sucrose, 10 mM Tris–HCl (pH 7.4). To isolate the membrane fraction, the homogenate was centrifuged (8000 × g, 10 min) and the supernatant was further centrifuged (105000 × g, 60 min). The final membrane pellet was resuspended in KRB and frozen in small aliquots (2.5–5.0 mg protein/ml). In some experiments the last centrifugation step was repeated once more. The 105000 × g supernatant (from unlabelled glands) was used as a source of cytosolic factor(s).

2.2. *Assay of PI breakdown*

Aliquots (100 μl) of the membrane suspension were added to 400 μl KRB buffer that included a desired concentration of neurotransmitters and/or cytosol. The membranes were then incubated at 37°C with shaking and the reaction was terminated in two different ways: (i) by adding 2 ml of 5% trichloroacetic acid and centrifugation (1500 × g, 10 min, in the cold). The pellet was washed once with 5% cold trichloroacetic acid and then directly counted in Scintiverse 1 (Fisher) in a liquid scintillation counter; (ii) by adding 4 ml chloroform:methanol:HCl (1:1:0.3%) for 30 min at room temperature, then 0.85 ml of 1 M HCl and isolation of the resulting lower phase. The lower phase was dried and counted in a liquid scintillation counter.

Protein was determined according to [15], using bovine serum albumin (Sigma, fraction V) as a standard. All chemicals were of analytical quality. All determinations were performed in replicates (3–5 determinations for each condition). Statistical analyses were performed using the t-test.

3. RESULTS

When [^{3}H]inositol-labelled parotid membranes were incubated for 15 min in KRB in the presence of 0.1 mM carbamylcholine (an acetylcholine esterase-resistant cholinergic agonist), a significant decrease in the label was observed (17.5%, see table 1). This effect of carbamylcholine was attenuated to an appreciable degree (by approx. 60%) by 1 μM of the specific muscarinic antagonist, atropine (table 1). This indicated that the cholinergic breakdown of [^{3}H]inositol-labelled PIs was mediated mainly by muscarinic receptors. We could not demonstrate a complete inhibition by atropine, since very high concentrations of this competitive inhibitor alone affected the breakdown of PIs. We cannot, however, exclude the possibility that a minor non-muscarinic effect accounted for this phenomenon.

Table 1

Neurotransmitter-stimulated phosphoinositide breakdown in parotid membranes

Additions	% Breakdown (mean ± SE)	Number of experiments
Carbamylcholine (0.1 mM)	17.5 ± 2.7	17
Carbamylcholine (0.1 mM) + atropine (1.0 μM)	7.3 ± 3.6	11
Epinephrine (0.1 mM) + propranolol (10 μM)	22.0 ± 2.2	9
Epinephrine (0.1 mM) + propranolol (10 μM) + phentolamine (10 μM)	NE	1
Propranolol	NE	9

The effects of agonists and antagonists were measured in membrane fractions as described in section 2. Both trichloroacetic acid precipitation and lipid extraction were used for the estimation of PIs. The radioactivities in trichloroacetic acid pellets, or in lipid extracts ranged usually between 2000 and 15000 dpm/tube (calculated by the external standard method). NE, no effect

A similar effect was observed when labelled parotid membranes were exposed to α-adrenergic stimulation (0.1 mM epinephrine, in the presence of 10 μM of the specific β-adrenergic blocker, propranolol). The specific α-adrenergic blocker, phentolamine, antagonized the effect of epinephrine (table 1).

Membrane-associated labelled inositides were routinely isolated by trichloroacetic acid precipita-

tion. In some experiments lipids were extracted with acidified chloroform:methanol. The two methods yielded essentially identical results, indicating that trichloroacetic acid-precipitable radioactivity does indeed represent the PI label (not shown).

The cholinergic breakdown of labelled PIs depended on the composition of the incubation medium. When labelled membranes were incubated in 0.27 M sucrose, 10 mM Tris–HCl (pH 7.4), a considerable breakdown of labelled PIs occurred in the absence of neurotransmitters (table 2), while virtually no such breakdown was observed in membranes incubated in KRB (see fig.1). Little if any additional effect was observed when labelled membranes were exposed to carbamylcholine in a low ionic strength medium (table 2). These results may imply that ion depletion promoted the loss of PIs, perhaps from the same pool that was acted upon by carbamylcholine.

Our data show that PI breakdown caused by muscarinic and α-adrenergic agonists occurs in thoroughly washed membranes. When cytosol was added to [^{3}H]inositol-labelled membranes, there was a time-dependent breakdown of PIs in the absence of the agonist (fig.1). The effect of carbamylcholine was not potentiated by the addition of cytosol (table 3). These results indicate that the neurotransmitter-sensitive enzymes were present in the washed membrane preparation, and that the cytosolic factors might not be required for the neurotransmitter-induced breakdown of PIs.

4. DISCUSSION

Our findings demonstrate that carbamylcholine or epinephrine induce significant loss of labelled PIs in isolated rat parotid membranes. The neurotransmitter-induced effects were blocked by the respective antagonists, atropine and phentolamine, indicating that the breakdown was an α-adrenergic or muscarinic receptor-mediated event.

Several reports describe hormone and neurotransmitter-stimulated PI breakdown in isolated membranes. Authors in [8] reported that α-adrenergic agonists caused an extensive (50%) breakdown of phosphatidylinositol in isolated liver plasma membrane. The effect was absolutely dependent on added cytosolic fraction [8]. Authors in [9,10] reported that vasopressin or α-adrenergic stimulation caused significant PI breakdown in rat liver plasma membranes. In their hands, this effect did not require cytosol [10]. Phosphatidylinositol-4,5-bisphosphate hydrolysis induced by vasopres-

Table 2

The effect of incubation medium on phosphoinositide breakdown

Additions	Radioactivity in trichloroacetic acid pellet (dpm/mg protein)		% Breakdown of phosphoinositides		
	KRB	S/T	Due to CCh in KRB	Due to S/T in S/T	Due to CCh in S/T
Expt 1					
None	8253	7402		10	
CCh	6372	6783	23		8
Expt 2					
None	8648	5232		40	
CCh	6464	4960	25		5
Expt 3					
None	3616	2815	–	22	–

Labelled parotid membranes were incubated either in KRB, or in 0.27 M sucrose, 10 mM Tris–HCl, pH 7.4 (S/T), as described in section 2. Carbamylcholine (CCh), 0.1 mM

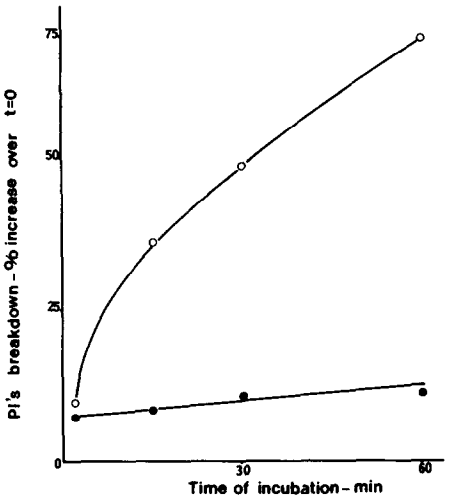

Fig.1. The effect of cytosol on PI breakdown. Labelled parotid membranes were incubated in KRB at 37 in the absence (●) and presence (○) of the 12000 × *g* post-mitochondrial supernatant (cytosol). At the indicated times aliquots were precipitated with 5% trichloroacetic acid by 5 min centrifugation at 12000 × *g*. The results are the means of 3–6 experiments, and are expressed as percent increase in supernatant radioactivity over corresponding controls (time 0, range of control radioactivities 800–1400 cpm). The protein ratio of cytosol to membranes ranged between 0.25 and 4.5, without affecting the rate of PI breakdown.

sin in rat liver plasma membrane in the presence of low Ca^{2+} concentrations (0.2 μM) and under isoosmotic conditions was reported in [11]. Significant serotoninergic PI breakdown in blowfly salivary gland membranes was demonstrated in [12]. Authors in [13] have reported a stimulated cell-free labelling of phosphatidylinositol 4,5-bisphosphate in rabbit iris, which might have reflected increased breakdown and turnover of this phospholipid.

The discrepancy, concerning the requirement for cytosol, between the results of authors in [8–10,12] and our own, may be attributed to the differences in membrane preparation techniques. Either authors in [8] removed the necessary membrane-associated factor(s) in their preparation, or the membrane preparations used by authors in [9,10, 12] and by ourselves retain sufficient contaminating cytosol to supply the requirements of the hormone or neurotransmitter-induced reaction. We observed no breakdown of PIs in unstimulated membranes. However, the addition of cytosol caused a significant breakdown of PIs in the absence of neurotransmitters. This finding argues for negligible cytosolic contamination. Possibly, the cytosol supplies factors other than the degradation enzymes. Moreover, cytosolic phosphatidylinositol-specific phospholipase C has been described in many tissues. This enzymatic activity, however, had little if any effect on membrane PIs

Table 3

The effect of cytosol on the cholinergic breakdown of PIs

Experiment	Additions	Radioactivity in lipids (dpm/mg protein)	% Breakdown
1	none	11406	
	CCh 0.1 mM	8291	27
	cytosol	10450	8
	CCh 0.1 mM + cytosol	10069	12
2	none	1993	
	CCh 0.1 mM	1253	37
	cytosol	1667	16
	CCh 0.1 mM + cytosol	1339	33

Parotid membranes were incubated with and without cytosol (105000 × *g* supernatant), as described in section 2. Lipid radioactivity was determined on trichloroacetic acid pellets in expt 1 and in chloroform:methanol extracts in expt 2. The protein ratio of cytosol to membranes was 1.0

[16]. Thus, it is possible that the hormonal breakdown of PIs uses a distinct, membrane-associated enzyme, that may, in some instances, require additional cytosolic factor(s) (see [8]).

The incubation of labelled membranes in low-ionic strength medium (sucrose-Tris) resulted in a relatively large loss of PIs. Hormones had little additional effect in this medium. This finding confirms reports in [10,17]. It was reported that the hydrolysis of PIs appeared to follow the removal of calcium from the membrane [10]. Removal of divalent cations was shown to increase membrane fluidity. The increase in fluidity, and the loss of membrane-associated calcium were observed when the membranes were exposed to norepinephrine [17].

It has been proposed that the hydrolysis of PIs follows an initial step of calcium removal from the membrane [10]. This is diametrically different from the mechanism proposed by authors in [7], who suggested that the initial hydrolysis of phosphatidylinositol 4,5-bisphosphate causes the release of inositol 1,4,5-trisphosphate and calcium mobilization.

These two hypotheses can be reconciled by assuming that the first step in hormonal stimulation is the hydrolysis of phosphatidylinositol 4,5-bisphosphate, followed by calcium mobilization from the intracellular non-mitochondrial calcium pool that, in turn, allows a further hydrolysis of PIs. This assumption may be tested by identifying the nature of PI breakdown products in cell-free systems. No information concerning the breakdown products of PIs in cell-free systems is available. In our hands, the observed breakdown was too limited to attempt the characterization of the breakdown products.

The demonstration of cell-free hormonal breakdown of PIs in several tissues should help in elucidating the molecular mechanism of this important biological pathway.

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