

Muscarinic stimulation of phospholipid turnover in dissociated avian salt gland cells

Stephen K. Fisher, Seth R. Hootman*, Anne M. Heacock, Stephen A. Ernst* and Bernard W. Agranoff⁺

*Neuroscience Laboratory, University of Michigan, 1103 East Huron, Ann Arbor, MI 48109 and *Department of Anatomy and Cell Biology, University of Michigan, Ann Arbor, MI 48109, USA*

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Addition of carbamylcholine to ³²P-prelabeled dissociated avian salt gland cells resulted in increased turnover of phosphatidic acid, phosphatidylinositol, phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate, which could be prevented by the inclusion of atropine. Carbamylcholine had no discernable effect on protein phosphorylation, measured either in the total preparation or in subcellular fractions. It is concluded that for the avian salt gland, no obligatory link is indicated between protein phosphorylation and either phospholipid turnover or salt secretion.

<i>Avian salt gland</i>	<i>Muscarinic receptor</i>	<i>Inositol phospholipid</i>	<i>Phosphatidate</i>
	<i>Protein phosphorylation</i>	<i>Secretion</i>	

1. INTRODUCTION

Activation of muscarinic cholinergic receptors in pancreas, parotid and lacrimal glands results in an increased secretory activity, an enhanced phosphorylation of specific proteins and an increased turnover of two quantitatively minor phospholipids, namely phosphatidate (PhA) and phosphatidylinositol (PhI) [1–6]. Recent evidence from thrombin-stimulated platelets suggests that the increased protein phosphorylation is a consequence of inositol phospholipid breakdown via the transient accumulation of diacylglycerol [7]. The latter is known to activate a widely distributed enzyme, protein kinase C [8], which has been proposed as a link between receptor-mediated changes in lipid and protein phosphorylation. Here, we have examined the possible interrelationship between lipid and protein phosphorylation, using a preparation of dispersed duck salt gland cells [9], physiologically responsive to muscarinic agonists [9,10]. The results indicate that muscarinic recep-

tor activation in these cells results in an increased turnover of phospholipids, as shown for salt gland slices [11], but has no discernable effect on protein phosphorylation.

2. EXPERIMENTAL

2.1. Preparation of cells

Duck salt glands were dissociated by a combination of enzymic digestion and mechanical dispersion [9]. After isolation, the cells were resuspended in a physiological salt solution (PSS) consisting of 142 mM NaCl, 5.6 mM KCl, 2.2 mM CaCl₂, 3.6 mM NaHCO₃, 1 mM MgCl₂, 5.6 mM D-glucose and 30 mM Hepes–NaOH buffer (pH 7.4).

2.2. Prelabeling of cells with ³²P_i

Dissociated cells were incubated in 10–20 ml PSS for 60 min at 37°C in air, in the presence of 1 mCi ³²P_i (carrier free, Amersham) in 450 µl. Separate portions of cells (~0.25 mg protein in 475 µl) were then transferred to tubes containing either PSS alone, or PSS and carbamylcholine

⁺ To whom correspondence should be addressed

(10^{-3} M) in 500 μ l final vol., incubated for a further 15 s to 15 min and then analyzed for changes in lipid and protein phosphorylation.

2.3. Extraction, separation and quantitation of phospholipids

Incubations were terminated by addition of chloroform-methanol, acidified, and the extracted 32 P-labeled phospholipids separated by thin-layer chromatography [12]. Phospholipids were localized by autoradiography, scraped from the chromatograms and scintillation-counted.

2.4. Analysis of phosphoprotein turnover

For incubations of ≤ 60 s, the reaction mixtures were layered over silicone oil (200 μ l) in microfuge tubes with 200 μ l 62.5 mM Tris-HCl buffer (pH 6.8) containing 1% SDS and 20% sucrose (TSS) as the bottom layer. Reactions were terminated by centrifugation of the cells into the SDS layer ($15000 \times g$ for 45 s) and removal of the overlying medium and silicone oil. For incubations of > 60 s, the reactions were terminated by rapid centrifugation in a microfuge ($15000 \times g$ for 10 s) followed by aspiration of the supernatant and addition of 150 μ l TSS. SDS-treated proteins were heated to 100°C for 5 min, then brought to 8 M final conc. in urea and 1% in β -mercaptoethanol.

2.5. Preparation of subcellular fractions

32 P-Labeled cells were homogenized in 1 ml ice-cold medium containing 50 mM potassium phosphate buffer (pH 7.0), 300 mM sucrose, 2 mM EDTA and 0.2 mM EGTA [2], then centrifuged at $5000 \times g$ for 15 min. The resulting pellet was washed and the centrifugation step repeated to obtain a $5000 \times g$ pellet (P1). The combined supernatants were then centrifuged at $100000 \times g$ for 60 min to obtain a pellet (P2) and supernatant (S2). Proteins in P1 and P2 were solubilized as described in 2.4. Proteins in S2 were precipitated by the addition of 10% trichloroacetic acid, washed with diethyl ether to remove trichloroacetic acid, and solubilized.

2.6. Separation of phosphoproteins and autoradiography

Phosphoproteins (200–400 μ g) were separated on 10% polyacrylamide-SDS slab gels as in [13]. Myosin, bovine serum albumin, ovalbumin, and

ribonuclease standards (200, 68, 45, and 14 kDa, respectively) were routinely run. Gels were stained, vacuum-dried and exposed to XRP-5 X-ray film for 3–8 days for preparation of autoradiograms.

3. RESULTS

More than 85% of the lipid radioactivity recovered from the prelabeled cells was located in four phospholipids: PhA, PhI, phosphatidylinositol 4-phosphate (PhIP) and phosphatidylinositol 4,5-bisphosphate (PhIP₂). Of these, PhIP and PhIP₂ contained the most label. The addition of carbamylcholine (10^{-3} M) resulted in a rapid loss of label from PhIP and PhIP₂ and a marked increase in the labeling of PhA (fig.1). Stimulated labeling of PhI was detectable only after 5 min incubation. Both the loss of radioactivity from PhIP and PhIP₂ and the increased labeling of PhA and PhI was prevented by inclusion of 10^{-5} M atropine (fig.1).

Duck salt glands also readily incorporated $^{32}\text{P}_i$ into phosphoproteins. Addition of carbamylcholine to the prelabeled preparation, however, did not result in detectable increases in phosphorylation or dephosphorylation of the pro-

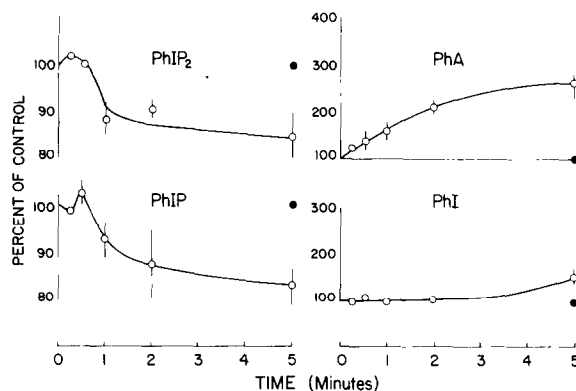


Fig.1. Radioactivity of prelabeled dissociated avian gland cells following addition of 10^{-3} M carbamylcholine. Results are means \pm range for 2–3 separate experiments and are expressed as % radioactivity relative to controls: (○) carbamylcholine; (●) carbamylcholine + atropine (10^{-5} M); 0.5–1% of the total $^{32}\text{P}_i$ added was incorporated into phospholipid during the 60 min prelabeling period. Of this, PhIP₂, PhIP, PhA and PhI contained 38, 20, 18 and 10%, respectively, of the total lipid radioactivity following preincubation.

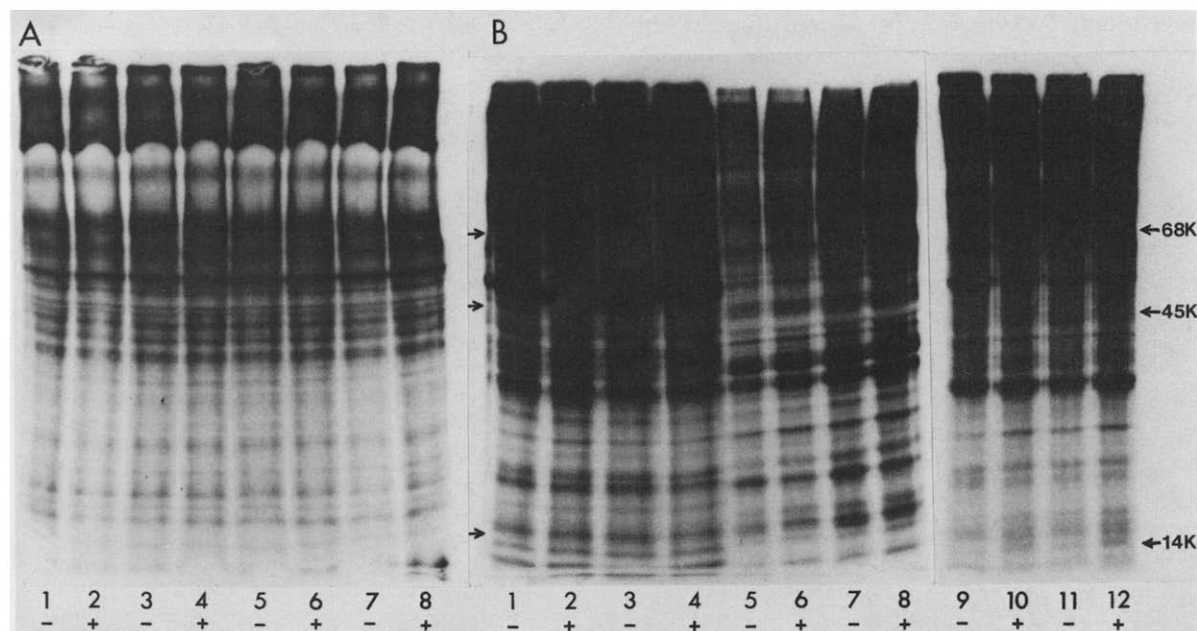


Fig.2. Autoradiogram of ^{32}P -labeled phosphoproteins. (A) Prelabeled salt gland cells were incubated in the presence (+) or absence (-) of 10^{-3} M carbamylcholine for 20 s (lanes 1-4) or 60 s (lanes 5-8). Each lane contained 30 μg of protein, and $\sim 1 \mu\text{Ci}$ protein-bound ^{32}P . (B) As above, following separation of subcellular fractions: 5000 $\times g$ pellet (lanes 1-4); 100000 $\times g$ supernatant (lanes 5-8); 100000 $\times g$ pellet (lanes 9-12). No change in labeling attributable to carbamylcholine were seen.

teins (fig.2A). To investigate whether possible changes in protein phosphorylation in a specific subcellular fraction might have been masked in gels prepared from whole cell protein, an analysis of subcellular fractions (P1, P2 and S2) was carried out. These also failed to reveal discernable alterations in protein phosphorylation following carbamylcholine addition, in any fraction investigated (fig.2B).

4. DISCUSSION

While several suggestions concerning the physiological significance of the phosphoinositide response have been advanced [14], its precise role in cellular functions remains an enigma. The pattern seen here, namely significant loss of radioactivity from the polyphosphoinositides, supports evidence from other systems [15,16] that their breakdown may be an early consequence of receptor-ligand interaction, and may precede stimulated labeling of PhA and PhI. This study also demonstrates that while muscarinic receptor

activation in the avian salt gland results in increased turnover of phospholipid, concomitant changes in protein phosphorylation are not detectable. Thus, while protein phosphorylation may be related to phosphoinositide turnover in some instances [7], it is not invariably the case. Unlike most other vertebrate exocrine organs, the avian salt gland elaborates a fluid secretion (hypertonic NaCl) containing little or no protein. The failure to observe muscarinic receptor-linked changes in protein phosphorylation here suggests that the increased protein phosphorylation observed following muscarinic stimulation of pancreas, parotid, and lacrimal gland [1-3] may be related to exocytosis rather than the fluid phase of secretion.

In assessing a role for phosphorylation of protein in stimulus-secretion coupling in exocrine glands, it is thus important to distinguish between exocytotic and fluid secretory mechanisms. Stimulated phospholipid labeling in the dissociated avian salt gland preparation described here suggests that these cells may constitute a convenient preparation for the study of those aspects of

phospholipid metabolism that are related to receptor-ligand interaction in the absence of those dealing with membrane fusion and extrusion of macromolecules in the exocytotic process.

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