PROTEIN PHOSPHORYLATION DURING SECRETION IN THE RAT LACRIMAL GLAND

A general role of EC-protein in stimulus–secretion coupling in exocrine organs?

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1. Introduction

We have recently shown that, in the parotid gland, cAMP-mediated secretion is associated with the phosphorylation of three membrane-bound proteins (protein I:35 000 dalton, protein II: 26 700 dalton and protein III:20 000 dalton [1]). The cAMP-dependent phosphorylation of protein I was confirmed independently by another group [2,3], which established its protein nature by tryptic digestion and determined a somewhat lower molecular weight (30 000). In contrast, cholinergic stimulation of secretion was associated with the phosphorylation of protein I (which we should prefer to designate 'EC-protein' from now on but not of proteins II and III [4,5]. We propose the name 'EC-protein' (= exocytosis coupled protein) since the former designation as protein I could lead to some confusion with 'protein I' described by Greengard's group (see for example [6]), which is certainly a different protein). The EC-protein is phosphorylated in close correlation with the enhancement of enzyme discharge independently from the kind of stimulus, be it β-adrenergic, cholinergic, a calcium ionophore or a derivative of cAMP [4,5] which supports the working hypothesis that it plays an important role in stimulus-secretion coupling. Our hypothesis would gain further reliability if it were possible to show that a phosphorylation of the EC-protein is not restricted to a special function in the parotid gland but would be also associated with stimulus-secretion coupling in other exocrine glands.

Several recent reports [7,8,9] have established the rat lacrimal gland as a powerful model for studying the physiology of stimulus-secretion coupling in vitro. Detailed analyses revealed that exocytosis can be induced by the action of muscarinic and α-adrenergic agonists, most probably by a rise in the intracellular Ca^{2+}-activity. In contrast to the parotid gland [10] but similar to the exocrine pancreas [11] there is no evidence for the existence of a cAMP-dependent mechanism involved in exocytosis.

In the present study we report the phosphorylation of two proteins during cholinergic stimulation of exocytosis in the rat exorbital lacrimal gland. One of them appears to be identical with the EC-protein found in the parotid gland. These results are in favour of the working hypothesis mentioned above.

2. Materials and methods

[^{32}P]Labelled inorganic phosphate (carrier free) and [^{3}H]leucine (132 mCi μmol^{-1}) were obtained from Amersham Buchler (Braunschweig); D,L-isoproterenol, carbamylcholine chloride and atropine were purchased from Sigma GmbH (Munich). Acrylamide and bisarcrylamide (twice recrystallized) were obtained from Serva (Heidelberg). Unisolve I from Zinsser Analytic (Frankfurt). All other reagents were of analytical grade and obtained from Merck (Darmstadt). Male Wistar rats (120–160 g, fed ad libitum) came from MUS-Rattus GmbH, Munich).

2.1. *Measurement of lacrimal secretion*

Protein discharge of rat lacrimal lobules was measured according to Putney et al. [12]. Portions (50 mg wet weight) of lobules were pulse labelled with 5 μCi [^{3}H]leucine for 20 min in a volume of 2.0 ml. The lobules were then incubated in 5 ml unlabelled Krebs–Ringer–Tris buffer [12] to allow for synthesis and

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package of secretory protein. Thereafter the medium was replaced by 5 ml of fresh buffer. Aliquots (0.5 ml) of the medium were removed at the times indicated and counted for radioactivity in a liquid scintillation spectrometer after addition of 5 ml Unisolve I. Precipitation of the secreted proteins with 10% (w/v) TCA revealed that all radioactivity released was precipitable. At the end of the incubation period, the lobules were homogenized in the incubation medium. After precipitation of the homogenate with 10% TCA the radioactivity incorporated into the pellet was determined as described previously [1]. The amount of radioactivity released into the medium was expressed as percentage of the total radioactivity incorporated into protein.

2.2. *Analysis of protein phosphorylation after stimulation of rat lacrimal lobules*

Lobules corresponding to about 100 mg wet weight were preloaded with 70–80 μCi [^{32}P]labelled inorganic phosphate for 30 min and stimulated as indicated. Twenty minutes after addition of the stimulus the tissue pieces were rapidly homogenized in 1 ml ice-cold stop solution (0.3 M sucrose, 50 mM potassium phosphate pH 7.0, 2 mM EDTA, 0.2 mM EGTA). The homogenate was centrifuged for 10 min at 15 000 × g_{max}. The resulting supernatant was spun again at 100 000 × g_{max} for 60 min. The 100 000 × g pellets were resuspended in small volumes of stop solution and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and autoradiography (for details see ref.1).

3. Results and discussion

Fig.1 illustrates the secretory response of rat lacrimal gland lobules to cholinergic and β-adrenergic stimulation. Protein discharge is enhanced about five fold in the presence of 2 × 10^{-6} M carbachol with respect to control incubations. The stimulation was abolished by the action of 10^{-5} M atropine. The β-adrenergic agonist isoproterenol did not induce any measurable protein discharge.

The phosphorylation patterns of the 100 000 × g pellets are shown in fig.2. Membrane fractions from mouse parotid gland lobules incubated in parallel were separated on the same gel allowing a comparison of the phosphorylation patterns of both glands.

Cholinergic stimulation of rat lacrimal gland slices

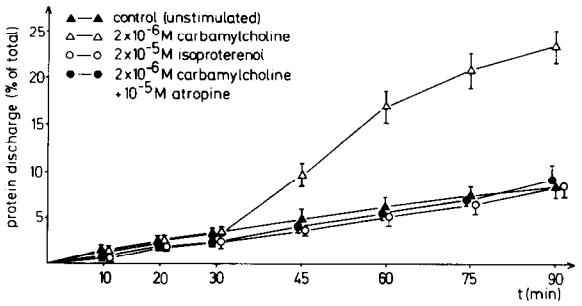

Fig.1. Protein discharge from rat lacrimal lobules after application of different stimuli. Secretion was measured by following the output of prelabelled protein (see section 2). Each point represents the mean of four experiments ± SEM.

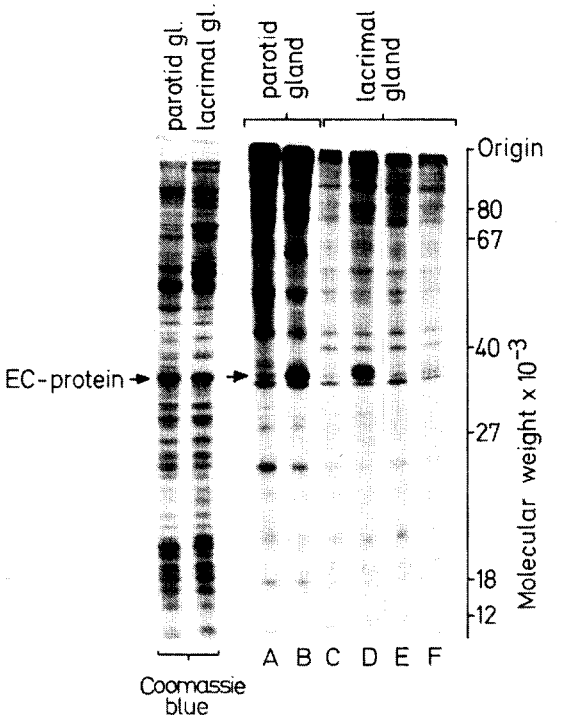

Fig.2. Stimulus-dependent phosphorylations during incubation of rat lacrimal gland slices. Mouse parotid gland slices were incubated and processed in parallel. Portions of 50 μg of protein derived from isolated 100 000 × g pellets were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and subjected to autoradiography. (Coomassie blue stains on the left, autoradiographs on the right). All samples were preloaded for 30 min with inorganic [^{32}P]phosphate and stopped 20 min after stimulation. A and C: unstimulated control incubations. B and D: 2 × 10^{-6} M carbachol. E: 2 × 10^{-5} M isoproterenol. F: 2 × 10^{-6} M carbachol + 10^{-5} M atropine.

resulted in a significant phosphorylation of a protein band corresponding to the EC-protein of mouse parotid gland (compare traces B and D in fig.2). In addition, a second band of a slightly higher molecular weight became phosphorylated yielding a phosphorylated double band which was not observed in the mouse parotid gland. Atropine inhibited the phosphorylation of both bands in the stimulated lacrimal gland (compare traces D and F in fig.2). In contrast to the parotid gland [1] no phosphorylation was induced in the lacrimal gland with isoproterenol (see trace E in fig.2). Although occasionally in the presence of carbachol phosphorylated bands could be detected in the high molecular weight range (e.g. trace D in fig.2) an analysis of other soluble or membraneous subfractions according to the method described previously [1] showed that only the EC-protein was consistently phosphorylated under cholinergic stimulation.

The finding that stimulation of exocytosis in the rat lacrimal gland is also associated with a phosphorylation of the EC-protein supports our working hypothesis that the phosphorylation of the EC-protein represents a link between receptor activation and exocytosis in exocrine glands which is secondary to the rise of the second messenger Ca^{2+} and/or cAMP and that the EC-protein plays a central role in the secretory process itself. It should be noted, however, that even a close correlation between phosphorylation of the EC-protein and secretion does not prove yet a causal relationship between these two phenomena. Further studies will not only have to prove such a causal relationship but also the nature of the additionally phosphorylated protein with a slightly higher molecular weight found in the lacrimal gland.

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