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Ca^{2+}- and calmodulin-dependent protein phosphorylation in rat lacrimal gland

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Ca^{2+}, in homogenized lacrimal glands, enhanced phosphorylation of several peptides. Phosphorylation of two of these peptides was further stimulated by addition of the Ca^{2+}-binding protein calmodulin and decreased by trifluoperazine, an inhibitor of Ca^{2+}–calmodulin-dependent activity. Thus, Ca^{2+}–calmodulin-dependent protein kinases and their substrates are present in lacrimal gland and could have an important role in lacrimal-gland function.

The lacrimal gland, an exocrine gland structurally and functionally similar to the exocrine pancreas and the salivary glands, secretes proteins, electrolytes and water, which play a major role in the nourishment and protection of the cornea. As in other exocrine, as well as endocrine, glands, Ca^{2+} has an essential role in lacrimal-gland stimulus–secretion coupling. Acetylcholine-stimulated rabbit lacrimal-gland fluid secretion, measured from the cannulated excretory duct, is blocked by decreasing the extracellular Ca^{2+} concentration with EGTA or by addition of verapamil, an inhibitor of Ca^{2+}-influx via voltage-sensitive channels (Botelho & Dartt, 1980). In the rat exorbital lacrimal gland, cholinergic and α-adrenergic stimulation of $^{86}Rb^{+}$ efflux (a marker of K^{+} efflux) and protein secretion are inhibited by decreasing the extracellular Ca^{2+} concentration with EGTA (Putney *et al.*, 1977, 1978; Herman *et al.*, 1978; Parod & Putney, 1978). In addition, in rat exorbital lacrimal gland, cholinergic and α-adrenergic agonists increase $^{45}Ca^{2+}$ uptake, content and efflux (Dreisbach, 1963; Keryer & Rossignol, 1976; Parod & Putney, 1978, 1979, 1980; Parod *et al.*, 1980), and the cholinergically stimulated increase in $^{45}Ca^{2+}$ content can be blocked by EGTA, $CoCl_2$, and to a small extent by compound D-600, a verapamil analogue (Parod & Putney, 1980).

The mechanism by which Ca^{2+} stimulates lacrimal-gland secretion has not yet been determined. In other secretory tissues there is evidence that calmodulin, a Ca^{2+}-binding protein, may play a role in Ca^{2+}-mediated secretion. For example, in pancreatic islets, insulinomas, the small intestine and polymorphonuclear leucocytes, the phenothiazines trifluoperazine and chlorpromazine, which are inhibitors of Ca^{2+}–calmodulin-activated processes, can inhibit secretion (Elferink, 1979; Ilundain & Naftalin, 1979; Krausz *et al.*, 1980; Schubart *et al.*, 1980; Smith & Field, 1980). One possible mechanism by which Ca^{2+} and calmodulin could control secretion is by activating protein kinases, which would phosphorylate specific proteins. Therefore, we investigated whether Ca^{2+} and calmodulin stimulate phosphorylation of specific proteins isolated from rat exorbital lacrimal-gland homogenate. We found that phosphorylation of two proteins (mol. wts 94000 and 50000) is dependent on Ca^{2+} and calmodulin.

Materials and methods

Adenosine 5′-[γ-^{32}P]triphosphate (triethylammonium salt; sp. radioactivity 2–10 Ci/mmol) was purchased from New England Nuclear (Boston, MA, U.S.A.). Calmodulin was prepared from bovine brain by using a modification of the method of Klee (1977) and was a gift from Dr. Paul Leavis. Trifluoperazine was a gift from Smith, Kline and French Laboratories (Philadelphia, PA, U.S.A.). All other reagents were analytical grade.

Exorbital lacrimal glands were bilaterally excised from decapitated male Wistar rats, minced and homogenized on ice in 5 ml of Tris/HCl buffer [50 mM-Tris/HCl, 10% (w/v) sucrose and 5 mM-$MgCl_2$; pH 7.5). Portions of homogenate containing approx. 70 μg of protein were incubated for 20 s at 37°C in a 100 μl reaction mixture containing Tris/HCl buffer, 5 μM-[γ-^{32}P]ATP, either 1.0 mM-EGTA alone or 1.0 mM-EGTA plus 1.1 mM-$CaCl_2$, 0.3–

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30 μM-calmodulin and 10–300 μM-trifluoperazine, where indicated. The reaction was terminated by addition of 40 μl of stopping solution containing 100 mM-EDTA, 5% (w/v) sodium dodecyl sulphate, 200 mM-dithiothreitol, 50 μg of pyronin Y/ml and heating for 2 min at 100°C. The samples were then solubilized by incubation at 37°C for 20 min. For each sample, 50 μl portions were used for electrophoresis.

To resolve the solubilized proteins, electrophoresis was performed by using a discontinuous sodium dodecyl sulphate/polyacrylamide-vertical-slab-gel system (Laemmli, 1970). The stacking gel contained 4% (w/v) acrylamide, 0.125 M-Tris (pH 6.8) and 0.1% (w/v) sodium dodecyl sulphate. The resolving gel contained a linear gradient of 5–15% acrylamide, 0.375 M-Tris (pH 8.8) and 0.1% sodium dodecyl sulphate. The electrode buffer contained 0.05 M-Tris, 0.37 M-glycine and 0.1% (w/v) sodium dodecyl sulphate. After electrophoresis at 50 V the gels were fixed and stained with hot 0.2% (w/v) Coomassie Brilliant Blue dissolved in 50% (v/v) ethanol and 10% acetic acid. The gels were destained by diffusion in hot 5% (v/v) ethanol and 7.5% (v/v) acetic acid and then dried.

Autoradiography was carried out with Kodak XAR-5 film. The molecular weights of the protein bands were estimated by comparison with five protein standards in the mol.wt. range 45000–200000 (Bio-Rad Laboratories, Richmond, CA, U.S.A.). Incorporation of phosphate into specific protein bands was determined by densitometry by using a scanning densitometer (Zeineh; Biomed Instruments, Chicago, IL, U.S.A.).

Results and discussion

Six experiments were performed for each experimental condition. In the absence of ionized Ca^{2+} (1.0 mM-EGTA) some ^{32}P incorporation was observed (Fig. 1). Addition of Ca^{2+} (1.1 mM-$CaCl_2$ and

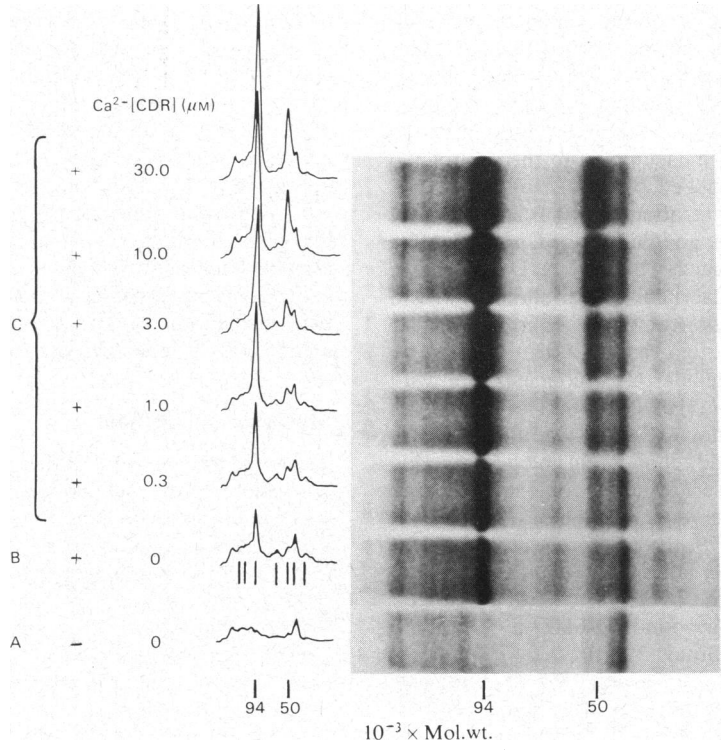

Fig. 1. *Effect of Ca^{2+} and calmodulin on phosphorylation of proteins isolated from lacrimal-gland homogenate*
Autoradiographs (right) and densitometric scans (left) are shown, illustrating phosphorylated proteins. Portions of homogenate were incubated for 20 s with [γ-^{32}P]ATP and (A) no free Ca^{2+} (1.0 mM-EGTA), (B) excess free Ca^{2+} (1.0 mM-EGTA and 1.1 mM-$CaCl_2$) plus endogenous calmodulin (CDR) or (C) excess free Ca^{2+} plus various concentrations of added calmodulin. Vertical lines on densitometric scan of B indicate proteins phosphorylated in the presence of free Ca^{2+} plus endogenous calmodulin. Molecular-weight calibration indicates the two proteins, which demonstrates a dose-dependent increase in phosphorylation in the presence of excess free Ca^{2+} and added calmodulin.

1.0mM-EGTA) in the presence of endogenous calmodulin caused an increase in phosphorylation of seven peptide bands, as well as a general stimulation of phosphorylation. In the presence of Ca^{2+}, phosphorylation of two of these peptide bands with mol.wt. 94000 and 50000 was increased markedly in a dose-dependent manner by the addition of 0.3–30.0 μM-calmodulin (Fig. 1). In the presence of Ca^{2+} and endogenous calmodulin, trifluoperazine caused a dose-dependent inhibition of the Ca^{2+}- and calmodulin-induced phosphorylation of both peptide bands, with inhibition of phosphorylation apparent at 10 μM-trifluoperazine (results not shown). When Ca^{2+} and 3.0 μM exogenously added calmodulin were present, trifluoperazine also inhibited Ca^{2+}- and calmodulin-induced phosphorylation of the two peptide bands in a dose-dependent manner (Fig. 2). Trifluoperazine (30 μM), in a calmodulin/trifluoperazine ratio of 1:10, was required to inhibit phosphorylation.

The existence of two peptide bands of mol.wt. 94000 and 50000 that were phosphorylated in the presence of Ca^{2+} and exogenous calmodulin demonstrates that the lacrimal gland contains Ca^{2+}–calmodulin-dependent protein kinase activity. Support for this conclusion comes from the demonstration that phosphorylation of the two bands was inhibited by decreasing the Ca^{2+} concentration with EGTA and by addition of trifluoperazine, a phenothiazine that in the presence of Ca^{2+} binds specifically to calmodulin and blocks Ca^{2+}–calmodulin-stimulated activities (Levin & Weiss, 1977). The two peptide bands were also phosphorylated by Ca^{2+} alone and this phosphorylation blocked by trifluoperazine. Although inhibition by trifluoperazine is in accord with the presence of Ca^{2+}–calmodulin-dependent protein kinases, it is known to have other effects. In particular, it has been reported that trifluoperazine inhibits Ca^{2+}/phospholipid-dependent protein kinases (Mori *et al.*, 1980; Schatzman *et al.*, 1981). From our data it appears that the lacrimal gland contains Ca^{2+}–calmodulin-dependent protein kinases. The presence of these kinases may provide insight into the role of Ca^{2+} in lacrimal-gland secretion, as well as secretion by other exocrine glands.

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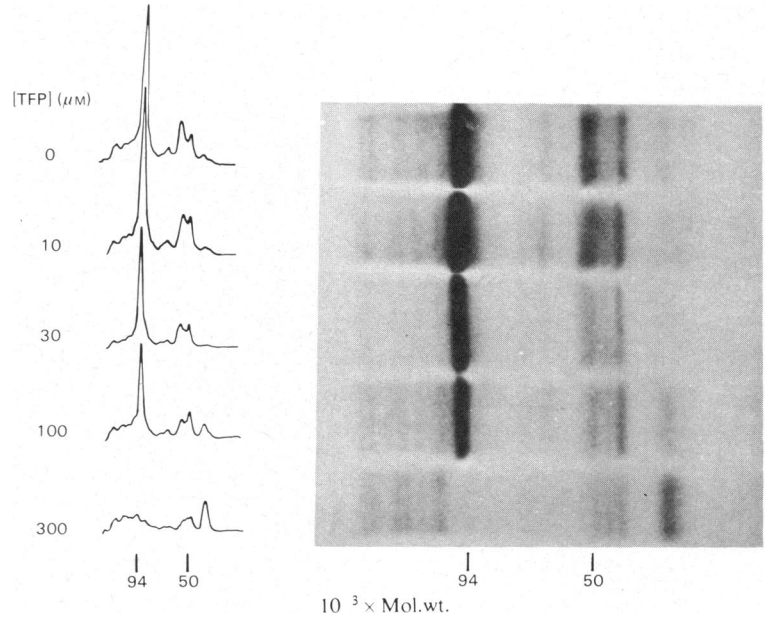

Fig. 2. *Inhibition of Ca^{2+} and calmodulin-dependent protein phosphorylation by trifluoperazine*
Autoradiographs (right) and densitometric scans (left) are shown illustrating phosphorylated proteins. Portions of homogenate were incubated for 20s with [γ-^{32}P]ATP, excess free Ca^{2+} (1.0mM-EGTA and 1.1mM-$CaCl_2$), 3.0 μM added calmodulin and various concentrations of trifluoperazine (TFP). Molecular-weight calibration indicates the two proteins, which demonstrated a dose-dependent increase in phosphorylation in the presence of excess free Ca^{2+} and added calmodulin.

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