Phosphorylation of the ribosomal protein S6 in response to secretagogues in the guinea pig exocrine pancreas, parotid and lacrimal gland

R. Jahn* and H.D. Söling

with the technical assistance of C. Bode and E. Rasenberger

Abt. für Klin. Biochemie, Zentrum Innere Medizin der Universität, Robert-Koch Str. 40, D-3400 Göttingen, FRG

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1. INTRODUCTION

It is now generally accepted that in the major exocrine glands secretion is regulated by rises of intracellular [Ca^{2+}] and/or cAMP in response to different hormones and neurotransmitters (review [1,2]). However, the molecular events following the increases of the second messenger concentrations are far less understood. Greengard [3] has proposed that a change in the degree of phosphorylation of specific proteins triggered by these second messengers may be a general mechanism by which the functional state of a cell is altered. According to this hypothesis, a number of studies has dealt with protein phosphorylation and dephosphorylation in response to secretagogues using functionally intact cell or tissue preparations of the exocrine pancreas [4–7], the lacrimal gland [8,9] and the parotid gland [10–16]. Although the data obtained are not easy to compare due to somewhat different methods applied, the following findings can be summarized from these papers:

(i) Only a few proteins are specifically phosphorylated or dephosphorylated under the influence of secretagogues. The phosphorylation pattern obtained during Ca^{2+}- or cAMP-dependent secretion is clearly different from each other, cAMP inducing apparently the phosphorylation of more proteins than Ca^{2+};

(ii) The degree of phosphorylation of most of these proteins correlates quite well with the degree of secretion induced under most conditions studied;

(iii) In all studies a major phosphoprotein (M_r 29000–35000) has been detected which is phosphorylated by Ca^{2+} as well as by cAMP-mediated secretion.

It has therefore been suggested that this protein may play a role in stimulus–secretion coupling leading to its designation as 'EC-protein' for 'exocytosis-coupled protein' [8]. The protein was found in a crude membrane fraction, the exact subcellular location being controversial [6,11,16]. In addition, the protein appeared as a double band after sodium dodecylsulfate gel electrophoresis of particulate fractions of the lacrimal gland and the exocrine pancreas suggesting that it may undergo multiple phosphorylation [9]. Here, we present

* Present address: Department of Pharmacology, School of Medicine, Yale University, 333 Cedar Street, New Haven, CT 06510, USA

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evidence that the 'EC-protein' is identical with the ribosomal protein S6 which is multiply phosphorylated under the influence of different secretagogues in three different exocrine glands.

2. MATERIALS AND METHODS

2.1. *Materials*

Male guinea pigs (body wt 250–350 g) were obtained from Lippische Versuchsanstalt (Lage). Sodium desoxycholate was purchased from Merck (Darmstadt), phenylmethylsulfonylfluoride from Boehringer (Mannheim). All other reagents were derived from sources in [8,11,14].

2.2. *[^{32}P]Phosphate incubation of the tissue and subcellular fractionation*

All animals were starved for 14 h prior to the experiments. Pancreatic lobules were prepared according to [17], lacrimal gland lobules and parotid gland slices as in [8,11]. Preloading of the tissue with [^{32}P]phosphate, incubation and stimulation was performed as in [11] using 400–500 mg wet wt instead of 100 mg. After addition of the agonists the incubation was carried out for additional 30 min and then finished by homogenization at 4°C in a stop-medium containing 0.3 M sucrose, 50 mM potassium phosphate (pH 7.0), 2 mM EDTA, 0.2 mM EGTA, 0.75 mM phenylmethylsulfonylfluoride and aprotinin (400 kallikrein inhibitor units/ml). A crude ribosomal fraction was then prepared as follows: The homogenate was spun at 15000 × g_{max} for 5 min. The resulting supernatant was centrifuged at 120000 × g_{max} for 60 min. The resulting microsomal pellet was resuspended in a buffer (= resuspension buffer) containing 0.3 M sucrose, 20 mM Tris–HCl (pH 7.5 at 4°C), 5 mM magnesium acetate, 0.1 M ammonium chloride, 1 mM dithioerythritol and 1.3% (w/v) sodium desoxycholate (2 ml/sample). After incubation on ice for 20 min the samples were layered over 3 ml 2 M sucrose which was dissolved in sucrose-free resuspension buffer. After centrifugation for 5 h at 62000 rev./min in a Beckman SW 65 rotor, the pellets were resuspended in 0.88 M KCl, 12.5 mM $MgCl_2$ and 50 mM Tris–HCl (pH 7.8 at 4°C) to remove adherent proteins and centrifuged at 100000 × g_{max} for 60 min. The final pellets were homogenized in small volumes of 5 mM $MgCl_2$, 80 mM KCl and 10 mM Tris–HCl (pH 7.6 at 4°C). The proteins were extracted with 67% (v/v) acetic acid, dialyzed against 0.5% acetic acid and lyophilized prior to electrophoresis. In later experiments the sucrose gradient step was replaced by a simple centrifugation in resuspension medium (100000 × g_{max} for 60 min) leading to somewhat higher yields.

2.3. *Electrophoresis and autoradiography*

One-dimensional sodium dodecylsulfate polyacrylamide gel electrophoresis and autoradiography were performed as in [11]. Two-dimensional separation of ribosomal proteins (~50–80 μg/sample) was carried out using a modification [18] of the procedure developed for ribosomal proteins in [19]. The gels were silver-stained according to [20], dried and subjected to autoradiography. In contrast to protein extracts derived from ribosomal fractions of lacrimal gland and pancreatic lobules, comparable extracts of parotid slices could not be directly subjected to two-dimensional electrophoresis due to some unidentified interfering material. Therefore, these samples were first run in one-dimensional sodium dodecylsulfate polyacrylamide gels. The bands were located by autoradiography of the unfixed gel, cut out, eluted and precipitated as in [21]. Rat liver ribosomal protein (100 μg) was added as protein carrier prior to the acetone precipitation step. The precipitates were extracted by 67% acetic acid, dialyzed and freeze-dried as given above; 70–80% of the radioactivity could be recovered.

3. RESULTS AND DISCUSSION

In [11,12,14,16] the M_r 35000 phosphoprotein was reported located in a microsomal pellet and it could be enriched by discontinuous sucrose density centrifugation in a fraction of low density [11] together with plasma membrane marker enzymes. However, after treatment of microsomes with the detergents sodium desoxycholate (1.3%, w/v) or Triton X-100 (up to 2%, v/v) ~90% of the recovered phosphoprotein was sedimented through a cushion of 2 M sucrose (not shown). Further washes at high ionic strength (0.88 M KCl) and subsequent extraction with 67% acetic acid (section 2.2) led only to minimal additional losses of the protein. Considering the basic isoelectric point reported earlier [14], these findings strongly sug-

gested a ribosomal location of the protein as supposed in [6]. Using the two-dimensional electrophoretic system [18] we could clearly identify the M_r 35000 phosphoprotein as the ribosomal protein S6. Fig.1 shows the protein pattern obtained after separation of a ribosomal fraction derived from guinea pig lacrimal glands. The position of S6 is indicated by the arrow. The effect of stimulation by carbachol on the protein pattern and on the phosphate incorporation is shown in fig.2. As compared to the control, S6 is partially shifted to its higher phosphorylated forms on the left side of the gel which is due to an incorporation of phosphate as demonstrated by the autoradiograph. The result of analogous incubations carried out with pancreatic lobules is given in fig.3. Here S6 also reaches a higher degree of phosphorylation during stimulation by carbachol which is again indicated by a shift to the left side of the gel. The effect of Bt_2cAMP is less pronounced, the lower phosphorylated forms of S6 being more prominent. This parallels the generally weaker effect of Bt_2cAMP on secretion (not shown, see also [2]). In the parotid a similar relation was found (fig.4), the effect of isoproterenol being as strong as that of carbachol.

Fig.1. Protein pattern derived from guinea pig lacrimal gland ribosomes after two-dimensional separation (section 2.3 for details). The framed area indicates the sector shown in fig.2–4. The arrow indicates the position of S6.

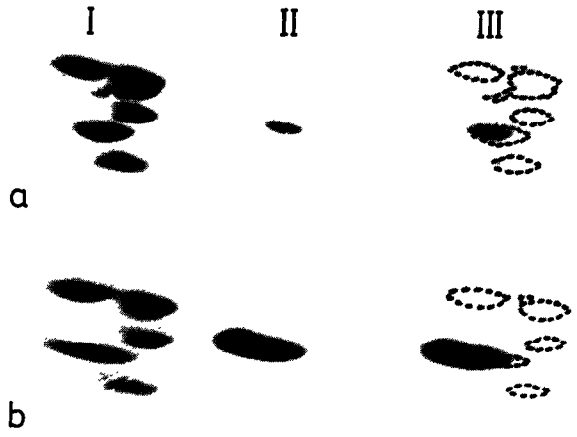

Fig.2. Influence of stimulation by carbachol on S6 phosphorylation in guinea pig lacrimal gland lobules. Two-dimensional separation was performed using crude lacrimal ribosomes prepared as given in the text: (I) protein stains; (II) autoradiographs; (III) projection of I over II; (a) control, unstimulated; (b) stimulation by 2×10^{-6} M carbachol.

Since the electrophoretic system allows a separation of the unphosphorylated and the singly and higher phosphorylated forms from one another, the degree of phosphorylation can be read from the gel and the autoradiograph. The number of phosphate residues per molecule never exceeds 3 or 4 (pancreas, carbachol stimulation, see fig.3). In most other cases, the 1- and 2-fold phosphorylated forms are predominant (pancreas, stimulation by Bt_2cAMP, parotid and lacrimal gland; cf. fig.2–4). This contrasts with other systems where an incorporation of up to 6 phosphates into the S6 protein has been observed (review [23]). The incorporation of > 1 phosphate/molecule easily explains the appearance of double bands in one-dimensional gel electrophoresis described in [8,9].

We had mentioned [22] that the 'EC-protein' in the parotid gland does not behave like the S6 protein. This had resulted from the effects of interfering substances in the guinea pig (but not the rat) parotid gland. This statement has to be corrected on the basis of the results presented here. Own unpublished experiments have revealed that the difficulties in finding the exact subcellular location were in part due to differences in the homogenization buffer used. The stop medium used in our ex-

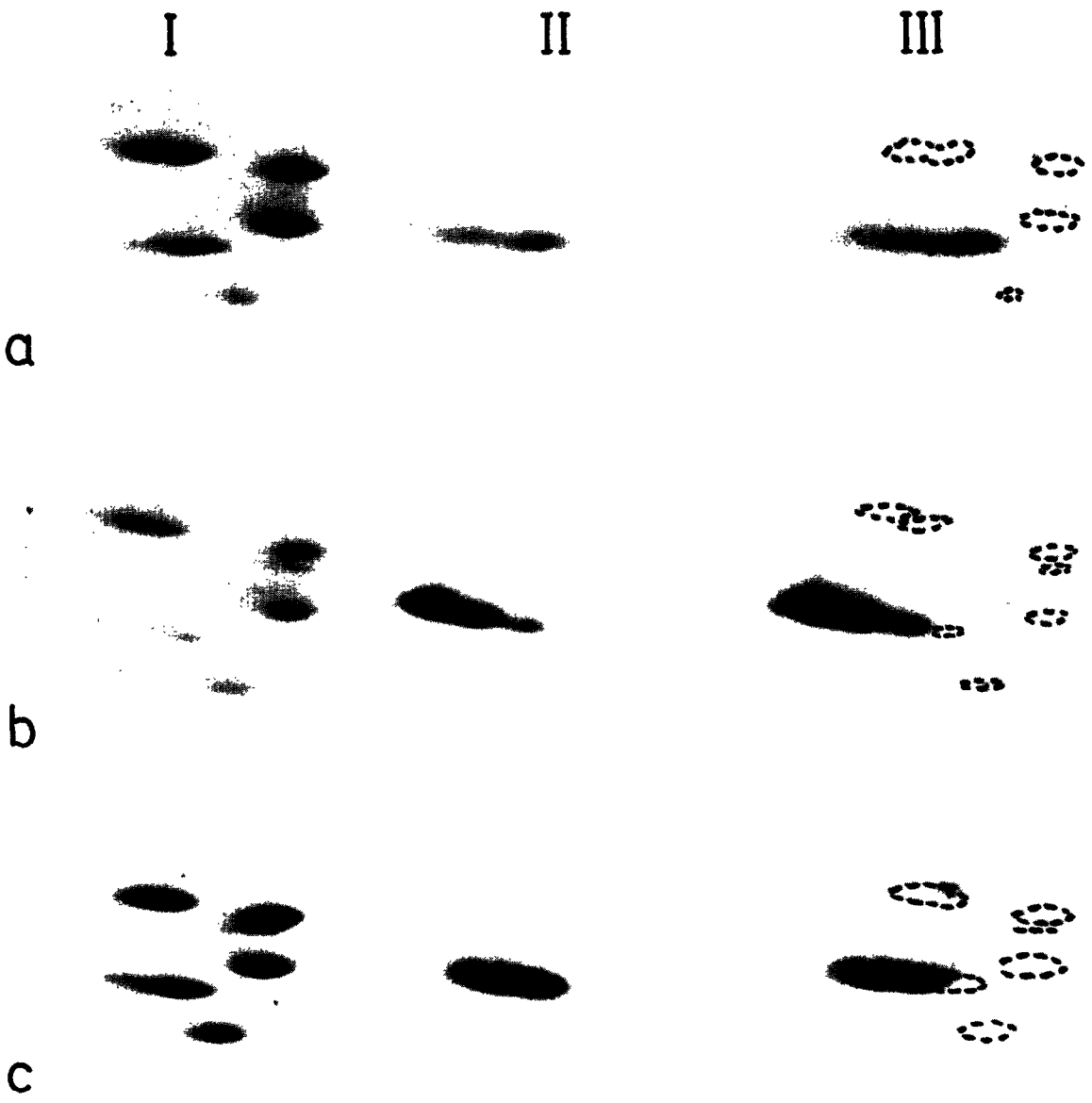

Fig.3. S6 phosphorylation in response to carbachol and Bt_2cAMP in pancreatic lobules. Preparation of the ribosomes before electrophoresis was performed as given in section 2.2. Columns I–III see legend to fig.2: (a) control, unstimulated; (b) stimulation by 2×10^{-6} M carbachol; (c) stimulation by 10^{-3} M Bt_2cAMP.

periments (section 2.2) in order to block protein kinases and phosphatases leads to partial stripping of the rough endoplasmic reticulum and sometimes to the loss of some ribosomal proteins which is probably due to the relatively high concentration of EDTA. The result was a highly untypical separation pattern in sucrose density gradients which led us to the erroneous assumption of a location in the plasma membrane [11].

The function of the phosphorylation of the S6 protein in response to secretagogues in exocrine glands is unclear at present. Our data correspond to an earlier report of the phosphorylation of S6 in a hamster islet cell tumor during cAMP-mediated insulin secretion [24]. Despite the close correlation between secretion and phosphorylation it is unlikely that the phosphorylation is involved in the molecular events underlying exocytosis. On the

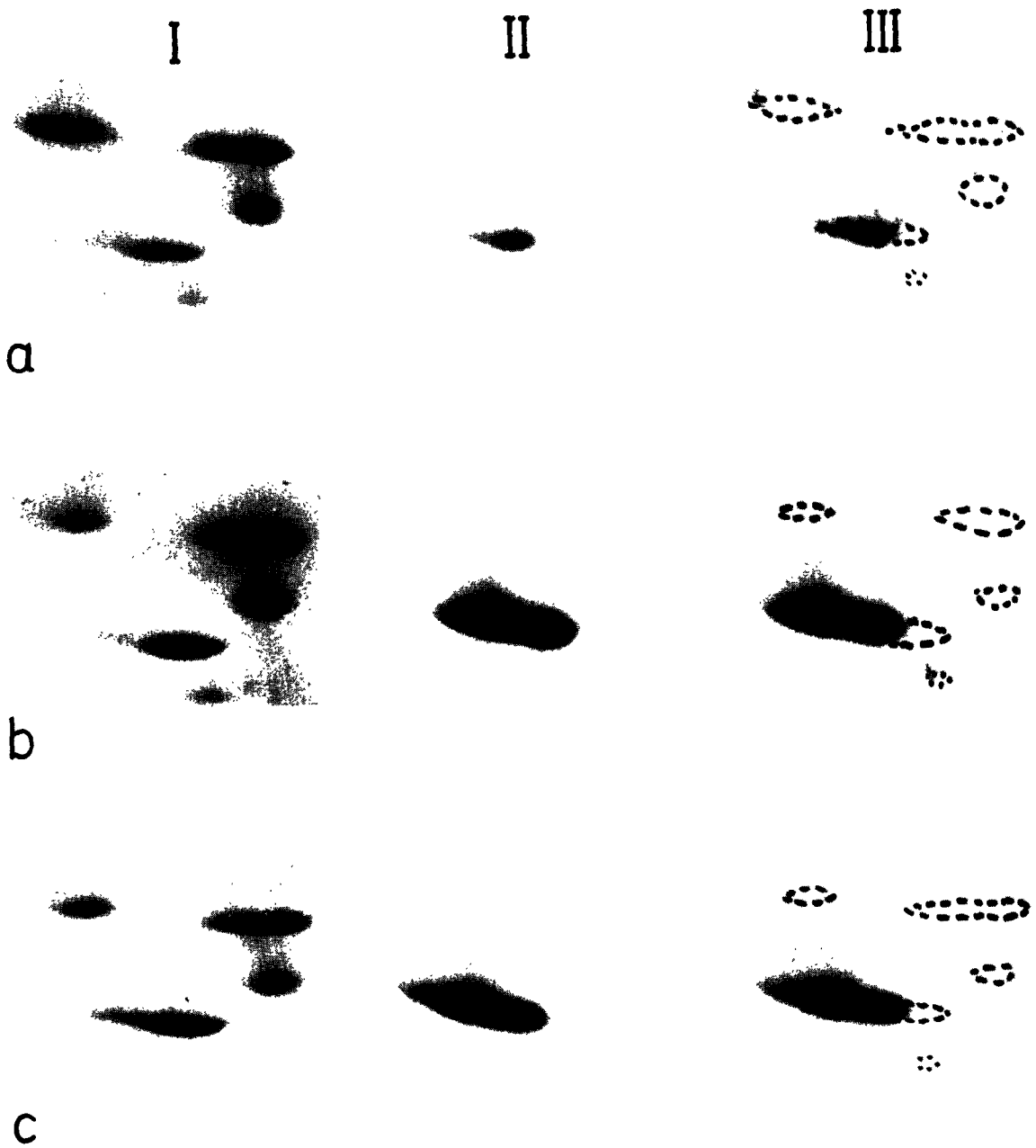

Fig.4. S6 phosphorylation in parotid gland slices during stimulation with isoproterenol and carbachol. The S6-containing fractions were eluted from a one-dimensional gel before two-dimensional electrophoresis. Columns I–III: see legend to fig.2: (a) control, unstimulated; (b) stimulated by 2×10^{-5} M isoproterenol; (c) stimulation by 2×10^{-6} M carbachol. Note that the shift of S6 in the protein pattern is partially overcome by the liver ribosomes added as standard during the extraction procedure (section 2.3).

other hand, a coupling between translation and secretion seems reasonable since each loss of protein due to secretion in these exocrine glands has to be restored by synthesis. In the exocrine pancreas [7] phosphorylation of an M_r 32500 protein which is apparently identical with S6 was also stimulated in the presence of exogenous insulin. We have confirmed this observation, but in the parotid gland

insulin was without effect (not shown). Since insulin has been reported to induce the synthesis of pancreatic amylase [25], these observations are in favour of an enhancing effect of S6 phosphorylation on translation. It should be noted, however, that despite many efforts a clear-cut relation between S6 phosphorylation and protein synthesis has not been found and is therefore questioned [23]. Due to their high degree of specialization as a machinery for the synthesis of exportable proteins which can be rapidly released by the action of secretagogues the exocrine glands may well serve as model systems to study the role of S6 phosphorylation on the regulation of the translational process.

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