Specific phosphorylation of a protein in calcium accumulating endoplasmic reticulum from rat parotid glands following stimulation by agonists involving cAMP as second messenger

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Stimulation of secretion in exocrine cells by agonists involving cAMP as second messenger is associated with the phosphorylation of a specific membrane-associated 22.4-kDa protein (protein III) (Jahn et al.). Here it is shown by subcellular fractionation of rat parotid gland lobules that protein III is associated with the endoplasmic reticulum. The submicrosomal fractions containing protein III, also contain the ATP-dependent microsomal calcium pump activity. Protein III in microsomal subfractions can be phosphorylated in vitro with catalytic subunit from cAMP-dependent protein kinase. Phosphorylated protein III contains exclusively P-serine. Protein III can be removed from ER-membranes with acid chloroform–methanol or Triton X-114, but not by high salt wash indicating that it is tightly associated with the membranes. Protein III is smaller than phospholamban and, in contrast to phospholamban, resistant to heating in SDS. A relationship between phosphorylation of protein III and microsomal calcium sequestration is discussed.

Protein phosphorylation *Parotid gland* *cAMP* *Endoplasmic reticulum* *Calcium*

1. INTRODUCTION

Stimulation of secretion in parotid gland from various species as well as in the rat lacrimal gland by agonists involving cAMP as second messenger is associated with the phosphorylation of 3 particular proteins with apparent molecular masses of 32, 25.7, and 22.4 kDa [1–6]. Agonists like carbamylcholine involving calcium as second messenger stimulate only phosphorylation of the 32-kDa protein. While the 32-kDa protein has been identified in the meantime as the ribosomal protein S6 [7,8], localization and function of the 24-kDa and the 22-kDa proteins are still unknown.

We can here show that the 22-kDa protein is a substrate for cAMP-dependent protein kinase and that it is localized in those fractions of the endoplasmic reticulum which exhibit the highest ATP-dependent calcium uptake activity.

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Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid

2. MATERIALS AND METHODS

2.1. *Incubation of rat parotid gland lobules*

Rat parotid gland lobules were prepared and incubated as in [1]. The incubation medium contained 100 μCi ^{32}P/100 mg wet tissue. No unlabelled phosphate was added together with the labelled phosphate. After preincubation in the presence of ^{32}P for 40 min, the lobules were stimulated by 2 ×

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10^{-5} M isoproterenol for 30 min. At the end of the incubation the lobules were separated from the medium and immediately homogenized with 10 vol. (v/w) of 0.3 M sucrose containing 5 mM EDTA and 5 mM triethanolamine–Cl (pH 7.0) by 3 strokes in a small glass-teflon Potter homogenizer.

2.2. *Subcellular fractionation of parotid gland tissue*

A flow scheme is depicted in fig.1. The homogenate was spun for 10 min at 180 × *g*. The pellet was resuspended with a glass Dounce homogenizer, and spun at 180 × *g* for 10 min. The supernatant was added to the first supernatant. This step was repeated once more. After centrifugation of the combined supernatants at 1000 × *g* for 10 min the pellet was further processed by discontinuous gradient centrifugation as in [9] as indicated in fig.1. The band in the 1.625 M sucrose layer represented mainly intact secretory granules. It was collected, diluted 10-fold with homogenizing medium, and the granules were sedimented by centrifugation at 20000 × *g* for 20 min. The 1000 × *g* supernatant was spun for 10 min at 5000 × *g*. The pellet containing mainly heavy mitochondria was further purified by discontinuous gradient centrifugation as described for secretory granules (fig.1). The heavy mitochondria were found above the 1.625 M sucrose layer. They were collected, diluted with homogenizing buffer and sedimented by centrifugation at 20000 × *g* for 20 min.

The 5000 × *g* supernatant was spun for 10 min at 10000 × *g* and the pellet designated as 'light mitochondrial fraction'. The 10000 × *g* supernatant was spun for 70 min at 50000 × *g*, and the pellet carefully suspended by hand in a glass-teflon Potter homogenizer with 3.6 ml homogenizing medium. Aliquots (1.2 ml) of this suspension were layered on top of a discontinuous sucrose gradient (3.6 ml) (fig.1). After centrifugation in a SW 65 swing-out rotor at 370000 × *g* for 120 min, the interphase fractions IF 1, IF 2 and IF 3, and the pellet (GP) were recovered, diluted with small volumes of homogenizing medium and collected by another centrifugation at 100000 × *g* for 60 min. The pellets were resuspended in homogenizing medium to give final protein concentrations of 5–10 mg/ml.

2.3. *Preparation of sarcoplasmic reticulum vesicles from dog heart*

This was performed as in [10].

2.4. *Electrophoresis and autoradiography*

Protein of the various fractions (50–100 μg) was precipitated with ice-cold 10% (w/v) trichloroacetic acid and the precipitated material sedimented by centrifugation (12000 × *g* for 2 min). The pellets were dried and the trichloroacetic acid removed by suspending the pellets in 0.5 ml acetone followed by another centrifugation. Subsequently 5 μl of 0.5 M K-phosphate (pH 7.6) and 1 μl of 2-mercaptoethanol were added to the pellets followed by addition of 20 μl of a solution containing 9% (w/v) SDS and 15% (v/v) glycerol. The mixture was shaken for 30 min in an Eppendorf shaker at room temperature followed by heating at 95°C for 4 min.

SDS-polyacrylamide electrophoresis as in [11] was carried out in 15% gels as in [1].

For two-dimensional electrophoresis, protein III was first resolved by one-dimensional electrophoresis as described. The area of the gel corresponding to protein III was dissected out of the gel and the protein eluted by shaking the gel pieces for about 1 h with 0.2 ml of 50 mM Tris–Cl (pH 7.9, 25°C) containing 0.1 mM EDTA, 5 mM dithioerythritol, 0.15 M NaCl, and 0.1% (w/v) SDS. Usually 0.2 ml of elution medium were used for one gel piece from one lane. After the first elution, the fluid was collected by centrifugation and the remaining gel pieces again eluted in the same way by shaking for 45 min. After another centrifugation the two supernatants were combined. Usually 60–70% of the protein was eluted in this way. Calibration proteins (50–100 μg) were added followed by precipitation of the proteins with 4 vol. of −80°C cold acetone. The mixture was kept for 30 min at −30°C and then sedimented at −20°C by centrifugation at 20000 × *g* for 10 min. After evaporation of the acetone 0.5 ml H_2O and 50 μl of the following solution were added: Tris–Cl 100 mM (pH 6.8, 22°C); $MgCl_2$ 1 mM; 2-mercaptoethanol 20% (v/v); SDS 3% (w/v). This mixture was incubated at 4°C for 24 h during which time the samples dissolved completely. The samples were lyophilized, resuspended in 50 μl H_2O, and submitted to two-dimensional electrophoresis as in [12]. The dried gels from one- or

two-dimensional electrophoresis were autoradiographed using Kodak X-Omat R films and Kodak X-Omatic enhancer screens.

2.5. *Protein phosphorylation in vitro*

Phosphorylation of microsomal membrane proteins by endogenous protein kinases was examined by incubating 50–100 μg of membrane protein in 100 μl of the following mixture (final concentrations given): Hepes 50 mM (pH 7.0); $MgCl_2$ 10 mM; EDTA 0.2 mM; dithioerythritol 0.1 mM; [γ-^{32}P]ATP 5 μM (20 mCi/μmol). The total volume was 100 μl. The reaction was started by addition of the membrane material. It was stopped after 10 s by addition of 100 μl of ice-cold 25% (w/v) trichloroacetic acid. When calcium was added, the final concentration of total calcium was 1 mM. When calmodulin was added, the final concentration was 40 μg/ml.

Phosphorylation of microsomal membranes, microsomal subfractions, or sarcoplasmic reticulum vesicles in the presence of exogenous protein kinase was performed as follows: the incubation (50 μl) consisted (final concentrations) of Hepes 50 mM (pH 7.0); $MgCl_2$ 10 mM; dithioerythritol 0.1 mM; [γ-^{32}P]ATP 50 μM (4 mCi/μmol) and 4000 units/ml of catalytic subunit from cAMP-dependent protein kinase. The incubation was started by addition of the membranes. It was performed at 37°C and stopped after 10 s by trichloroacetic acid as described above. Where indicated, the membrane fractions had been heated for 2 min at 95°C as in [3] in order to destroy most of the membrane ATPase activities.

2.6. *Determination of ATP-dependent calcium uptake*

This was performed by a filtration method as in [22].

2.7. *Determination of enzyme activities*

Amylase activity was determined with the Monoamyl test system as described by the manufacturers (Biomed, Munich). Succinic dehydrogenase activity was determined as in [13], *N*-acetylglucosaminidase activity as in [14], thiamine pyrophosphatase activity as in [15] with the following modification: the incubation was carried out in 30 mM Tris–Cl (pH 7.4, 37°C) with 0.37 mM thiamine pyrophosphate and 5 mM $MgCl_2$. Unspecific phosphatase activity was determined in the same reaction mix except that thiamine pyrophosphate was replaced by glycerophosphate. Alkaline phosphodiesterase was measured by a modification [1] of the method in [16].

2.8. *Further determinations*

RNA was determined as in [17], protein with the Coomassie blue binding assay in [18].

2.9. *Materials*

Male Wistar rats (150–190 g) were kept on a standard laboratory diet (Altromin R). Substrates and coenzymes for enzyme activity determinations came from Boehringer-Mannheim. Acrylamide, bisacrylamide and Hepes were purchased from Serva (Heidelberg), L-isoproterenol from Sigma (Munich). Tris came from J.T. Baker (Deventer). All other chemicals (analytical grade) were from E. Merck (Darmstadt). The radioactive compounds (^{45}Ca, ^{32}P and [γ-^{32}P]ATP) came from the Amersham Buchler Company (Braunschweig). Catalytic subunit of cAMP-dependent protein kinase was purified as in [19]. Calmodulin was purified by Dr Jürgensmeister in our laboratory from bovine testes as in [20].

3. RESULTS

3.1. *Subcellular fractionation of parotid gland cells after phosphorylation of protein III in intact lobules*

It had been shown in pilot experiments that the fractionation medium inhibited protein phosphatases completely. The addition of EDTA to the medium led to some differences in comparison to fractionation performed in the absence of EDTA. The high concentration of EDTA affects especially the integrity of the ribosomes as indicated by the broad distribution of RNA. However, the separation of the various particulate subcellular fractions is sufficient to allow an estimation of a given fraction with material from the other fractions. The results are summarized in table 1.

In contrast to the method in [21], the separation technique used here allows the enrichment of secretory granules without a simultaneous enrichment of mitochondria and lysosomes. The low enrichment factor for the secretory granules results

Table 1

Distribution of marker enzyme activities and RNA in subcellular fractions from rat parotid glands after in vitro stimulation of parotid gland lobules with isoproterenol (2×10^{-5} M) for 30 min (mean values ± SD, $n = 3$)

Cell fraction	Amylase (munits/ mg · min)	Succinic dehydrogenase (nmol/ mg · min)	*N*-Acetylglucosaminidase (μmol/ mg · 30 min)	Thiaminepyrophosphatase (μg P_i/ mg · 150 min)	β-Glycerophosphatase (μg P_i/ mg · min)	Phosphodiesterase (nmol/ mg · min)	RNA (μg/ mg)
Homogenate	9056 ± 1656	13.5 ± 2.4	0.32 ± 0.02	18.1 ± 1.5	11.8 ± 1.5	41.1 ± 9.8	130 ± 6
Secretory granules	19 247 ± 6418	19.2 ± 2.3	0.16 ± 0.03	11.7 ± 3.0	5.2 ± 1.1	15.6 ± 4.9	28 ± 8
Heavy mitochondria	4421 ± 1789	124.2 ± 18.9	0.93 ± 0.06	29.4 ± 3.0	6.6 ± 1.4	33.8 ± 10.1	64 ± 3
Light mitochondria	1705 ± 147	32.7 ± 3.7	0.82 ± 0.05	28.2 ± 4.9	6.2 ± 0.9	105.6 ± 23.5	116 ± 6
IF 1	513 ± 52	8.9 ± 1.1	0.20 ± 0.03	35.6 ± 4.9	13.6 ± 1.9	319.5 ± 89.9	71 ± 7
IF 2	406 ± 53	18.9 ± 3.8	0.30 ± 0.02	27.3 ± 5.1	4.8 ± 1.0	56.9 ± 6.5	127 ± 2
IF 3	550 ± 23	16.5 ± 2.8	0.42 ± 0.08	23.8 ± 6.5	4.7 ± 2.7	35.7 ± 8.0	113 ± 4
Pellet fraction (PF)	420 ± 158	4.1 ± 0.2	0.27 ± 0.10	22.2 ± 7.2	7.0 ± 5.3	11.2 ± 3.2	106 ± 21
Cytosol	12 575 ± 1441	0.5 ± 0.1	0.21 ± 0.04	26.3 ± 10.4	18.6 ± 7.2	7.5 ± 2.3	117 ± 3

Note that the inclusion of 5 mM EDTA in the homogenizing and separation media has led to some distortion of the marker distribution as is especially obvious from the distribution of RNA. The inclusion of EDTA was necessary in order to inhibit protein dephosphorylation during the separation process. For details see section 2. Mean values ± SD

at least partially from the fact that the subcellular fractionation was performed 30 min following stimulation of secretion by isoproterenol during which time a considerable percentage of the secretory granules had undergone exocytosis.

The heavy mitochondrial fraction is characterized by the enrichment of succinic dehydrogenase but also contains a considerable percentage of the lysosomal fraction as indicated by the high activities of *N*-acetylglucosaminidase. The light mitochondrial fraction represents a mixture of mitochondria, lysosomes, plasma membranes and endoplasmic reticulum as indicated by the various marker enzymes and RNA. The post-mitochondrial supernatant was subfractionated on a stepwise sucrose gradient into 4 particulate fractions as described in fig.1. IF 1 represents mainly plasma membranes as indicated by the high alkaline phosphodiesterase activity. IF 2 and IF 3 consist predominantly of endoplasmic reticulum as indicated by the high RNA content and the high ATP-dependent azide resistant calcium-accumulating activity (86.2 + 22.2 and 50.6 + 21.9 nmol calcium · mg^{-1} · 10 min^{-1}, respectively). This fits well with our earlier observation [22] that IF 2 and IF 3 exhibit the highest capacity for ATP-driven calcium accumulation. The pellet fraction (GP) contains almost the same amount of RNA and is almost free from contamination with mitochondria or plasma membranes (table 1). On the other hand, this fraction shows a rather low ATP-dependent calcium uptake capacity (11.6 + 2.4 nmol calcium · mg^{-1} · 10 min^{-1}) which contrasts with our earlier observation [22] that the pellet fraction resulting from the microsomal subfractionation had a high calcium-accumulating

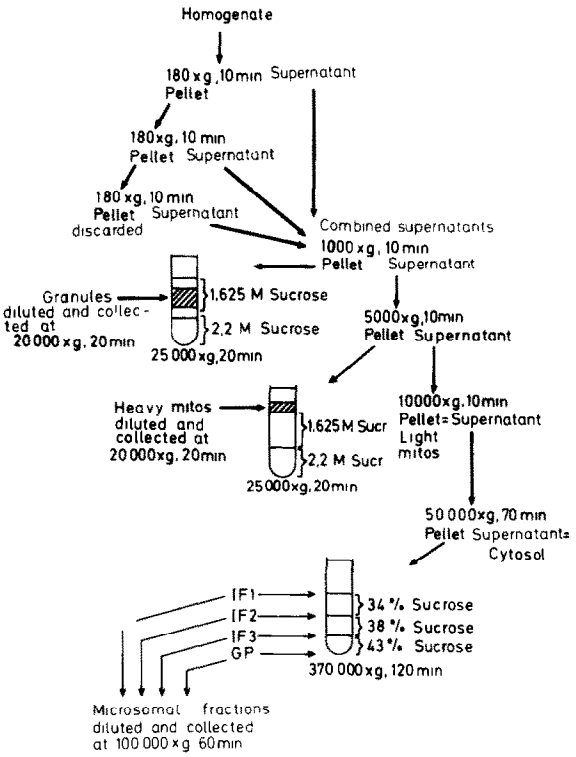

Fig.1. Flow scheme of preparation of subcellular fractions from rat parotid gland lobules.

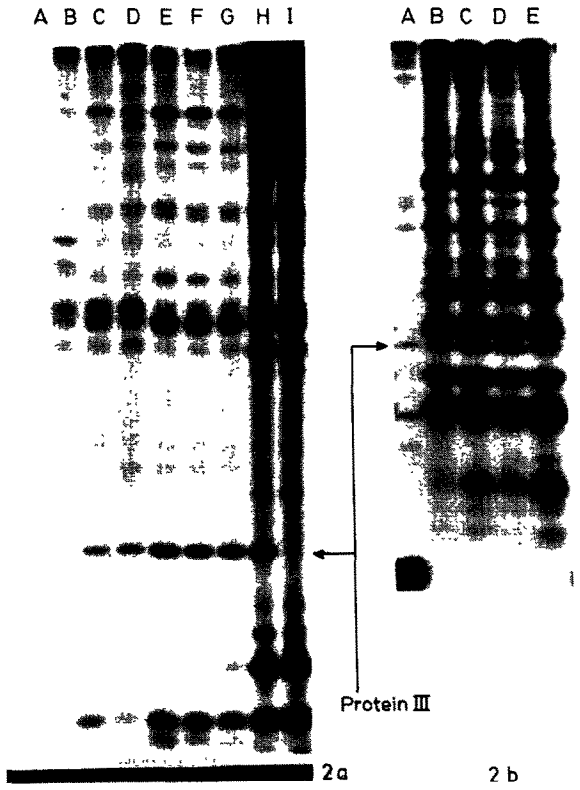

Fig.2. (a) Distribution of ^{32}P-labelled protein III between different subcellular fractions from rat parotid glands. Isolated parotid gland lobules had been incubated with ^{32}P and stimulated with isoproterenol. After stimulation for 30 min the lobules were homogenized and the homogenate subfractionated as given in section 2. Aliquots from each subcellular fraction corresponding to 60 μg protein were separated by polyacrylamide gel electrophoresis. (A) secretory granules; (B) heavy mitochondria; (C) light mitochondria; (D) IF 1 (representing mainly plasma membranes); (E, F and G) IF 2, IF 3 and GP, respectively (representing mainly endoplasmic reticulum); (H, I) 50000 $\times$ g pellets from total homogenates of parotid gland lobules which had been incubated with ^{32}P in the presence or absence of isoproterenol, respectively. (b) In vitro phosphorylation of microsomal subfractions from rat parotid glands with catalytic subunit of cAMP-dependent protein kinase. Phosphorylation was carried out as given in section 2. Aliquots corresponding to 100 μg of protein were used from each fraction. They were heated for 2 min at 95°C prior to phosphorylation. (A) 50000 $\times$ g pellet from parotid gland lobules which had been labelled in intact cells in the presence of isoproterenol (25 μg protein). This lane serves as control. (B–E) IF 1, IF 2, IF 3 and GP fraction (see fig.1 and text).

capacity. Most likely under the present conditions these membranes have become leaky due to the EDTA-treatment.

When the subcellular fractions characterized biochemically in table 1 were subjected to polyacrylamide gel electrophoresis in such a way that the same amount of protein was used from each fraction, the phosphorylated protein III could be detected in all fractions except the secretory granules (fig.2a). There were however considerable quantitative differences: the highest amount was detected in IF 2. Fractions IF 3 and GP also contained high amounts of phosphorylated protein III, as did the light mitochondrial fraction, while the plasma membranes-enriched fraction (IF 1) contained much less.

3.2. *Phosphorylation in vitro*

Rat parotid gland cells were subfractionated as described but without prior labelling with ^{32}P. Fractions IF 1, IF 2, IF 3 and GP were subse-

quently used as substrates for in vitro phosphorylation with [γ-^{32}P]ATP and catalytic subunit of cAMP-dependent protein kinase. The membrane preparations had been heated as in [3] to remove endogenous ATPase activity. The results are depicted in fig.2b. Accordingly a protein with the same apparent molecular mass as protein III from intact cells became phosphorylated in all 4 fractions, the highest incorporation being found in IF 2 (fig.2b). The identity of the in vitro labelled protein and phosphorylated protein III was supported by two-dimensional electrophoresis (fig.3a). The in vitro labelled phosphoprotein migrated to the same position as protein III, and a mix of in vitro labelled protein with phosphorylated protein III from intact cells resulted in only one spot (fig.3a).

In the absence of catalytic subunit a significant phosphorylation of many proteins occurred, but protein III remained unlabelled. Addition of calcium resulted in the phosphorylation of an additional 34-kDa protein (not 56) and further addition of calmodulin led to the phosphorylation of proteins of 56, 33, and 28 kDa, but not protein III (fig.3b).

3.3. *Nature of phospho amino acid and extraction of protein III*

The only phospho amino acid which could be found in vivo or in vitro labeled protein III was P-serine.

Protein III could not be extracted from microsomal membranes with either 0.5 M NaCl, 100 mM Na_2CO_3, or 0.5% desoxycholate. It could however be completely extracted with acid chloroform–methanol and partially with Triton X-114 (fig.4a). The partial extraction with Triton X-114 (Bordier (23)) together with the inefficiency of high salt solutions indicates that protein III is firmly attached to the membranes (probably by hydrophobic regions of the protein) and might be an integral membrane protein.

3.4. *Comparison of protein III with phospholamban*

Sarcoplasmic reticulum from dog heart which had been incubated in vitro with [γ-^{32}P]ATP and catalytic subunit of cAMP-dependent protein kinase exhibited on the electropherogram two prominent phosphorylated bands ($PL_{(a)}$ and $PL_{(b)}$ in fig.4b). These bands represent phospholamban ($PL_{(a)}$) and phospholamban 'subunits' ($PL_{(b)}$). Co-electrophoresed phosphorylated protein III migrated with a lower apparent molecular mass (fig.4b). Furthermore, while phospholamban was converted to a significant extent to smaller phosphopeptides with an app. M_r of about 7 kDa ($PL_{(b)}$) during heating at 95°C in 4% SDS, protein III was not affected by this treatment (not shown).

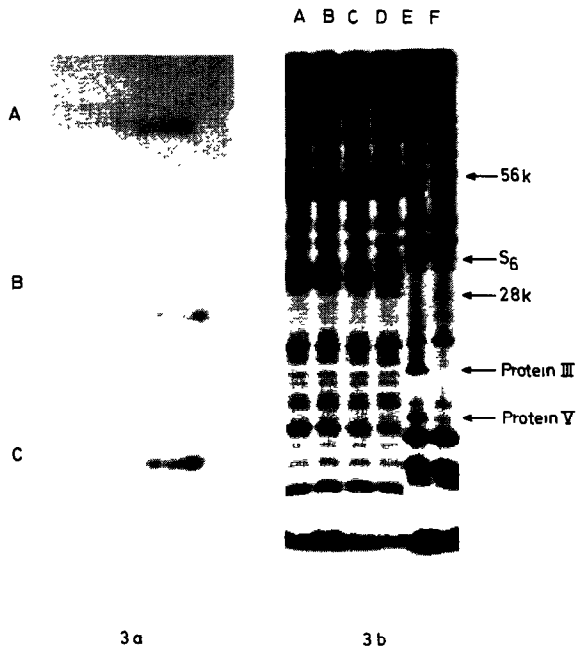

Fig.3. (a) Autoradiographs following two-dimensional electrophoresis of protein III. (A) Protein III after labelling in intact cells in the presence of isoproterenol. (B) Protein III after in vitro labelling with catalytic subunit of cAMP-dependent protein kinase. (C) Two-dimensional co-electrophoresis of a mixture of equal amounts (with respect to radioactivity) of A and B. Whether the multiple spots obtained for A and B reflect multi-site phosphorylation is under investigation. (b) Autophosphorylation of total microsomal membranes in vitro. The phosphorylation was carried out as given in section 2. (A) Control; (B) plus calmodulin, no calcium; (C) plus calcium, no calmodulin; (D) plus calmodulin, plus calcium; (E,F) 50000 × *g* pellet from parotid gland lobules after labelling with ^{32}P in intact cells in the presence or absence of isoproterenol, respectively. Protein V corresponds to the 16.5-kDa protein in [5]. It is not located in the endoplasmic reticulum.

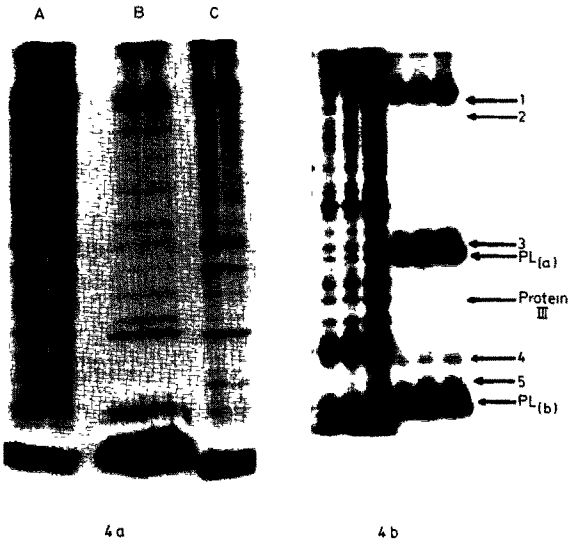

Fig.4. (a) Extraction of protein III from parotid gland microsomes with Triton X-114; 50000 × *g* pellets from parotid gland lobules labelled in intact cells with ^{32}P in the presence of isoproterenol (2×10^{-5} M) were extracted with Triton X-114 as in [22]. After phase separation the samples were spun in an Eppendorf centrifuge and aliquots were taken from the upper (aqueous) phase (A), from the lower (Triton X-114) phase (B), and from the sediment (C). A comparison of A and B shows that protein III is incompletely but rather selectively extracted into the Triton phase. Autoradiographs following SDS-polyacrylamide gel electrophoresis are shown. (b) Comparison of protein III and phospholamban. Parotid gland microsomes or sarcoplasmic reticulum vesicles from dog heart were phosphorylated in vitro with catalytic subunit of cAMP-dependent protein kinase as given in the text. (A) Parotid gland microsomes; (B) sarcoplasmic vesicles. $PL_{(a)}$, phospholamban; $PL_{(b)}$, breakdown products of phospholamban (subunits). The numbered arrows refer to the positions of marker proteins (1, conalbumin; 2, albumin; 3, chymotrypsinogen; 4, myoglobin; 5, cytochrome *c*. Autoradiographs following SDS-polyacrylamide gel electrophoresis are shown.

4. DISCUSSION

According to our results, protein III resides in the endoplasmic reticulum. Subfractionation of microsomes shows that those fractions containing the highest amount of protein III possess also the highest capacity for ATP-dependent calcium accumulation.

Association of phosphoproteins with the calcium sequestration process is known from other systems: phosphorylation of phospholamban in cardiac muscle sarcoplasmic reticulum is associated with an increased affinity of the calcium transport ATPase for calcium [24]. In platelets cAMP-dependent phosphorylation of a 24.3-kDa protein in intracellular membrane vesicles sedimenting at 100000 × *g* is associated with an increased calcium efflux [25].

Although a function of protein III phosphorylation has not yet been proven the following facts justify the working hypothesis that such a relationship might exist:

(i) We have shown [2] that the stimulation of exocytosis by the β-agonist isoproterenol remains unaffected in the absence of exogenous calcium and presence of EGTA while stimulation of exocytosis by carbamylcholine is completely blocked under the same conditions. This indicates that sufficient amounts of calcium must have been released by isoproterenol from endogenous stores to permit the fusion-fission process;

(ii) Stimulation of secretion from exocrine glands is associated with an increased phosphorylation of the ribosomal protein S6 [7,8]. This occurs with agonists involving calcium as well as with agonists involving cAMP as second messenger. The phosphopeptide pattern of the ribosomal protein S6 indicates that even though cAMP serves as second messenger for β-agonists, isoproterenol-induced phosphorylation of the protein S6 is not or not exclusively catalyzed by a cAMP-dependent protein kinase, but rather by a calcium-dependent protein kinase [26].

It therefore seems possible that part of the effects of agonists involving cAMP as second messenger is exerted by the creation of small intracellular calcium transients. The possible role of the endoplasmic reticulum for this process, in particular the possible function of phosphorylation of protein III in this context are under investigation.

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