

## PHOSPHORYLATION OF PROTEIN COMPONENTS OF ISOLATED ZYMOGEN GRANULE MEMBRANES FROM THE RAT PANCREAS

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### 1. Introduction

Our earlier results have already documented some aspects of protein phosphorylation in rat pancreas fragments [1]. The stimulation of amylase secretion by pancreozymin and caerulein was accompanied in vitro by an increase in  $^{32}\text{P}$  orthophosphate incorporation into proteins, and especially so in the membrane proteins of zymogen granules. The mechanism involved in the activation by pancreozymin of protein kinase(s) directing these phosphorylations is not clearly established. The role of cyclic AMP as an intracellular mediator is controversial [2–4] and recent evidence supports the participation of cyclic GMP [4]. The aim of the present investigation was to examine some properties of the isolated membranes of zymogen granules from the rat pancreas. We were able to detect an endogenous protein kinase activity capable of phosphorylating 8 to 10 membrane protein constituents as well as added histones. Cyclic AMP, and cyclic GMP to a lesser extent, exerted moderate stimulatory effects. These effects of cyclic nucleotides on membrane phosphorylation became more apparent upon addition of a partially purified soluble protein kinase also extracted from the rat pancreas.

### 2. Materials and methods

Zymogen granules were isolated from rat pancreas as described previously [1]. Secretory proteins were released in 0.2 M bicarbonate at pH 8.2. Membranes were purified by discontinuous gradient centrifugation according to Meldolesi [5]. They were utilized within the same day for the phosphorylation tests.

A crude protein kinase was also prepared. Rat pancreases were homogenized in 5 mM phosphate buffer (pH 7.0) containing 4 mM EDTA and 75 mM  $\epsilon$ -aminocaproate. The homogenate was centrifuged at  $27\,000 \times g$  for 30 min. Ammonium sulfate was added to the supernatant to a final concentration of 30%. The precipitate was collected, dissolved in 5 mM phosphate (pH 7.0), 2 mM EDTA, 75 mM  $\epsilon$ -aminocaproate, and dialyzed overnight against the same buffer. This preparation was kept at  $-90^\circ\text{C}$ .

The basal assay mixture for studying membrane phosphorylation contained, in a total volume of 0.2 ml, 200  $\mu\text{g}$  membrane proteins (as determined by the Folin assay), 10–20  $\mu\text{M}$  [ $\gamma$ - $^{32}\text{P}$ ] ATP (1 to  $4 \cdot 10^3$  cpm/pmole; the Radiochemical Centre, Amersham, England), 50 mM Tris-HCl (pH 7.5), 1 mM aminophylline, Trasylol (Bayer, Leverkusen, Germany) 500 UIK/ml, and 10 mM  $\text{MgSO}_4$ . When specified, the medium was supplemented with 10 mM NaF, histones II A (Sigma, St. Louis, Missouri),  $10^{-6}$  M cyclic or  $5 \cdot 10^{-6}$  M cyclic GMP. The phosphorylation was initiated by the addition of radioactive ATP and conducted at  $30^\circ\text{C}$ .

### 3. Results and discussion

Fig. 1 shows that phosphorylation proceeded rapidly and that after 60 sec a plateau was maintained for at least 5 min. This reaction was not limited by the hydrolysis of [ $\gamma$ - $^{32}\text{P}$ ] ATP by membranous ATPase. Indeed, the addition of an ATP-regenerating system made of 7.5 mM phosphoenolpyruvate and 25 U/ml pyruvate kinase did not improve the kinetics (data not shown). On the other hand, such a membrane preparation

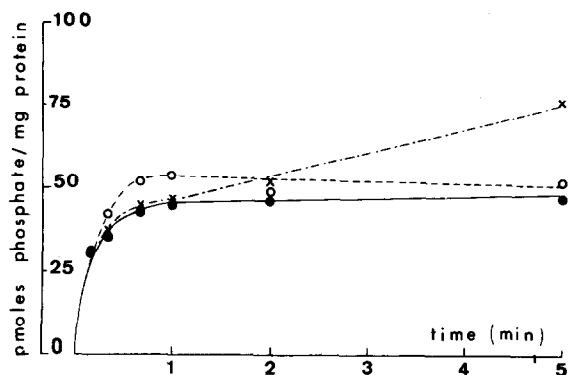


Fig. 1. Time-study of autophosphorylation of the membrane of zymogen granules with  $[\gamma\text{-}^{32}\text{P}]$  ATP. This experiment was conducted as described under Materials and methods and is representative of 3 similar experiments. The phosphorylations were stopped by the addition of 10%  $\text{CCl}_3\text{COOH}$  and the pellets were filtered on Millipore AAWP (Bedford, U.S.A.). Results of one representative experiment ( $n=3$ ). (●-●-●) basal medium; (x-x-x) 10 mM NaF; (○-○-○)  $1\cdot 10^{-6}$  M cyclic AMP.

had a moderate phosphoprotein phosphatase activity which was inhibited by 50% by 10 mM NaF (P. Robberecht, personal communication). Fig. 1 illustrates that in the presence of 10 mM NaF, protein phosphorylation exceeded control values. The plateau appears therefore to result from an equilibrium between the opposite activities of protein kinase and phosphoprotein phosphatase in the zymogen granule membrane.  $1\cdot 10^{-6}$  M cyclic AMP induced a moderate but consistent stimulation of autophosphorylation.

Table 1 shows a comparison of the endogenous activity of membranes with a preparation of soluble protein kinase(s) partially purified from the rat pancreas. The autophosphorylation of membranes was only slightly stimulated by  $1\cdot 10^{-6}$  M cyclic AMP or  $5\cdot 10^{-6}$  M cyclic GMP and the protein kinase activity exerted by these membranes on added histones II A was unaffected by cyclic nucleotides. On the other hand, the activity of soluble protein kinase(s) was markedly stimulated by  $1\cdot 10^{-6}$  M cyclic AMP and  $5\cdot 10^{-6}$  M cyclic GMP. When this soluble preparation was added to membranes, the aggregate phosphorylation was lower than the sum of each autophosphorylation activities considered separately. The marked effect of cyclic nucleotides on this mixture suggests that the activity of soluble protein kinase(s) was predominating and contributed to the phosphorylation of membrane proteins. It can be hypothesized that a component of the crude soluble protein kinase preparation inhibited membrane protein kinase activity. It is more likely however that the affinity of semi-purified soluble protein kinase(s) for endogenous substrates was lower than that for membrane proteins. Competitive inhibition might render the autophosphorylation of soluble protein negligible in the presence of zymogen granule membranes.

As shown in Table 2, extraction of membranes with 0.5 M KCl did not decrease the protein kinase activity measured on endogenous substrates or on added histones. In addition, this treatment did not affect the capacity of membranes to be phosphorylated in the

Table 1  
Comparison of endogenous and exogenous protein kinase activities

Proteins added			pmoles phosphate/mg protein. 10 min		
Zymogen granule membrane (158 $\mu\text{g}$ )	Soluble protein kinase (250 $\mu\text{g}$ )	Histones II A (250 $\mu\text{g}$ )	no cyclic nucleotide added	cyclic AMP $1\cdot 10^{-6}$ M	cyclic GMP $5\cdot 10^{-6}$ M
+	0	0	47	58	52
0	+	0	21	33	26
+	+	0	27	59	44
+	0	+	125	125	116
0	+	+	531	1004	811

Assays were performed in the presence of 10 mM NaF. Results of one representative experiment ( $n=4$ ).

Table 2  
Protein kinase activity of the membrane of zymogen granules  
after treatment with 0.5 M KCl

Proteins added	pmoles phosphate/mg protein, 5 min	
	Control membranes	Washed membranes
No addition	48	73
Soluble protein kinase (250 $\mu$ g)	110	129
Histones II A (250 $\mu$ g)	64	66

Purified membrane pellets were suspended at 0°C in 50 mM Tris-HCl (pH 7.5) and Trasylol 500 UIK/ml in the presence or absence (control) of 0.5 M KCl. These suspensions were recentrifuged for 10 min at 2500  $\times$  g. The recovered membranes were then utilized for phosphorylation tests in the presence of 10 mM NaF. Results of one representative experiment (n=3).

presence of exogenous protein kinase(s). Thus it appears that protein kinase activity and protein substrates for this enzyme were intrinsic parts of the zymogen membrane and can not be assimilated to exogenous proteins simply contaminating the membrane.

Individual protein components of the zymogen granule membrane were analyzed by polyacrylamide gel electrophoresis in sodium dodecylsulfate. Staining with Coomassie blue revealed at least 26 distinct bands (fig. 2A). The crude soluble protein kinase preparation when submitted to similar electrophoresis showed 10 to 12 bands, the main component having a mol.wt of 66 000 (fig. 2B).

As can be seen in fig. 3, 8 to 10 components of

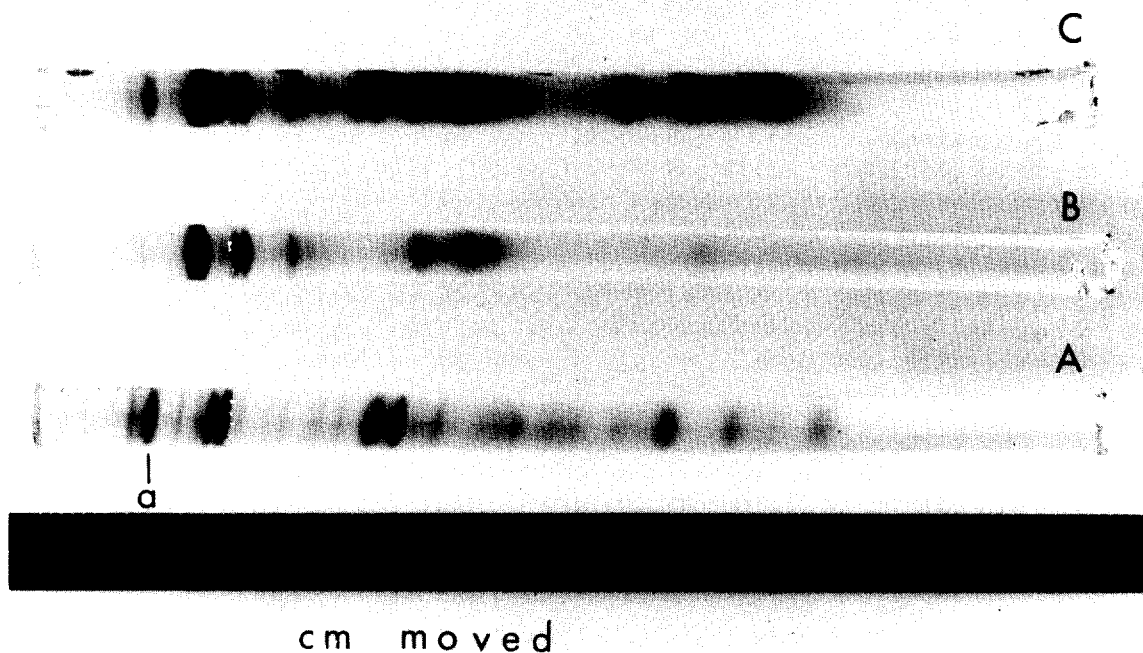


Fig. 2. Polyacrylamide gel electrophoresis in 0.1% sodium dodecylsulfate after 10 min incubation of: A: zymogen granule membrane from rat pancreas (a indicates the protein band most markedly phosphorylated in the high molecular weight range, as illustrated in fig. 3). B: semi-purified soluble protein kinase preparation from rat pancreas. C: a mixture of the two preceding extracts. The standard incubation medium described under Materials and methods was supplemented with 10 mM NaF. Each incubation was stopped by the addition of 0.1 ml 0.03 M phosphate buffer (pH 7.2), 3% (v/v)  $\beta$ -mercaptoethanol, 24% sucrose, 6% sodium dodecylsulfate. These samples were heated for 2 min at 100°C. 25  $\mu$ l samples were then applied to polyacrylamide gels containing 10% acrylamide, 0.1% sodium dodecylsulfate, 0.1 M sodium phosphate (pH 7.2), 0.125% persulfate, and 0.0135% Temed (from Fluka, Buchs, Switzerland). Electrophoresis was carried out for 270 min at 8 mA/tube. These gels were stained with Coomassie blue in 10% acetic acid-25% isopropanol and destained in 10% acetic acid-10% isopropanol.

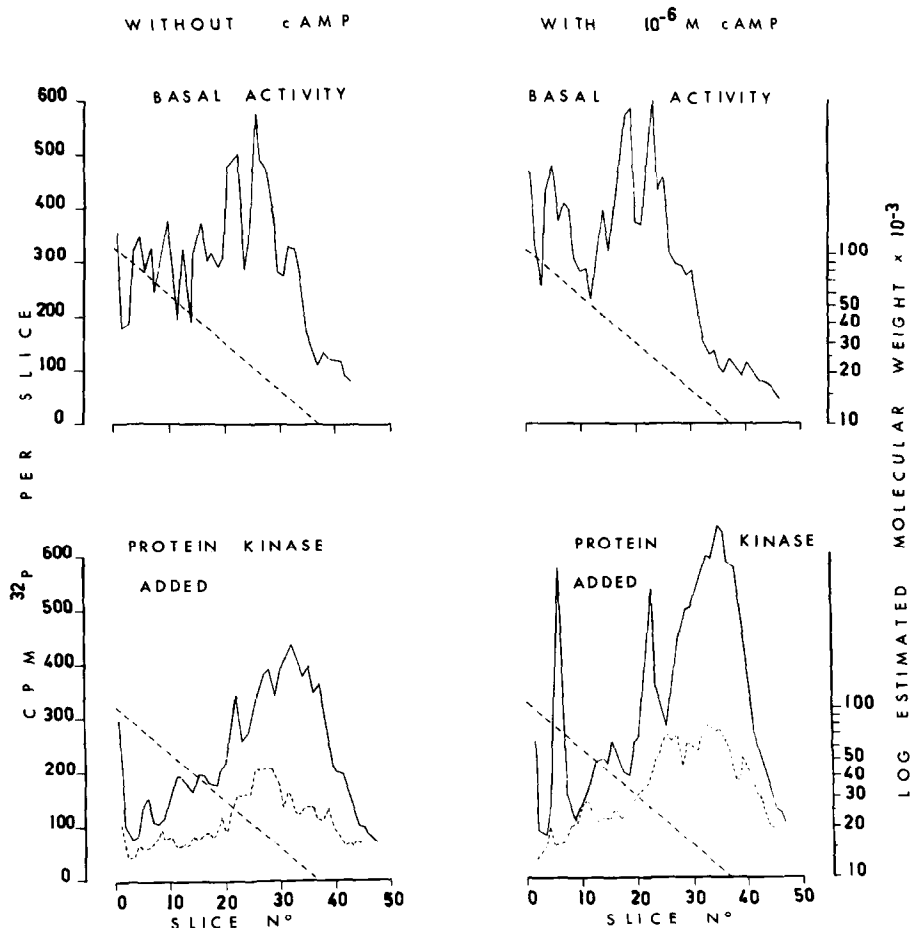


Fig. 3. Distribution of  $^{32}\text{P}$  among the proteins of phosphorylated zymogen granule membranes, after separation by polyacrylamide gel electrophoresis in 0.1% sodium dodecylsulfate. After 10 min incubation carried out under the same general conditions as those in fig. 2, in the presence of 10 mM NaF and with or without  $1 \cdot 10^{-6}$  M cyclic AMP, 100  $\mu\text{l}$  of SDS-solubilized proteins were applied to each gel. After electrophoresis, the gels were fixed overnight in 10% acetic acid-25% isopropanol, washed in 10% acetic acid-10% isopropanol and cut into 2 mm slices. The radioactivity was measured by scintillation counting. The autophosphorylation profile of the isolated crude soluble protein kinase preparation is illustrated by broken lines in the two lower panels. The linear relationship between molecular weight and the distance of migration was standardized with bovine serum albumin, hen ovalbumin, bovine carboxypeptidase A, bovine ribonuclease and horse heart cytochrome *c*.

the membrane with mol.wt ranging from 12 000 to 70 000 were phosphorylated by endogenous kinase. There was no specific effect of cyclic AMP on a particular protein species. These results are at variance with those reported by MacDonald and Ronzio [6] while our work was in progress. These authors noticed that more than 70% of  $^{32}\text{P}$  was incorporated into a single protein of approximate mol.wt 130 000 in zymogen granule membranes incubated for 5 sec

without aminophylline and NaF (this component was isolated by SDS polyacrylamide gel electrophoresis at pH 2.4). After incubation in the presence of the exogenous kinase, we detected 8 phosphorylated components. Cyclic AMP increased the incorporation of  $\gamma\text{-}^{32}\text{P}$  in all molecular species but its effect was particularly pronounced for a protein with a mol.wt of approx. 70 000 (Band a in fig. 2A).

In conclusion, it appears that 8 to 10 proteins in

the zymogen granule membrane can be phosphorylated by an endogenous protein kinase. In addition, soluble protein kinase(s) can also phosphorylate the membrane of zymogen granules. The endogenous membrane protein kinase activity was only slightly dependent on cyclic nucleotides. If we assume that this kinase remained unaltered during membrane isolation, this observation might suggest that the *in vivo* phosphorylation of zymogen granule membrane is not under hormonal control. This conclusion would contradict our previous hypothesis, based on intact cell preparation [1], according to which phosphorylation of specific proteins could change granule membrane properties and facilitate the fusion with the plasma membrane during exocytosis. It is however possible to reconcile the data obtained on pancreas fragments and pancreas extracts if we admit that rises in the intracellular levels of cyclic GMP or cyclic AMP entail a dissociation of cytoplasmic protein kinase(s) and that the liberated catalytic subunit(s) are secondarily transferred on zymogen granule membranes, resulting in 'in situ' phosphorylation of 8 to 10 constituent proteins. This hypothesis finds some support in recent observations. Korenman et al. [7] demonstrated a translocation of cytoplasmic protein kinase towards microsomes, in a cyclic AMP-independent form, after stimulating rat uterus with isoproterenol. Our own preliminary results (unpublished data) indicate an important rise in membrane bound protein kinase activity when zymogen granule membranes of

rat pancreas were incubated at 0°C in the presence of semi-purified soluble protein kinase and of  $1 \cdot 10^{-6}$  M cyclic AMP.

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#### References

- [1] Lambert, M., Camus, J. and Christophe, J. (1973) *Biochem. Biophys. Res. Commun.* 52, 935–942.
- [2] Case, R. M., Johnson, M., Scratcherd, T. and Sherratt, H. S. A (1972) *J. Physiol.* 223, 669–684.
- [3] Rutten, W. J., de Pont, J. J. H. M. and Bonting, S. L. (1972) *Biochim. Biophys. Acta* 274, 201–213.
- [4] Robberecht, P., Deschodt-Lanckman, M., de Neef, P., Borgeat, P. and Christophe, J. (1974) *FEBS Lett.* 43, 139–143.
- [5] Meldolesi, J., Jamieson, J. D., Palade, G. E. and Evans, L. M (1971) *J. Cell. Biol.* 49, 109–129.
- [6] MacDonald, R. J. and Ronzio, R. A. (1974) *FEBS Lett.* 40, 203–206.
- [7] Korenman, S. G., Bhalla, R. C., Sanborn, B. M. and Stevens, R. H. (1974) *Science* 183, 430–432.