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THE EFFECT OF CYCLIC NUCLEOTIDES AND CHOLERA TOXIN ON IN VIVO AND IN VITRO PHOSPHORYLATION OF SMALL INTESTINAL BRUSH BORDER MEMBRANES

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The effect of cyclic nucleotides and cholera toxin on the phosphorylation of the brush border membrane proteins of the rat jejunum was studied. Phosphorylation was analyzed by autoradiography of brush border membrane proteins separated by SDS-polyacrylamide gel electrophoresis. Phosphorylation was performed either in vivo by perfusion of the jejunum with [^{32}P]orthophosphate followed by an analysis of the isolated membranes or in vitro by phosphorylation of isolated brush border membranes by [γ-^{32}P]ATP in the presence of saponin. The addition of cholera toxin (10 μg/ml) or dibutyryl-cAMP (5 mmol/l) to the perfusate was unable to produce significant changes in the phosphoprotein pattern. On the other hand, cAMP (at 5 μmol/l) induced an increase of the phosphorylation of a 86 kDa protein when freshly isolated brush border membranes were phosphorylated by [γ-^{32}P]ATP. However, the same effect could also be induced by low concentrations of cGMP (0.1 μmol/l). It is concluded that brush border membranes from rat jejunum do not contain cAMP-dependent protein kinase activity and that cAMP-dependent protein phosphorylation of this membrane does probably not represent the final event of cholera toxin-induced secretion.

Introduction

Different stimuli (e.g. cholera toxin, several hormones) leading to secretion of water and electrolytes in the small intestine are accompanied by an increase in the intracellular concentration of cAMP. cAMP has been suggested to provoke an inhibition of the electroneutral Na^+/Cl^- cotransport in the villus cells and an increase of electrogenic secretion of chloride localized in the crypt cells [1]. Since many effects of cAMP are suggested to be mediated through regulation of protein kinase activity [2,3], it could be speculated that phosphorylation and dephosphorylation reactions of single membrane proteins could lead to altered brush border membrane functions (transport properties and/or membrane structure).

Evidence was provided for an effect of cyclic nucleotides on phosphorylation of the small intestinal brush border membrane [4–6,13,14]. Lucid and Cox [5] reported enhanced incorporation of ^{32}P into the brush border membrane under the influence of cholera toxin by an in vivo method; De Jonge [13] and Shlatz et al. [4,6] reported increased ^{32}P incorporation into a single polypeptide when isolated brush border membranes were phosphorylated by [γ-^{32}P]ATP in the presence of cAMP and cGMP.

With respect to transmembrane transport, cholera toxin- and dibutyryl-cAMP-induced changes are retained at the level of the isolated brush border membrane [7]. Therefore, it seems reasonable to analyze also alterations in protein

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phosphorylation at the level of the isolated membrane after the small intestine has been perfused with cholera toxin or dibutyryl-cAMP in the presence of [^{32}P]orthophosphate.

In the present study, brush border membranes were phosphorylated either in vivo by perfusion with [^{32}P]orthophosphate or in vitro by phosphorylation of freshly isolated vesicles (without prior perfusion) by [γ-^{32}P]ATP. The effect of the cyclic nucleotides cAMP and cGMP and of cholera toxin on the brush border membrane protein phosphorylation was analyzed by SDS gel electrophoresis.

Materials and Methods

In vivo phosphorylation

Rats (male Wistar, approx. 200 g) were anesthezised with Inactin (100 mg/kg) and loops of jejunum (approx. 20 cm) were perfused with a Ringer solution containing (in mmol/l): 140 Na^+, 4 K^+, 1.25 Ca^{2+} and 145.5 Cl^-. [^{32}P]Orthophosphate was added to the perfusion solution as $K_2H^{32}PO_4$ (NEN, NEX 060) up to 5 μCi/ml. Due to the addition of the isotope, the concentration of free phosphate was 0.2 mmol/l. After 90 min (see Results), 5 mmol/l dibutyryl-cAMP (Sigma) was added to the perfusate and the perfusion was continued for another 30 min. Cholera toxin (Schwarz Mann) was added up to 10 μg/ml at the beginning, and the perfusion was carried out for 120 min. The intestinal segments were then rinsed with ice-cold Ringer solution and brush border membranes were prepared starting from the scraped mucosa as described in Ref. 8. Immediately after purification, the membranes were denaturated as described below.

In vitro phosphorylation

Brush border membranes referred to in the text as freshly isolated membranes were prepared from rat jejunum by a $CaCl_2$ precipitation method as described by Kessler et al. [8]. The purified membranes were resuspended in 60 mmol/l mannitol, 5 mmol/l $MgCl_2$ and 12 mmol/l Tris-HCl (pH 7.0). Phosphorylation by [γ-^{32}P]ATP was carried out at a concentration of 2.5 mg protein per ml in a final volume of 80 μl. Before the addition of [γ-^{32}P]ATP, the membranes were preincubated in 60 mmol/l mannitol, 5 mmol/l $MgCl_2$, 12 mmol/l Tris-HCl (pH 7.0) either containing 0.1% saponin or not for 1 min at 25°C. [γ-^{32}P]ATP (NEN, NEG 002) was then added up to 20 μmol/l (10 μCi) and after 20 s the reaction was stopped by adding an aliquot of a denaturation buffer (Tris buffer, pH 6.8) to obtain the final concentrations of 2% SDS and 0.7 mol/l β-mercaptoethanol. The samples were then boiled for 2 min.

SDS-polyacrylamide gel electrophoresis was performed on 8.4% slab-gels according to Laemmli [9]. To each well the same amount of protein (100 μg) was applied. For autoradiography, Coomassie blue stained gels were dried under vacuum and exposed to Kodak X-ray films (SO-282) for several days at −80°C. Electrophoresis was calibrated using the standard protein kit from Pharmacia. Scan traces were recorded with the Ultro Scan Laser Densitometer from LKB (Sweden). Protein was determined according to Bradford [10] using the dye reagent and the standard protein mixture from Bio-Rad.

Results

Brush border membranes of rat jejunum were phosphorylated by an in vivo perfusion with [^{32}P]orthophosphate. Incorporation of ^{32}P into the polypeptides of the isolated brush border membranes was found to increase up to 60 min and remained constant afterwards (data not shown). Therefore, in order to analyze possible changes of the protein phosphorylation relative to an equilibrium situation, dibutyryl-cAMP was added after a perfusion time of 60 min. To circumvent a possible variability existing between different animals, brush border membranes of three independent experiments were pooled and analyzed. As shown in Fig. 1, the addition of dibutyryl-cAMP (5 mmol/l) to the perfusate did not change the phosphorylation of the brush border membrane proteins significantly (compare Figs. 1A and 1B). Also no changes of the phosphorylation pattern could be observed when the perfusion was performed in the presence of cholera toxin. In particular, no increase of protein phosphorylation was observed after perfusion with cholera toxin or dibutyryl-cAMP. Occasionally a small but rather general dephosphorylation was observed after perfusion with these drugs.

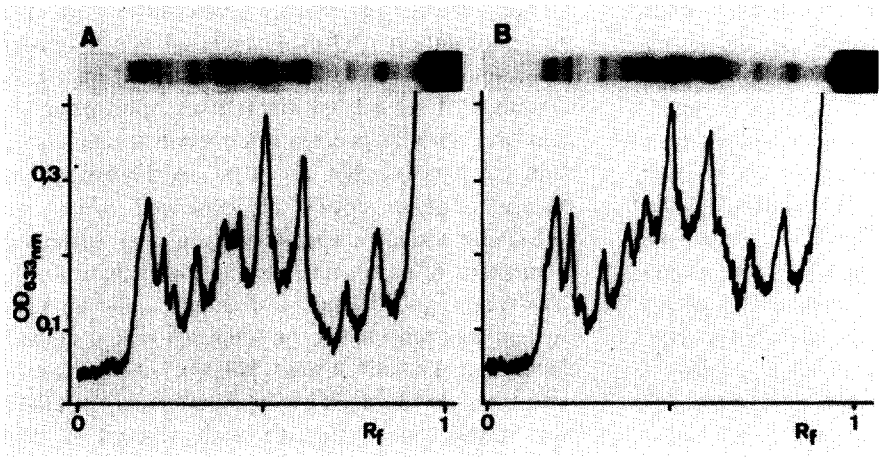

Fig. 1. In vivo phosphorylation of rat jejunum brush border membrane proteins. Loops of rat jejunum were perfused with [^{32}P]orthophosphate for 120 min either in the absence (A) or in the presence (B) of cholera toxin (see Methods). The isolated brush border membranes were analyzed by SDS-polyacrylamide gel electrophoresis. Autoradiograms and scan traces of the phosphoproteins are presented. Perfusion with 5 mmol/l dibutyryl-cAMP resulted in the same phosphoprotein pattern as presented in (B). OD, absorbance.

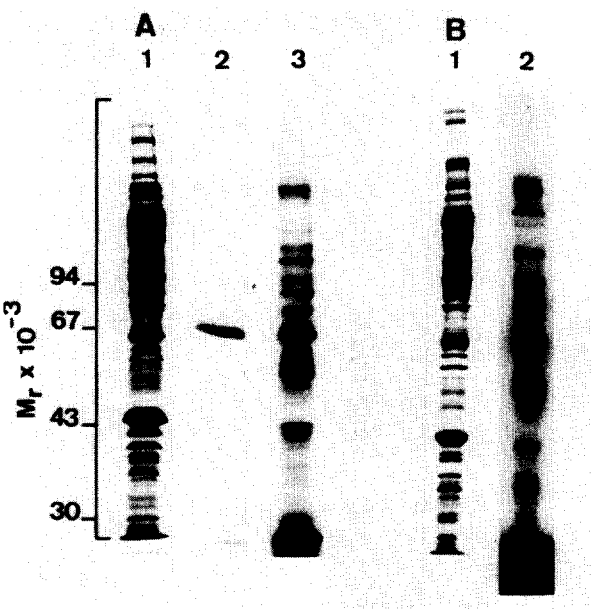

Fig. 2. Comparison of the in vivo and in vitro phosphorylation of rat jejunum brush border membranes. (A) Autoradiography of freshly isolated brush border membranes phosphorylated by [γ-^{32}P]ATP either in the absence (2) or in the presence (3) of 0.1% saponin. (1) Coomassie blue stain. (B) Phosphorylation pattern (2) and Coomassie blue stain (1) of brush border membranes labeled with [^{32}P]orthophosphate by in vivo perfusion.

The effect of cAMP on the brush border membrane phosphorylation was also analyzed by phosphorylating freshly isolated (without prior perfusion) brush border membrane vesicles by [γ-^{33}P]ATP. Under isosmotic conditions, only one polypeptide could be labeled by [γ-^{32}P]ATP (Fig. 2A). This band showed an apparent molecular weight of approx. 70000 and might correspond to the subunit of alkaline phosphatase [11]. In the presence of 0.1% saponin, however, several polypeptides were labeled by [γ-^{32}P]ATP. As reported by Haase et al. [12], brush border membranes prepared by a divalent cation precipitation technique are obtained as closed right-side-out oriented vesicles. Therefore it is suggested that the brush border membrane is rather impermeable for ATP and that most of the ^{32}P acceptor sites (and protein kinases) are located at the inner membrane surface (cytoplasmic site) of the vesicles.

Routinely, phosphorylation was carried out in the presence of 0.1% saponin for 20 s. Longer incubation times led to considerable dephosphorylation reactions (not shown). The ^{32}P-pattern obtained by phosphorylation of freshly isolated brush border membranes with [γ-^{32}P]ATP is compared with the phosphoprotein pattern obtained by an in vivo labeling (Fig. 2). Although certain proteins

are labeled by both labeling procedures, other proteins are labeled only by one or the other procedure. These differences might be explained as follows: (i) Differences in the membrane structure/organization (protein kinases, acceptor sites). (ii) The in vivo labeling measures a steady-state phosphorylation, whereas phosphorylation of the isolated membranes by [γ-^{32}P]ATP measures initial rates of the labeling of (partially prephosphorylated) proteins. (iii) Time-dependent changes in phosphorylation levels during membrane preparation. To examine this possibility, the phosphoprotein patterns of a homogenate sample denaturated at time zero was compared to that of a sample denaturated after 3 h incubation at 4°C. The result of this control experiment showed a slight yet general dephosphorylation with time, suggesting that although a certain amount of dephosphorylation occurs during membrane preparation this should not influence our basic findings. This conclusion does not take into account that dephosphorylation reactions might take place already within the time needed for preparing the mucosal scraping.

The effect of cyclic nucleotides on the phosphorylation of freshly isolated brush border membranes by [γ-^{32}P]ATP is illustrated in Fig. 3. Cyclic AMP clearly stimulated the phosphorylation of a 86 kDa protein at concentrations higher than 0.5 μmol/l, but no other cAMP-dependent phosphorylation could be observed. Also no cAMP-dependent phosphorylation was observed in proteins of molecular weights lower than 30000 (analyzed by a 15% gel; not shown). The 86 kDa protein was reported to be identical with a cGMP dependent protein kinase [13,14]. In agreement with the data reported by De Jonge [13,14], cGMP was much more effective with respect to the phosphorylation of the 86 kDa protein than cAMP. Phosphorylation of this protein was already clearly stimulated at 0.1 μmol/l cGMP, but the phosphorylation of the other proteins was not affected.

Discussion

In many systems cAMP has been described as a modulator of protein kinase activity [2,3]. There-

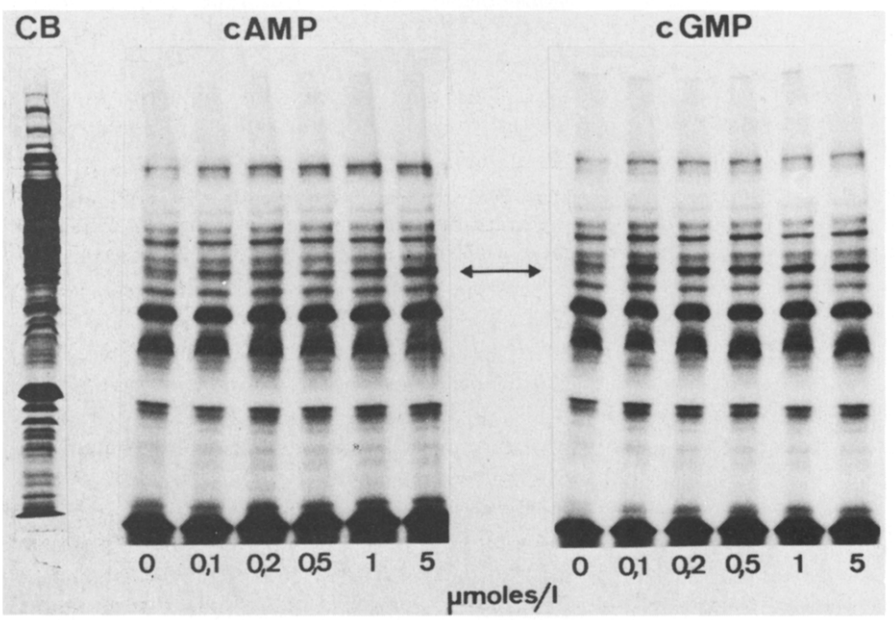

Fig. 3. Effect of the cyclic nucleotides cAMP and cGMP on brush border membrane phosphorylation. Autoradiograms of brush border membranes phosphorylated by 20 μmol/l [γ-^{32}P]ATP (for 20 s at 25°C) in the presence of 0.1% (w/v) saponin and in the presence of the indicated concentrations (in μmol/l) of the cyclic nucleotides are shown. CB, Coomassie blue stain. The arrow marks the 86 kDa protein.

fore, it may well be that protein phosphorylation regulated by different levels of intracellular cAMP [1] would represent a final step of cholera toxin induced inhibition of NaCl absorption and/or secretion in the small intestine. Indeed, it has been shown that cholera toxin stimulates the incorporation of ^{32}P into the brush border membranes in vivo [5]. However, only total ^{32}P-incorporation was analyzed by these authors and therefore no predictions can be made concerning the phosphorylation of single proteins. Additionally, these authors suggested that cholera toxin stimulates the phosphorylation of microvillous core proteins. In our experiments, however, which do not show an effect of cholera toxin, it is unlikely that the core proteins are lost during the membrane preparation, since the isolated vesicles still contain core proteins like actin and villin.

In the present study, brush border membranes of rat jejunum were labeled with ^{32}P by an in vivo perfusion either in the presence or in the absence of dibutyryl-cAMP or cholera toxin. On the basis of an analysis by one-dimensional gel electrophoresis, no significant changes of the phosphorylation of the brush border membrane proteins due to the addition of dibutyryl-cAMP or cholera toxin could be detected (see Fig. 1). However, it should be mentioned that the resolving power of a one-dimensional gel might not be high enough for the detection of minor changes within a single (multisite phosphorylated) protein or of changes in a minor component of the membrane.

On the other hand, phosphorylation studies using brush border membranes isolated without prior perfusion of the small intestine jejunum were performed. As reported by Shlatz et al. [4,6], cAMP clearly stimulated total incorporation of ^{32}P from [γ-^{32}P]ATP into these membranes. Furthermore, it has been shown that cAMP stimulates the phosphorylation of a single protein which has been identified as a cGMP-dependent protein kinase [13]. However, as indicated in Fig. 3, cAMP only simulates the effect of cGMP with respect to the autophosphorylation of the cGMP-dependent protein kinase (see also Refs. 13 and 14), but these authors found a protein of a molecular weight of about 103000 to be affected by cAMP. This discrepancy might be due to the different gel systems used in these studies. Our data, which are in agreement with those of De Jonge [13,14], suggest that the isolated brush border membranes from rat jejunum do not contain protein kinase activity specifically dependent on cAMP. However, in addition to the detection limits of an analysis by one-dimensional gel electrophoresis, it cannot be completely ruled out that the catalytic subunit C might be inactivated during the preincubation (1 min) of the membranes in the presence of 0.1% saponin and 0.1 mM cAMP as shown by Shaltiel et al. [15]. But if so, the degradation of subunit C in the presence of cAMP should not be more than about 50% (see Ref. 15) during the time needed (80 s) for the presented phosphorylation experiments.

In conclusion, no evidence for a cAMP-dependent brush border membrane protein phosphorylation could be obtained either by perfusion of rat jejunum in vivo with [^{32}P]orthophosphate in the presence of cholera toxin or dibutyryl-cAMP or by in vitro phosphorylation of freshly isolated brush border membranes by [γ-^{32}P]ATP. Therefore, it is rather unlikely that alterations in NaCl absorption induced by intoxication of the enterocyte with cholera toxin is directly mediated via cAMP protein kinase activity which would lead to an altered protein phosphorylation of the brush border membrane.

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References

1 Field, M. (1981) in Physiology of the Gastrointestinal Tract (Johnson, L.R., ed.), Vol. 2, pp. 963–982, Raven Press, New York
2 Greengard, P. (1978) Science 199, 146–152
3 Rasmussen, H. and Waisman, D.M. (1981) Biochem. Actions Horm. 8, 1–115
4 Shlatz, L.J., Kimberg, D.V. and Cattieu, K.A. (1978) Gastroenterology 76, 293–329
5 Lucid, S.V. and Cox, A.C. (1972) Biochem. Biophys. Res. Commun. 49, 1183–1186
6 Shlatz, L.J., Kimberg, D.V. and Cattieu, K.A. (1978) Gastroenterology 75, 838–846
7 Murer, H., Lücke, H. and Kinne, R. (1980) in Secretory Diarrhea (Field, M., Fordtran, J.S. and Schultz, S.G., eds.), pp. 31–43, Am. Physiol. Soc., Washington

8 Kessler, M., Acuto, O., Storelli, C., Murer, H., Müller, M. and Semenza, G. (1978) Biochim. Biophys. Acta 506, 136–154
9 Laemmli, U.K. (1970) Nature 227, 680–685
10 Bradford, M.M. (1976) Anal. Biochem. 72, 248–254
11 De Jonge, H.R., Ghijsen, W.E.J.M. and Van Os, C.H. (1981) Biochim. Biophys. Acta 647, 140–149
12 Haase, W., Schafer, A., Murer, H. and Kinne, R. (1978) Biochem. J. 172, 57–62
13 De Jonge, H.R. (1981) Adv. Cyclic Nucleotide Res. 14, 315–333
14 De Jonge, H.R. (1976) Nature 262, 590–592
15 Shaltiel, S., Alhanaty, E., Cesla, R., Jiménez, J.S., Kupfer, A. and Zick, Y. (1981) in Protein Phosphorylation (Rosen, O.M. and Zick, Y., eds.), pp. 82–101, Cold Spring Harbour Conf.