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Effects of Ca^{2+}, theophylline and promethazine on protein phosphorylation in intact cells of rabbit ileum

Correlation with active Na and Cl absorption

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The effects of Ca^{2+}, theophylline and promethazine on the phosphorylation of microvillus membrane proteins have been studied in rabbit ileal epithelial cells, using intact cell phosphorylation techniques followed by purification of microvillus membranes, separation of peptides by two-dimensional polyacrylamide gel electrophoresis, and quantitation of phosphorylation by computerized densitometry of autoradiograms. The Ca^{2+} ionophore A23187 caused increased phosphorylation of four and possibly five polypeptides; theophylline increased phosphorylation of three peptides, two of which had the same M_r and pI values as the peptides altered by the Ca^{2+} ionophore; promethazine decreased the phosphorylation of one of the peptides increased by Ca^{2+} ionophore. The phosphorylated peptides, which respond similarly to more than one agent which affect ileal Na and Cl absorption, could be involved in the regulation of NaCl absorption either as transport proteins or regulators of transport proteins.

Electrolyte absorption; Ca^{2+} calmodulin-dependent protein kinase; Phosphorylation; cyclic AMP-dependent protein kinase; (Rabbit intestine)

1. INTRODUCTION

The regulation of Na and Cl absorption in mammalian small intestine occurs at the brush border membrane of the intestinal epithelial cells [1]. Na and Cl absorption is regulated by Ca^{2+} and cAMP in these cells and an increase in the concentration of either will cause a decrease in the linked neutral NaCl absorptive process, the major pathway of active Na absorption in the intraprandial state [1–5]. Since cAMP always [6] and Ca^{2+}, acting through Ca^{2+}/calmodulin or protein kinase C, usually [7] act via protein phosphorylation, studies on protein phosphorylation should be useful to our understanding of the mechanisms controlling Na and Cl absorption in the intestine.

Consequently, we have examined the effects of cAMP and Ca^{2+} on protein phosphorylation in purified microvillus membranes which have been prepared after phosphorylation in intact epithelial cells, in order to correlate the phosphorylation state with changes in Na and Cl transport. The only previous study on whole intestinal cell phosphorylation, which used one-dimensional SDS-polyacrylamide gel electrophoresis (SDS-PAGE), failed to detect any changes in phosphorylation in purified brush border membranes from rat ileum following exposure to cholera toxin [8]. We report here, by the use of the

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higher resolution two-dimensional PAGE, that both cyclic nucleotides and Ca^{2+} stimulate the phosphorylation of specific microvillus membrane proteins in rabbit ileal epithelial cells. In addition, by correlating changes in phosphorylation of these substrates with the effects of Ca^{2+} and cAMP on Na and Cl absorption, suggestions can be made as to which phosphorylated substrates might be involved in the regulation of Na and Cl absorption.

2. MATERIALS AND METHODS

Five sequential techniques were used: (i) the intracellular nucleotide pools were labeled by exposure of the cells to [^{32}P]orthophosphate; (ii) the cells were exposed to test agents under conditions and for the times which change active Na and Cl absorption; (iii) microvillus membranes were purified; (iv) the membranes were solubilized and proteins separated by two-dimensional gel electrophoresis with isoelectric focusing in the first dimension followed by SDS-PAGE as the second dimension; (v) autoradiograms were prepared from the gels and the incorporation of ^{32}P into specific proteins quantitated by computerized densitometry.

2.1. *Membrane preparation*

Male New Zealand albino rabbits weighing approx. 2–2.5 kg were killed by intravenous injection of pentobarbital. Strips of ileal mucosa (5 cm each), with the serosa and muscularis propria removed [4], were incubated with 3 ml Krebs Ringer'-HCO_3 (KRB) containing 5 mCi $H_3{}^{32}PO_4$ for 90 min at 25°C to label the intracellular phosphate pool. The tissues were then washed in KRB, transferred to 3 ml KRB and exposed for 10 min to either the agents to be studied or, as a control, the vehicle used to dissolve the agent (exposure to the agents for this time produced maximum changes in active Na and Cl absorption) [5,9]. The mucosal cells were scraped off using glass slides, homogenized and brush border membranes prepared by a modification of the method of Hopfer et al. [10]. All procedures were carried out at 4°C unless otherwise stated. Homogenization was in 3 ml of a solution containing 5 mM EDTA, 1 mM Hepes (pH 7.5) with 1 mM iodoacetic acid and 1 mM phenylmethylsulfonyl fluoride (PMSF) as protease inhibitors, using a Virtis homogenizer for three 10-s periods at maximum speed with 30-s cooling intervals. Homogenates were centrifuged at 500 × *g* for 10 min at 4°C. The supernatant was discarded and the pellet was washed three times in the same buffer, and then suspended in 3 ml of 90 mM NaCl, 1 mM Hepes and 0.8 mM EDTA, pH 7.4, containing the same protease inhibitors. The suspension was vortex-mixed and left to stand on ice for 20 min. The supernatant from this step was centrifuged at 500 × *g* for 10 min at 4°C. The pellet was then suspended in 1.35 ml of 100 mM sorbitol, 1 mM Hepes (pH 7.4), vortex-mixed and mixed with 150 μl of 4.7 M NaSCN (final concentration 0.47 M) and homogenized on ice by 10 strokes in a glass-teflon homogenizer. 10.5 ml sorbitol buffer was added and microvillus membranes separated from cytoskeletal material by centrifugation at 5000 × *g* for 20 min at 4°C. The microvillus membranes were pelleted by centrifugation of the supernatant at 34000 × *g* for 30 min. The resulting pellet of purified microvillus membranes was suspended in a lysis solution consisting of 9.5 M urea, 2% NP-40, 100 mM DTT, 2% ampholytes (pH 3–10) and 0.005% Evans Blue dye and stored at −80°C until subjected to electrophoresis. The final membrane suspension and the initial homogenate were compared as to sucrase activity, as described [11], with protein determined by the method of Lowry et al. [12].

2.2. *Separation of phosphorylated proteins*

Two-dimensional gel electrophoresis was performed according to the method of O'Farrell [13]. First dimension: isoelectric focusing (IEF) was performed on phosphorylated membranes in 3 mm i.d. × 12 cm tubes containing ampholyte carriers (pH 3–10). Their composition was 9.5% urea, 4% acrylamide, 2% NP-40, and 2% ampholytes. Second dimension: SDS-PAGE: SDS equilibrated gels from the IEF run were placed on top of a continuous gradient SDS-PAGE slab gel and subjected to electrophoresis for 3 h at 30 mA per gel. The resolving gel contained a linear gradient of 5–15% acrylamide in 0.375 M Tris-HCl, pH 8.8, 0.1% SDS. The stacking gel contained 4% acrylamide in 0.125 M Tris-HCl, pH 6–8. The M_r values for individual phosphoproteins were determined by the method of Weber and Osborn [14] as modified for gradient gels [15], by using M_r stan-

dards (Sigma) which were loaded in a separate well of each gel.

At the end of the electrophoresis, the gels were fixed in 25% isopropanol and 10% acetic acid, stained with 0.1% Coomassie brilliant blue and destained with 10% isopropanol and 10% acetic acid, dried and autoradiographed at −80°C for 2 weeks using Kodak XAR-5 film and intensifying screens.

The autoradiograms were analyzed using the apparatus and software described by Mariash et al. [16]. The equipment consists of a video camera for data acquisition in scanning the autoradiograms, a digitizing circuit for translation of the data and a microcomputer for analysis. The intensity of each phosphorylated peptide was taken as a percentage of the individual fields scanned for purpose of comparison, and the changes in intensity reported are changes in the percentage of the total intensity of the scanned area provided by each spot of interest between control and test conditions. Statistical analysis of the data was performed by Student's paired *t*-test. Data are presented as means ± SE.

Table 1

Percent change in phosphorylation of microvillus membrane peptides

Agent	M_r	p*I*	% change
Ca^{2+} ionophore A23187			
(10 μM) (*N* = 6)	116000	6.6	↑ 95 ± 18
	110000	5.6	↑ 37 ± 7
	53000	5.7	↑107 ± 7
	32000	6.3	↑ 95 ± 18
Theophylline			
(10 mM) (*N* = 5)	53000	5.7	↑141 ± 33
	32000	6.3	↑169 ± 42
	32000	5.9	↑129 ± 25
Promethazine			
(10 μM) (*N* = 5)	116000	6.6	↓ 47 ± 6

Results are means ± SE of percent change in phosphorylation determined in individual experiments, with control taken as 100%. Data for the Ca^{2+}-ionophore response of the peptide of M_r 77000 are not included due to failure to increase in all experiments.
N, number of experiments

3. RESULTS

3.1. *Characterization of plasma membrane preparation*

The microvillus membrane preparation contained 0.58 ± 0.07% of the initial protein and 12.9 ± 3.0% of the initial sucrase activity (data from 8 separate membrane preparations). There was a 16.0 ± 4.3-fold increase in sucrase specific activity relative to the whole homogenate (sucrase specific activity in the homogenate was 0.13 ± 0.01 ng glucose per ng protein and in the microvillus membrane 2.22 ± 0.27).

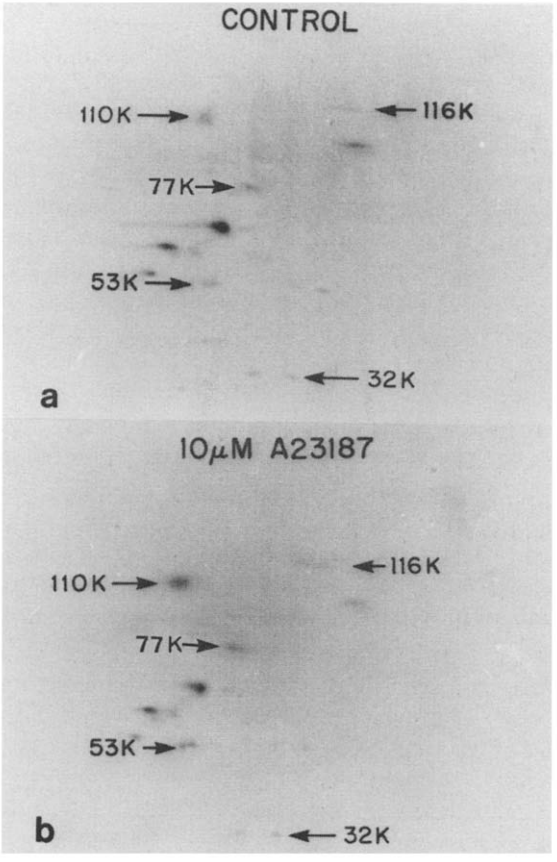

Fig.1. Autoradiograms obtained from microvillus membranes purified from rabbit ileal cells which had been loaded with $^{32}PO_4$ and subjected either to control conditions (a), or to 10 μM A23187 (b). The microvillus membranes after purification were solubilized and subjected to isoelectric focusing (pH 3–10) and polyacrylamide (5–15%) gel electrophoresis. Arrows indicate peptides, the phosphorylation of which was increased by exposure to Ca^{2+} ionophore A23187.

3.2. *Whole-cell phosphorylation*

The Coomassie blue staining in SDS-PAGE revealed a pattern that contained polypeptides with M_r ranging from 10000 to 200000 and a p*I* range between 4 and 9. No changes were detected in the Coomassie brilliant blue pattern of purified microvillus membranes after two-dimensional PAGE in response to the Ca^{2+} ionophore A23187, theophylline or promethazine treatment.

3.3. *Effects of the Ca^{2+} ionophore A23187*

In fig.1 we show autoradiograms of two-dimensional gels of microvillus membranes purified from intestinal villus epithelial cells under control conditions (a) and after treatment with 10 μM ionophore A23187 (b). Under both conditions it can be seen that there are many phosphorylated peptides with M_r between 140000 and 100000 and with isoelectric points from 4 to 8. The pattern of phosphorylated peptides in the autoradiogram obtained from ionophore-treated tissue is similar to that from control tissue except for five peptides. Increased phosphorylation in response to Ca^{2+} ionophore was detected in peptides with M_r 116000, 110000, 53000 and 32000 with isoelectric points of 6.6, 5.6, 5.7 and 6.3, respectively. A fifth peptide, with M_r 77000 and p*I* 5.8, was less reproducibly increased by the Ca^{2+} ionophore. The results of six similar experiments are summarized in table 1 in which the percent increases in phosphorylation are expressed after quantitation by densitometry. As it has been suggested that some effects of ionophore A23187 are mediated via activation of the cyclooxygenase pathway and possible prostaglandin-induced changes in cAMP levels [17], which would also cause increased phosphorylation, the experiments were repeated in the presence of 10 μM indomethacin. Indomethacin had no effect on the ability of the ionophore to increase the phosphorylation of the same peptides (not shown).

Table 2

Correlation of changes in active NaCl absorption and in whole cell microvillus membrane phosphorylation in rabbit ileum

	Effect on Na and Cl absorption	Effect on whole-cell phosphorylation
Ca^{2+} ionophore A23187 (10 mM)	↓	↑ 116000; 110000; (77000) 53000; 32000
Theophylline (10 mM)	↓	↑ 53000; 32000; 32000
Promethazine (10 μM)	↑	↓ 116000

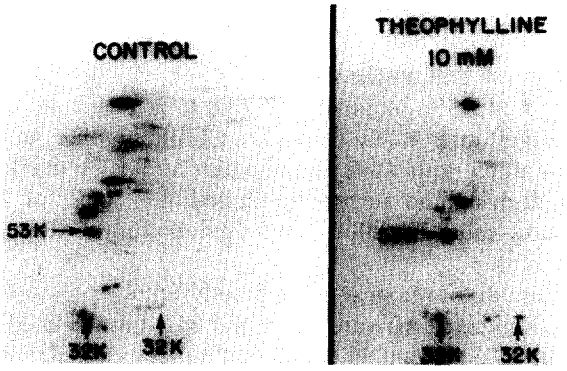

Fig.2. Autoradiograms obtained from microvillus membranes purified from rabbit ileal cells which had been loaded with $^{32}PO_4$ and subjected either to control conditions, or to 10 mM theophylline. Arrows indicate peptides, the phosphorylation of which was increased by exposure to theophylline.

3.4. *Effects of theophylline*

In fig.2 are shown autoradiograms of two-dimensional gels of microvillus membranes purified from intestinal villus cells under control conditions and after the intestinal cells had been treated with 10 mM theophylline to raise the intracellular cyclic nucleotide levels. The patterns of phosphorylated peptides under control conditions are similar to those shown in fig.1a. Treatment with theophylline caused an increase in phosphorylation of three peptides, two of which appear to be similar to those affected by Ca^{2+} ionophore, based on M_r and p*I*. Increased phosphorylation was detected in the 53 kDa peptide with p*I* of 5.7, in the 32 kDa peptide with p*I* of 6.3 and in a second 32 kDa peptide with p*I* of 5.9.

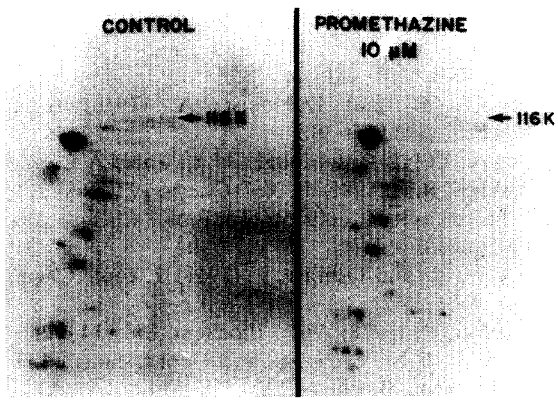

Fig.3. Autoradiograms obtained from microvillus membranes purified from rabbit ileal cells which had been loaded with $^{32}PO_4$ and subjected either to control conditions, or to 10 μM promethazine. The arrow indicates the 116 kDa peptide, the phosphorylation of which was decreased by exposure to promethazine.

3.5. *Effect of promethazine*

Promethazine, which previously has been shown to stimulate active Na and Cl absorption [8], also affected whole-cell phosphorylation. As shown in fig.3, 10 μM promethazine decreased the phosphorylation of a single microvillus membrane peptide, with M_r 116000 and isoelectric point 6.6. This peptide has the same molecular mass and p*I* as the 116 kDa peptide the phosphorylation of which was increased by the Ca^{2+} ionophore.

4. DISCUSSION

These studies demonstrate that treatment of rabbit ileal epithelial cells with calcium ionophore A23187, to raise intracellular Ca^{2+}, and with theophylline to raise intracellular cyclic nucleotide levels, increases the phosphorylation of specific peptides in microvillus membranes. In a previous publication [8], and in our own preliminary studies, no increased ileal phosphorylation in response to cAMP could be detected in such studies when peptide separation was performed by single dimensional PAGE. The use of two-dimensional PAGE to provide improved resolution of individual peptides appears to be the key to the successful detection of changed phosphorylation in the microvillus membrane.

The important question is whether any of these phosphorylated proteins are involved in the control of NaCl absorption by Ca^{2+} and by cyclic nucleotides. Some information can be gained by correlating whole-cell phosphorylation studies with changes in active NaCl absorption caused by the agents studied which affect transport. A summary and comparison of the effects of these agents on phosphorylation and on electrolyte transport as previously reported are shown in table 2. Increased phosphorylation of the peptides with M_r of 116000, 110000, 53000, 32000 and perhaps 77000 is associated with a decrease in NaCl absorption. Decreased phosphorylation of the 116 kDa peptide by promethazine is associated with increased NaCl absorption [9,18]. From the results obtained, we can draw the following conclusions about proteins with increased phosphorylation. The 116 kDa protein could be involved in NaCl absorption. Increased Ca^{2+}, which decreases NaCl absorption, increased its phosphorylation. Promethazine, which stimulates NaCl absorption, decreased its phosphorylation. The 53 kDa and one of the 32 kDa proteins are phosphorylated by both Ca^{2+} ionophore and cyclic nucleotides (raised by treatment with theophylline). Given that both these agents affect NaCl absorption similarly, and that they both appear to increase the phosphorylation of the same proteins, the 53 and 32 kDa proteins are also candidates for involvement in NaCl absorption. There is as yet no additional support for a role in transport of the remaining peptides, the 110 kDa peptide affected by Ca^{2+} ionophore and the second 32 kDa peptide affected by theophylline.

This demonstration of phosphorylated proteins in the microvillus membrane of rabbit ileal cells – thought to be the site of the regulation of NaCl absorption [1] – leads to the hypothesis that one or more of these proteins may be transport proteins or regulators of transport proteins. Additional studies are needed to identify the proteins definitively.

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REFERENCES

[1] Field, M. (1981) in: Physiology of the Gastrointestinal Tract, edn I (Johnson, L.R. ed.) pp.963–982, Raven, New York.

[2] Bolton, J.E. and Field, M. (1977) J. Membrane Biol. 35, 159–174.

[3] Donowitz, M. (1983) Am. J. Physiol. 245, G165–G177.

[4] Donowitz, M. and Asarkof, N. (1982) Am. J. Physiol. 243, G28–G35.

[5] Donowitz, M. and Welsh, M.J. (1986) Annu. Rev. Physiol. 48, 135–150.

[6] Nestler, E.S., Walaas, S.I. and Greengard, P. (1984) Science 225, 1357–1364.

[7] Nishizuka, Y. (1984) Nature 308, 693–698.

[8] Scalera, V., Biber, J. and Murer, H. (1983) Biochim. Biophys. Acta 730, 321–326.

[9] Cohen, M.E., Sharp, G.W.G. and Donowitz, M. (1986) Am. J. Physiol. 251, G710–G717.

[10] Hopfer, U., Crowe, T.D. and Tandler, B. (1983) Anal. Biochem. 131, 447–452.

[11] Messer, M. and Dahlquist, A. (1966) Anal. Biochem. 14, 376–392.

[12] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1981) J. Biol. Chem. 193, 265–275.

[13] O'Farrell, P.H. (1975) J. Biol. Chem. 250, 4007–4013.

[14] Weber, K. and Osborn, J. (1969) J. Biol. Chem. 244, 4406–4411.

[15] Lambin, P. (1978) Anal. Biochem. 85, 114–125.

[16] Mariash, C.N., Seelig, S. and Oppenheimer, J.H. (1982) Anal. Biochem. 130, 388–394.

[17] Martens, H., Tobey, N.A., Rollin, R.E., Berschneider, H.M. and Powell, D.W. (1985) Gastroenterology 88, 1490.

[18] Donowitz, M., Wicks, J., Madara, J.L. and Sharp, G.W.G. (1985) Am. J. Physiol. 248, G726–G740.