CALCIUM AND CALMODULIN-DEPENDENT PROTEIN PHOSPHORYLATION IN RABBIT ILEUM

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Received 13 July 1981

1. Introduction

Evidence is accumulating to suggest that calcium is a physiological regulator of intestinal electrolyte transport [1–3]. Conditions which increase intracellular calcium such as the use of the calcium ionophore A23187 [1,2] or exposure to neurohumoral substances such as serotonin [4] or carbachol [1] cause stimulation of intestinal chloride secretion and/or inhibition of Na^+ and Cl^- absorption. To the contrary, conditions which decrease intracellular calcium, such as exposure to the calcium channel blocker verapamil, stimulate Na^+ and Cl^- absorption [3]. Calmodulin may be involved in these effects since the anti-psychotic drug trifluoperazine (an inhibitor of the calcium–calmodulin complex) inhibited intestinal secretion caused by the calcium ionophore A23187 [5,6].

The intracellular mechanisms by which calcium and calmodulin affect intestinal ion transport are not known. However, as calcium and calmodulin affect phosphorylation and function of specific proteins in several other systems [7,8], phosphorylation is a potential control mechanism for ileal electrolyte transport. These studies are the first demonstration that calcium and calmodulin can cause phosphorylation of intestinal peptides.

2. Materials and methods

Fed white male New Zealand rabbits (2–2.5 kg body wt) were anesthetized with sodium pentobarbital and the distal ileum removed. After washing with cold Ringer's-HCO_3 solution, mucosal scrapings were obtained on ice using glass slides. This technique has been demonstrated to obtain primarily villous tip cells and some crypt cells [9]. 150 mg tissue was homogenized in 7.5 ml 5 mM $MgCl_2$, 10% sucrose, 50 mM Tris–HCl (pH 7.5) using a ground-glass hand homogenizer. The homogenate, containing ~200 μg protein, was preincubated in a 100 μl reaction mixture containing 1 mM EGTA, 5 mM $MgCl_2$, 10% sucrose, 50 mM Tris–HCl (pH 7.5) and, where indicated, 1.1 mM $CaCl_2$, and different concentrations of calmodulin and trifluoperazine, for 1 min at 37°C.

The phosphorylation reaction was started by the addition of [^{32}P]ATP at a final concentration of 5 μM. The reaction was stopped after 20 s by the addition of 50 μl of a solution containing 0.1 mM EDTA, 5% SDS, 200 mM dithiothreitol and 50 mg/ml pyronin Y followed immediately by immersion in a boiling water bath for 2 min. The mixture was then placed in a 37°C water bath for 20 min. Samples, containing ~40 μg protein, were subjected to electrophoresis on a polyacrylamide continuous gradient slab gel (5–15% acrylamide) according to [10]. The gels were subjected to autoradiography using XAR-5 Kodak film (Eastman Kodak, Rochester NY) with exposure times ranging from 4–48 h. The autoradiographs were subsequently analyzed by use of a Zeneih scanning densitometer (Biomed Products, Chicago IL). Amount of phosphorylation was assumed proportional to peak height of the densitometry scan [11]. Between 6 and 8 experiments were performed for each experimental condition.

[γ-^{32}P]Adenosine triphosphate (spec. act. 3–10 Ci/mmol) was obtained from New England Nuclear

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Published by Elsevier/North-Holland Biomedical Press
00145793/81/0000–0000/$02.50

(Boston MA); trifluoperazine was a gift from Smith, Kline and French Co (Philadelphia PA). Calmodulin was purified from bovine brain according to [12]. Molecular mass standards consisted of ovalbumin (M_r 43 000), bovine serum albumin (M_r 68 000), phosphorylase B (M_r 94 000), β-galactosidase (M_r 116 500) and myosin (M_r 200 000) (Bio-Rad Lab, Richmond CA).

3. Results and discussion

The effect of calcium on phosphorylation was determined by comparing phosphorylation in the presence of 1 mM EGTA plus 1.1 mM calcium (~100 μM free Ca^{2+} [13]) to that in the presence of 1 mM EGTA and no added calcium. As shown in fig.1, the presence of calcium caused an increase in ^{32}P incorporation into at least 3 ileal peptides having M_r-values 94 000, 70 000 and 50 000. This calcium effect on all 3 peptides was reversed with 100 μM trifluoperazine suggesting that the increase in phosphorylation of these peptides was a result of the action of calcium with endogenous calmodulin or with phospholipid-dependent kinases [14]. In fig.1 calcium increased phosphate incorporation in an additional peptide of M_r 54 000. However, this result was not seen consistently.

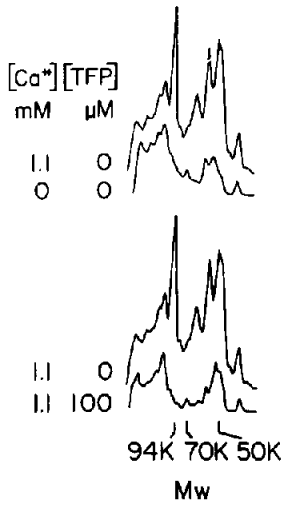

Fig.1. Calcium-induced phosphorylation of whole homogenate of rabbit ileal mucosa (above) and inhibition by trifluoperazine (below). All phosphorylation reactions contained 1 mM EGTA. The densitometry scan of an autoradiograph is shown.

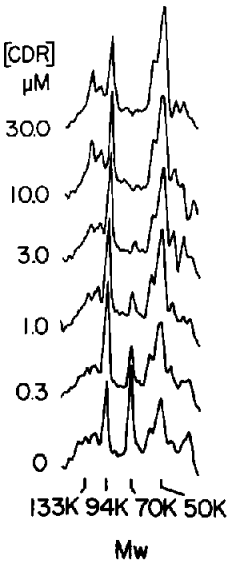

Fig.2. Densitometry scan demonstrating effect of exogenous calmodulin on phosphorylation of whole homogenate of rabbit ileal mucosa. All phosphorylation reactions contained 1.1 mM Ca^{2+} and 1 mM EGTA.

The addition of calmodulin to the ileal homogenate containing 100 μM free Ca^{2+} resulted in a dose-dependent increase in ^{32}P incorporation in 2 peptides having M_r 133 000 and 50 000 and a decrease of phosphate incorporation in one peptide, with M_r 70 000 (fig.2). The 133 000 M_r peptide did not show enhancement of phosphorylation with the addition of calcium suggesting that it was unaffected by the level of endogenous calmodulin under these experimental conditions. However, ^{32}P incorporation increased with added calmodulin, with an initial increase between 0.3–3 μM and a maximum effect at 30 μM. An

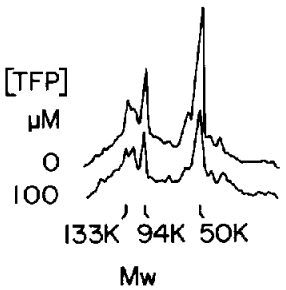

Fig.3. Trifluoperazine inhibition of calcium–calmodulin-induced phosphorylation in whole homogenate of rabbit ileal mucosa. All phosphorylation reactions contained 1.1 mM calcium, 1 mM EGTA, and 10 μM calmodulin. The densitometry scan taken from an autoradiograph is shown.

increase in ^{32}P incorporation in the 50 000 M_r protein occurred in a dose-dependent manner with added calmodulin between 0.3–10 μM (fig.2). In contrast, phosphate incorporation into the 94 000 M_r peptide was frequently maximal without added calmodulin. When added calmodulin increased phosphorylation of this peptide, as little as 0.1 or 0.3 μM calmodulin led to maximal phosphorylation. This suggests that phosphorylation of the 94 000 M_r peptide was close to maximal with the endogenous calmodulin present in the whole homogenate. The ability of calcium–calmodulin to increase ^{32}P incorporation in the 133 000, 94 000 and 50 000 M_r peptides was inhibited by trifluoperazine (fig.3), thus supporting the view that the phosphorylation seen was due to calcium–calmodulin-sensitive protein kinase activity in the ileal homogenate. Trifluoperazine was effective when present in concentrations tenfold those of calmodulin.

Decreased incorporation of ^{32}P into the 70 000 M_r protein was observed at as low as 0.1 μM calmodulin. The decrease in ^{32}P incorporation was concentration-dependent such that with 10 μM calmodulin there was no detectable ^{32}P incorporation (fig.2). The effect of calmodulin to decrease ^{32}P incorporation was unaffected by trifluoperazine.

These results, using a whole homogenate of rabbit ileal mucosa, demonstrate calcium and calmodulin-dependent phosphorylation of several proteins which can be blocked by trifluoperazine. The cellular location and function of the calmodulin-sensitive proteins remain to be determined and placed in the context of the control of electrolyte transport by the ileum.

Acknowledgements

Supported by NIH grant AM 26523, NIH Training grant AM 07024 and the John A. Hartford Foundation. M.D. is the recipient of NIH Research Career Development Award KO4-00588.

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