# Ca²⁺–calmodulin-, cyclic AMP- and cyclic GMP-induced phosphorylation of proteins in purified microvillus membranes of rabbit ileum

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Evidence is available to suggest that $Ca^{2+}$–calmodulin and cyclic nucleotides are involved in the regulation of ion transport in rabbit ileum. Since both $Ca^{2+}$–calmodulin and cyclic nucleotides exert many of their effects by phosphorylation, the effects of $Ca^{2+}$–calmodulin and cyclic nucleotides on phosphorylation of purified microvillus membrane from rabbit ileal mucosa were evaluated. $Ca^{2+}$–calmodulin increased phosphorylation of five microvillus-membrane peptides, with $M_r$ values of 137000, 77000, 58000, 53000 and 50000. The increases in phosphorylation caused by $Ca^{2+}$–calmodulin were: $M_r$-137000 peptide, $111 \pm 26\%$; $M_r$-77000 peptide, $71 \pm 17\%$; $M_r$-58000 peptide, $51 \pm 8\%$; $M_r$-53000 peptide, $113 \pm 20\%$. These increases were maximal at 1 μM-calmodulin and 0.3–0.9 μM free $Ca^{2+}$; concentrations of $Ca^{2+}$ causing half-maximal effects on phosphorylation for the different peptides were 0.06–0.12 μM. Cyclic AMP and cyclic GMP increased phosphorylation of two peptides, of $M_r$ 137000 and 85000. The concentrations of cyclic nucleotides giving half-maximal phosphorylation of the $M_r$-137000 peptide were 0.3 μM-cyclic AMP and 4.6 μM-cyclic GMP, and for the $M_r$-85000 peptide, 3.9 μM-cyclic AMP and 0.05 μM-cyclic GMP. The maximal increase in phosphorylation of the $M_r$-137000 peptide was 200% for cyclic AMP and 95% for cyclic GMP, and that of the $M_r$-85000 peptide was 220% for cyclic AMP and 120% for cyclic GMP. These studies demonstrate the existence of $Ca^{2+}$–calmodulin-, cyclic AMP- and cyclic GMP-dependent protein kinases and substrate proteins in purified rabbit ileal microvillus membranes and that $Ca^{2+}$ can regulate phosphorylation of these proteins over the presumed physiological concentration range of cytosol free $Ca^{2+}$.

There is increasing evidence for a role of $Ca^{2+}$ as an intracellular regulator of active intestinal $Na^+$ and $Cl^-$ transport (Donowitz, 1983). Increased intracellular $Ca^{2+}$ is associated with a decrease in $Na^+$ and $Cl^-$ absorption and/or an increase in $Cl^-$ secretion in rabbit ileum (Bolton & Field, 1977; Ilundain & Naftalin, 1979; Donowitz *et al.*, 1980, 1982*b*; Donowitz, 1983). These changes are similar to those seen with increased tissue cyclic AMP and also cyclic GMP (Field, 1981). These ion-transport

changes occur after increases in intracellular $Ca^{2+}$, whether artificially increased by the $Ca^{2+}$ ionophore A23187 or by neurohumoral substances which increase ileal calcium content (those identified to date include 5-hydroxytryptamine, carbachol, neurotensin and substance P) (Bolton & Field, 1977; Donowitz *et al.*, 1980, 1982*a*). Conversely, decreased intracellular $Ca^{2+}$ is associated with an increase in $Na^+$ and $Cl^-$ absorption. This occurs whether the decreased $Ca^{2+}$ is caused by the 'Ca²⁺ channel blocker' (−)-verapamil, exposure to $Ca^{2+}$-free bathing solutions or by neurohumoral substances such as dopamine (Donowitz & Asarkof, 1982; Donowitz *et al.*, 1982*b*). Since

Abbreviation used: SDS, sodium dodecyl sulphate.

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changes in the rate of ileal ion transport can usually be explained by changes in the apical-membrane permeability to $Na^+$ and $Cl^-$ in epithelial cells involved in electrolyte absorption, and to $Cl^-$ in epithelial cells involved in secretion, $Ca^{2+}$ might alter ion transport by affecting brush-border permeabilities (Frizzell et al., 1979; Field, 1981).

The intracellular $Ca^{2+}$-binding protein calmodulin is involved in multiple actions of intracellular $Ca^{2+}$. Its involvement in intestinal transport is suggested, since the $Ca^{2+}$–calmodulin inhibitors trifluoperazine and chlorpromazine blocked the stimulation of intestinal secretion (Ilundain & Naftalin, 1979; Smith & Field, 1980; Field, 1981), and the $Ca^{2+}$–calmodulin inhibitor W13, one of the naphthalenesulphonamides, stimulated active ileal $Cl^-$ absorption (Donowitz et al., 1983).

Many actions of intracellular $Ca^{2+}$ and calmodulin and all actions of cyclic nucleotides occur by phosphorylation of substrate proteins. We have previously demonstrated the presence of $Ca^{2+}$–calmodulin protein kinases and their endogenous protein substrates in homogenates of rabbit ileum (Taylor et al., 1981). We now describe $Ca^{2+}$–calmodulin- and cyclic nucleotide-dependent protein kinase activity and endogenous substrates in purified microvillus membranes of rabbit ileum

## Materials and methods

Fed male New Zealand White rabbits (2–2.5 kg) were anaesthetized with sodium pentobarbital, and the distal 30–40 cm of ileum was removed. All procedures were carried out at 0–4°C unless otherwise stated. After washing of the ileum with cold Ringer bicarbonate medium (Donowitz et al., 1980), mucosal scrapings were obtained on ice by using glass slides, and purified microvillus membrane was prepared by a modification of the methods of Forstner et al. (1968) followed by that of Hopfer et al. (1973). Approx. 5 g of rabbit ileal mucosa was homogenized in 70 vol. of 5 mM-EDTA/5 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], pH 7.5, in a Waring blender by using three 5 s homogenization periods with intervening 10 s cooling intervals. A crude brush-border pellet was collected by centrifugation for 10 min at 700 g (Sorvall rotor SS34) and washed three times with 40 ml of 5 mM-EDTA/5 mM-Hepes, pH 7.5, and once with 250 mM-sorbitol/5 mM-Hepes, pH 7.5, by centrifugation at 25 000 g for 20 min. The membranes were then suspended in 250 mM-sorbitol/5 mM-Hepes, pH 7.5, by homogenization; $MgSO_4$ was added (final concn. 10 mM), and the suspension was left for 10 min and then centrifuged at 5000 g for 10 min. The pellet was discarded and the supernatant centrifuged at 25 000 g for 20 min. This final microvillus-membrane pellet was resuspended in 250 mM-sorbitol/5 mM-Hepes and stored at $-80°C$. The membranes were used within 14 days, during which there was no significant change in enzyme activity or pattern of phosphorylation compared with freshly prepared membranes.

In separate experiments, membranes were prepared as described above, or in addition were washed once with 0.14 M-, 0.28 M- or 0.56 M-NaCl before phosphorylation in order to remove loosely bound proteins. The proteinase inhibitors phenylmethanesulphonyl fluoride (0.3 mM) and aprotinin (100 kallikrein-inhibitor units/ml) were present in all solutions except during homogenization.

Microvillus-membrane suspensions containing 50–65 μg of protein were incubated in a 100 μl phosphorylation reaction mixture containing 5 μM-$[\gamma\text{-}^{32}P]ATP$, 3 mM-EGTA, 5 mM-$MgCl_2$, 10% (w/v) sucrose and indicated concentrations of $Ca^{2+}$, calmodulin, cyclic AMP, cyclic GMP and the $Ca^{2+}$–calmodulin inhibitor trifluoperazine (Taylor et al., 1981). Reactions were usually carried out at 0°C for the indicated times. In studies with cyclic AMP and cyclic GMP, the membranes were preincubated for 5 min at 27°C. In some experiments phosphorylation was performed with simultaneous sonication (Branson Sonifier, microprobe; Branson Sonic Power Co., Danbury, CT, U.S.A.) for 30 s on ice, and in others alamethacin (2 μg/ml) was included in the phosphorylation reaction mixture. The reaction was stopped by addition of 50 μl of a solution of 0.1 M-EDTA, 5% (w/v) SDS, 200 mM-dithiothreitol and 50 μg of Pyronin Y/ml, followed immediately by immersion in boiling water for 2 min and then by incubation at 27°C for 20 min. In some experiments, hydroxylamine was added to the stopping solution (final concn. 1 M).

Samples (50 μl) of phosphorylated proteins were subjected to electrophoresis in a discontinuous SDS/polyacrylamide vertical-slab-gel system (Laemmli, 1970). The resolving gel contained a linear gradient of 5–15% acrylamide in 0.375 M-Tris/HCl (pH 8.8)/0.1% SDS, though in some experiments 6% acrylamide was used. The stacking gel contained 4% (w/v) acrylamide, 0.125 M-Tris/HCl (pH 6.8) and 0.12% SDS. The electrode buffer contained 0.05 M-Tris/HCl, 0.37 M-glycine and 0.1% SDS. After electrophoresis at 26 mA, the gels were stained with Coomassie Brilliant Blue, dried, and subjected to autoradiography with Kodak XAR-5 film (Eastman Kodak Co., Rochester, NY, U.S.A.). The autoradiographs were analysed with a Zeineh densitometer (Biomed Products, Chicago, IL, U.S.A.), with the amount of phosphorylation assumed to be proportional to the peak height of the densitometry scan (Avruch et al., 1976). $M_r$ values for individual peptides were determined by the method of Weber & Osborn (1969), as modified

for gradient gels (Lambin, 1978), by using $M_r$ standards studied simultaneously on the same gels.

Ca$^{2+}$ concentrations were varied by adding different amounts of CaCl$_2$ to a fixed EGTA concentration. The concentrations of free Ca$^{2+}$ in the phosphorylation reaction solutions were calculated as described by Bartfai (1979), by using a computer program. These calculations were confirmed by measurements with a Ca$^{2+}$-selective electrode as described by Becker et al. (1980).

Marker-enzyme assays used for characterizing the membrane preparation were: sucrase, for microvillus membrane (Messer & Dahlquist, 1966); Na$^+$ + K$^+$-dependent ATPase, for basolateral membrane (Albers et al., 1965); monoamine oxidase, for mitochondria (Wurtman & Axelrod, 1963); cytochrome c reductase, for endoplasmic reticulum (Omura & Takesue, 1970). Protein was measured by the method of Lowry et al. (1951).

At least four to six experiments were performed for each test condition. The results reported represent means ± S.E.M. Statistical analyses were performed by Student's t test for paired and unpaired data.

[γ-$^{32}$P]ATP (sp. radioactivity 6–10 Ci/mmol) and [$^{32}$P]P$_i$ (sp. radioactivity 200 Ci/μmol) were obtained from New England Nuclear. Calmodulin was purchased from Calbiochem, La Jolla, CA, U.S.A., and produced a single band on SDS/polyacrylamide gels. Troponin C was purified from rabbit skeletal muscle (Leavis & Lehrer, 1978) and was generously given by Dr. P. Leavis, Boston Biomedical Research Institute, Boston, MA, U.S.A. Cyclic AMP, cyclic GMP, hydroxylamine and phenylmethanesulphonyl fluoride were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Aprotonin was obtained from FBA Pharmaceuticals (New York, NY, U.S.A.). Trifluoperazine was a gift from Smith, Kline and French Co., Philadelphia, PA, U.S.A., and alamethacin was a gift from Upjohn Co., Kalamazoo, MI, U.S.A. $M_r$ standards (Bio-Rad, Richmond, CA, U.S.A.) were: lysozyme ($M_r$ 14400), soya-bean trypsin inhibitor (21500), carbonic anhydrase (31000), ovalbumin (45000), bovine serum albumin (66200), phosphorylase b (97400), β-galactosidase (116250) and myosin (200000).

## Results

### Membrane preparation

The microvillus-membrane preparations contained 0.6 ± 0.2% of the initial protein and 15.4 ± 2.4% of the initial sucrase activity (mean ± S.E.M. for six preparations). There was a 26-fold increase in sucrase specific activity relative to the whole homogenate. The membranes were purified 12-fold compared with the basolateral-membrane marker Na$^+$ + K$^+$-dependent ATPase, 31-fold

compared with the mitochondrial marker monoamine oxidase, and 19-fold compared with the endoplasmic-reticulum marker cytochrome c reductase. This is similar to previously reported microvillus-membrane preparations of rat ileum made by similar techniques (Mircheff & Wright, 1976; Weiser et al., 1978; De Jonge, 1981). Electron-microscopic examination showed that the preparations consisted of vesicles in which breaks were visible in 10%. There was little contamination by core or terminal web as judged by electron microscopy. The Coomassie Blue-stained banding pattern of microvillus proteins (Fig. 4a) was similar to that reported for rat intestine (Mircheff & Wright, 1976; Weiser et al., 1978). Compared with previously reported preparations of brush border, the amounts of several proteins identified by Coomassie Blue staining were greatly decreased. These proteins included the terminal web protein myosin and the core protein actin (Mooseker & Howe, 1982). The $M_r$-110000 protein which links the core to microvillus membrane was present in this preparation.

During labelling with [$^{32}$P]ATP, there was linear incorporation with time of $^{32}$P into all peptides for 180 s, except for a peptide of $M_r$ 77000. Incorporation of radioactivity into this peptide was linear for 30 s followed by loss of $^{32}$P from the band. Consequently, all studies were carried out in duplicate, with phosphorylation reactions of 20 s and 2 min. Addition of 1 M-hydroxylamine to the stopping solution had no effect on the labelling patterns, indicating that $^{32}$P incorporation was not due to formation of acyl phosphate bonds (Tada et al., 1975). If [$^{32}$P]P$_i$ was substituted for [$^{32}$P]ATP, no labelling of protein was observed.

### Ca$^{2+}$–calmodulin-induced phosphorylation

The effect of Ca$^{2+}$–calmodulin on microvillus-membrane phosphorylation was determined by comparing phosphorylation in the presence of 3 mM-EGTA alone with that resulting from addition of Ca$^{2+}$ and/or calmodulin in varying concentrations. Ca$^{2+}$–calmodulin caused increased phosphorylation of five peptides, of $M_r$ 137000, 77000, 58000, 53000 and 50000 (Table 1, Fig. 1). In addition, Ca$^{2+}$–calmodulin increased phosphorylation of a 47000-$M_r$ peptide in 50% of the cases. Extraction of the membrane with 0.14 M-NaCl completely removed the 47000-$M_r$ peptide from the membrane in the absence and presence of Ca$^{2+}$–calmodulin, but washing with concentrations up to 0.56 M did not prevent the effects of Ca$^{2+}$–calmodulin on the other five peptides. Since Ca$^{2+}$–calmodulin increases phosphorylation of a peptide with the same estimated $M_r$ in the cytosol (results not shown), we assume that the 47000-$M_r$ peptide represents loose sticking of a cytosol protein to the
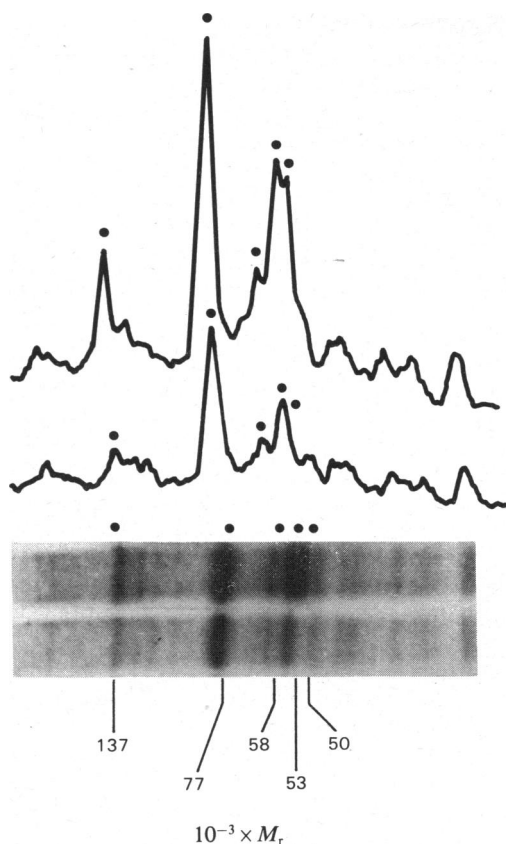
Fig. 1. *Ca²⁺-calmodulin-induced stimulation of phosphorylation of purified microvillus membranes*

Phosphorylation of microvillus-membrane peptides was linear for at least 3 min when done at 0 °C for all peptides except for the $M_r$-77000 peptide, for which phosphorylation was linear for 30 s. The phosphorylation shown was performed for 120 s, and thus a submaximal effect of phosphorylation of the $M_r$-77000 peptide is demonstrated. For both densitometry scans and autoradiographs the higher $M_r$ values are presented on the left. The upper lane is the effect of $Ca^{2+}$ plus calmodulin and the bottom lane represents phosphorylation in the $Ca^{2+}$-free condition (i.e. EGTA). $Ca^{2+}$-calmodulin increased phosphorylation of five microvillus-membrane peptides indicated by the vertical lines. The $M_r$-50000 peptide usually only appeared as a shoulder on the densitometry scan, whereas all the other peptides appeared as distinct peaks.

membrane, and at this time we do not consider this phosphorylation further.

Of the peptides phosphorylated by $Ca^{2+}$-calmodulin, only the $137000$-$M_r$ peptide occurred in an area with significant Coomassie Blue staining. The percentage increase in phosphorylation of the different peptides caused by $Ca^{2+}$-calmodulin

Table 1. *Ca²⁺-calmodulin-increased ileal microvillus-membrane phosphorylation*

Results are means ± s.e.m.; numbers in parentheses are numbers of animals studied. Concentrations of free $Ca^{2+}$ and exogenous calmodulin (1 μM) were maximum for phosphorylation of all peptides. Free $[Ca^{2+}]$ was 0.3 μM for all studies except for the $M_r$-77000 peptide, which was studied at 0.9 μM. Because the $M_r$-50000 peptide appeared on the densitometry scan as a shoulder, quantification is not presented.

| $10^{-3} \times$ Estimated $M_r$ | Increase of phosphorylation caused by $Ca^{2+}$-calmodulin (%) |
|---|---|
| 137.0 ± 3.1 (18) | 111 ± 26 |
| 77.0 ± 1.4 (15) | 71 ± 17 |
| 58.0 ± 1.6 (15) | 51 ± 8 |
| 53.0 ± 1.3 (15) | 113 ± 20 |
| 50.0 ± 1.0 (13) | — |

varied from 51 to 113% (range 21–206%) (Table 1). Phosphorylation of the $50000$-$M_r$ peptide could not be quantified, since it usually appeared as a shoulder in the scan.

$Ca^{2+}$ alone without added calmodulin caused increased phosphorylation of the same peptides as did $Ca^{2+}$-calmodulin, although the effects were quantitatively less, and were more variable; this might be explained by the presence of endogenous calmodulin in the membrane preparation in spite of washing with EDTA, since (*a*) a Coomassie Blue-stained band was present in the membrane with the same estimated $M_r$ as standard calmodulin (see Fig. 4*a*), and (*b*) calmodulin was measurable in the membrane by radioimmunoassay at 290 pmol/mg of protein by using unheated standards (kindly performed by C. C. Fan and D. W. Powell, University of North Carolina School of Medicine, Chapel Hill, NC, U.S.A., with the New England Nuclear radioimmunoassay kit for calmodulin). This amount of endogenous calmodulin represents less than 15% of the exogenous calmodulin added in these experiments. When studied at a fixed free $Ca^{2+}$ concentration of 0.3 μM or 0.9 μM, calmodulin caused a concentration-dependent increase in phosphorylation of each of the peptides listed in Table 1, with maximal phosphorylation occurring at 1 μM added calmodulin. Half-maximal increases in phosphorylation were caused by 0.002–0.02 μM exogenous calmodulin. However, because of the presence of calmodulin in the membrane preparation, formal determinations of maximal and half-maximal effects of the calmodulin on phosphorylation were not attempted; 1 μM-calmodulin was used in all subsequent experiments. Troponin C (10 μM) in the presence or absence of $Ca^{2+}$ did not alter phosphorylation of any peptide.

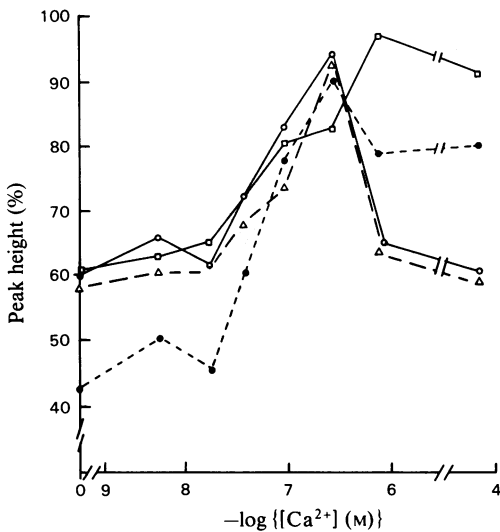Fig. 2. *Ca²⁺-dose–response of Ca²⁺–calmodulin-induced phosphorylation*

All studies were done in the presence of 1 $\mu$M exogenous calmodulin. The free $Ca^{2+}$ concentrations were calculated as described by Bartfai (1979). The magnitude of phosphorylation is plotted on the ordinate, in which the maximum phosphorylation for each experiment was taken as 100%. These data represent mean results for seven separate $Ca^{2+}$-dose–response curves. $Ca^{2+}$ concentrations ($\mu$M) giving half-maximal effect were: $M_r$-137000 peptide, 0.06 (○); $M_r$-77000, 0.12 (□); $M_r$-58000, 0.08 (△); $M_r$-53000, 0.06 (●). For all peptides except the $M_r$-77000 peptide, maximum phosphorylation occurred at 0.3 $\mu$M, with a decrease in phosphorylation at higher $Ca^{2+}$ concentrations. Maximum phosphorylation of the $M_r$-77000 peptide was reached at 0.9 $\mu$M; this phosphorylation did not significantly decrease when the $Ca^{2+}$ concentration was further increased. The amount of $Ca^{2+}$ required for half-maximal $Ca^{2+}$-induced phosphorylations was determined in the presence of 1 $\mu$M-calmodulin.

In the presence of 1 $\mu$M added calmodulin, $Ca^{2+}$ caused a dose-dependent increase in phosphorylation in all five peptides (Fig. 2). In these experiments, known amounts of $Ca^{2+}$ were added with 3 mM-EGTA to give a range of free $Ca^{2+}$ concentrations both as calculated (Bartfai, 1979) and as checked by a $Ca^{2+}$-selective electrode (Becker *et al.*, 1980). Because of the possibility that membranes might contain significant amounts of $Ca^{2+}$, solutions containing membrane were incubated for several hours, then the $Ca^{2+}$ added to the solution from the membrane was determined in the absence of EGTA. The total calcium added to the phosphorylation solution by this membrane was determined by using a $Ca^{2+}$-selective microelectrode (Lee, 1981) (kindly performed by Dr. C. O. Lee, Cornell University School of Medicine, New York, NY, U.S.A.) and found to contribute less than 0.1% of the added calcium at a free $Ca^{2+}$ concentration of 0.3 $\mu$M. For all peptides except the $M_r$-77000 peptide, maximum phosphorylation occurred at 0.3 $\mu$M free $Ca^{2+}$, whereas phosphorylation of the $M_r$-77000 peptide reached a maximum at 0.9 $\mu$M-$Ca^{2+}$ (Fig. 2). For all peptides except the $M_r$-77000 peptide, a decrease in phosphorylation occurred at 0.9 $\mu$M free $Ca^{2+}$ and higher concentrations. No similar significant decrease in phosphorylation with increased free $Ca^{2+}$ was seen for the $M_r$-77000 peptide at $Ca^{2+}$ concentrations above 10 $\mu$M. For all phosphorylations increased by $Ca^{2+}$–calmodulin, the concentrations of $Ca^{2+}$ resulting in half-maximal phosphorylation in the presence of 1 $\mu$M added calmodulin were between 0.06 and 0.12 $\mu$M. Similar patterns in magnitude of $Ca^{2+}$–calmodulin-induced phosphorylation occurred when the vesicle phosphorylation was compared with phosphorylation during sonication or in the presence of alamethacin, both of which were done to allow increased access of added ATP to the inside of closed vesicles. This indicated that the vesicles were not sealed.

The involvement of $Ca^{2+}$–calmodulin in these changes in phosphorylation was further suggested by studies using trifluoperazine, an inhibitor of $Ca^{2+}$–calmodulin-dependent processes. Trifluoperazine at 10 times the added calmodulin concentration (10 $\mu$M-trifluoperazine) significantly inhibited the $Ca^{2+}$–calmodulin-increased phosphorylation (Fig. 3). Trifluoperazine by itself caused a slight but non-significant increase in phosphorylation in the absence of $Ca^{2+}$ and calmodulin (control in Fig. 3). This indicates that basal phosphorylation is not dependent on $Ca^{2+}$–calmodulin. All phosphorylations increased by $Ca^{2+}$–calmodulin were inhibited by trifluoperazine and were not significantly different from the phosphorylations in the absence of $Ca^{2+}$–calmodulin. Thus $Ca^{2+}$–calmodulin failed to increase significantly the phosphorylation of any peptide in the presence of trifluoperazine, and 1 $\mu$M-trifluoperazine also inhibited $Ca^{2+}$–calmodulin-induced phosphorylation, but the changes were more variable than the inhibition with 10 $\mu$M-trifluoperazine.

*Cyclic AMP- and cyclic GMP-induced phosphorylation*

Cyclic AMP and cyclic GMP stimulated phosphorylation of two peptides with $M_r$ values of 137000 and 85000 ($n = 11$ and 7 for cyclic AMP and cyclic GMP respectively). Both phosphorylated peptides were in areas of significant Coomassie Blue staining. Cyclic AMP induced an
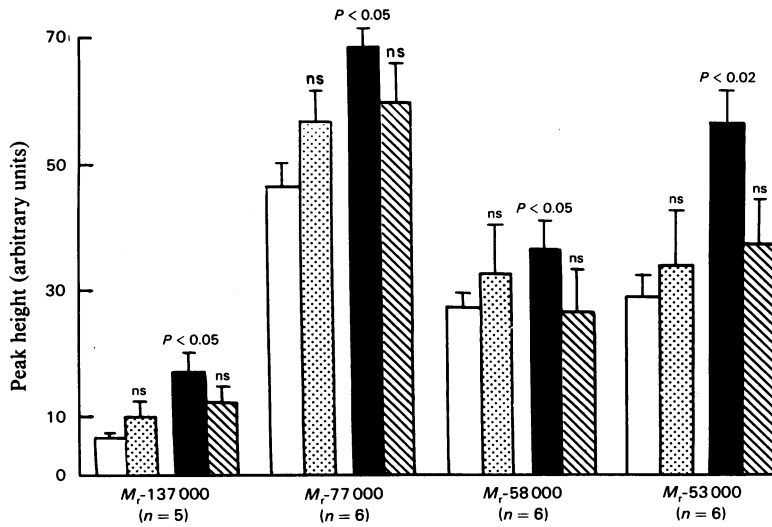
Fig. 3. *Trifluoperazine inhibition of* $Ca^{2+}$*–calmodulin-induced phosphorylation*

The magnitude of phosphorylations was compared in the presence and absence of $Ca^{2+}$ plus calmodulin. Control (□) represents phosphorylation in the absence of $Ca^{2+}$. The effects of $Ca^{2+}$ plus calmodulin (■) were studied in tissues from the same animals in the presence and absence of $10\,\mu M$-trifluoperazine, a concentration 10 times that of the added calmodulin. Trifluoperazine by itself (▣) caused a slight increase in phosphorylation of different peptides, but had no statistically significant effect on phosphorylation of any peptide. $Ca^{2+}$–calmodulin in the absence of tri-fluoperazine caused a statistically significant increase in phosphorylation of each peptide. $Ca^{2+}$–calmodulin in the presence of trifluoperazine (▨) did not significantly increase phosphorylation of any peptide compared with either the control or the control plus trifluoperazine. Results are means $\pm$ S.E.M. $P$ values are in comparison with control conditions (paired $t$ test); ns, not significant.
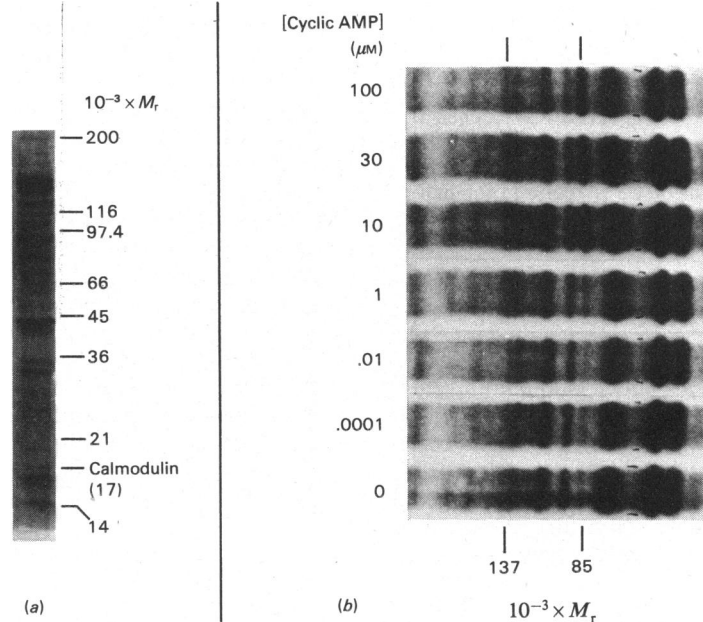


Fig. 4. *Cyclic AMP-induced phosphorylation of microvillus-membrane peptides*

(*a*) Coomassie Blue-staining pattern of microvillus-membrane peptides in the presence of $3\,mM$-EGTA and in the absence of cyclic AMP. (*b*) Autoradiograph showing cyclic AMP effect on phosphorylation performed at 27°C for 120s. Cyclic AMP increased phosphorylation, in a dose-dependent manner, of two microvillus-membrane peptides with $M_r$ 137000 and 85000. The differences in patterns of basal phosphorylation in Figs. 1 and 4 are probably due to differences in conditions of phosphorylation (0°C versus 27°C).

increase in phosphorylation of these peptides in a dose-dependent manner (Fig. 4), with a half-maximal effect at 0.3 $\mu$M for the $M_r$-137000 peptide and at 3.9 $\mu$M for the $M_r$-85000 peptide. Maximal phosphorylation of the $M_r$-137000 peptide was seen in the presence of 3 $\mu$M-cyclic AMP (200% increase) and of the $M_r$-85000 peptide in the presence of 10 $\mu$M-cyclic AMP (220% increase). Phosphorylation of these peptides was linear with time for 2–3 min at 0° and 27°C. Studies reported were performed at 27°C for 120 s. Addition of the phosphodiesterase inhibitor isobutylmethylxanthine (0.1 mM) had no effect on the extent of cyclic AMP-induced phosphorylation, nor did addition of 3 mM-EGTA.

Cyclic GMP stimulated phosphorylation of the same two peptides in a dose-dependent manner. Cyclic GMP gave a 95% increase in phosphorylation of the $M_r$-137000 peptide, with a half-maximal effect at 4.6 $\mu$M and a maximal effect at 30 $\mu$M, and a 120% increase in phosphorylation of the $M_r$-85000 peptide, with a half-maximal effect at 0.05 $\mu$M and a maximal effect at 0.46 $\mu$M. No additive effect on the phosphorylation of the $M_r$-137000 or -85000 peptides was seen when maximal concentrations of cyclic AMP and cyclic GMP were added together.

Similar to results with phosphorylation increased by $Ca^{2+}$–calmodulin, cyclic nucleotide-induced changes in membrane phosphorylation were qualitatively and quantitatively similar when compared with similar studies performed during sonication and in the presence of alamethacin to increase the vesicle permeability and to increase accessibility of the inside of the vesicles to ATP. Extraction of membranes with 0.56 M-NaCl did not alter the cyclic AMP- or cyclic GMP-induced phosphorylations.

## Discussion

Although there is no single mechanism for the action of intracellular $Ca^{2+}$, many of the physiological effects of $Ca^{2+}$ are mediated by changes in protein phosphorylation (Schulman et al., 1980). This is different from the actions of cyclic nucleotides, which appear to be mediated entirely by protein phosphorylation (Greengard, 1978). In the present work we demonstrate the presence of endogenous $Ca^{2+}$–calmodulin-, cyclic AMP- and cyclic GMP-dependent protein kinases and substrates for these kinases in microvillus membrane purified from rabbit ileal-mucosal cells.

We chose to study phosphorylation of purified microvillus membranes separate from microvillus core, rather than in intact brush border, because the membrane is the most likely area at which transport protein permeabilities are regulated.

Phosphorylation was studied in a vesicle preparation that was leaky and allowed entry of ATP. Vesicles similarly prepared are thought to be oriented right-side out (Hopfer et al., 1973). ATP had ready access to the inside of our vesicles, since phosphorylation was comparable when vesicles were studied with or without disruption by sonication during phosphorylation or by alamethacin exposure. We had predicted the leakiness of the vesicles because of the initial homogenization in an EDTA-containing solution (Hopfer et al., 1973). Demonstration of disruption in 10% of vesicles by electron microscopy in one plane suggests the presence of breaks in a much larger fraction, as was shown functionally.

$Ca^{2+}$ and calmodulin caused increased phosphorylation of five microvillus-membrane peptides, with $M_r$ values of 137000, 77000, 58000, 53000 and 50000. The phosphorylation of the $M_r$-77000 peptide appears to be regulated differently from that of the other four peptides. It has different $Ca^{2+}$ concentrations for half-maximal and maximal effects and turns over much more rapidly. This could be explained by at least two separate $Ca^{2+}$–calmodulin-dependent kinases in the microvillus membrane, or by a protein phosphatase associated with this peptide.

Both $Ca^{2+}$ and calmodulin are required for the increase in phosphorylation of these peptides. These phosphorylations could be physiologically significant, since the increases in phosphorylation occur with half-maximal concentrations of free $Ca^{2+}$ which approximate to the $Ca^{2+}$ concentrations that are present in the cytosol of intact cells of several tissues. For instance, free $[Ca^{2+}]$ of rabbit heart muscle and in proximal tubules of the salamander Necturus has been reported to be approx. 0.1 $\mu$M (0.11 and 0.34 $\mu$M respectively) (Lee et al., 1980a,b; Lee, 1981). This would allow small changes in cytosol free $Ca^{2+}$ to regulate the function of the membrane protein kinases. The calmodulin involvement in these $Ca^{2+}$-increased phosphorylations was established, since calmodulin increased phosphorylation in a concentration-dependent manner and troponin C could not substitute in this calmodulin effect. Since $Ca^{2+}$ is also capable of activating a phosphatidylserine-sensitive protein kinase (so-called C kinase) (Takai et al., 1979), as well as acting through other protein kinase-dependent and -independent mechanisms, and because trifluoperazine inhibits $Ca^{2+}$–phosphatidylserine-induced phosphorylation (Takai et al., 1979) as well as $Ca^{2+}$–calmodulin-dependent processes, it was necessary to demonstrate the dependency on the concentration of calmodulin to demonstrate clearly involvement of calmodulin.

The shape of the $Ca^{2+}$–phosphorylation dose-response curve was characterized by a narrow range

over which $Ca^{2+}$ caused maximum phosphorylation, and by a decrease in phosphorylation when $Ca^{2+}$ was increased above this concentration. Although no explanation for such a response has been found in the present studies, similar-shaped $Ca^{2+}$-dose–response curves have been reported for other $Ca^{2+}$–calmodulin-dependent processes, including phosphorylase kinase activity in rat liver. (Chrisman et al., 1980), suggesting some mechanism of action common to a class of $Ca^{2+}$–calmodulin functions. One possibility is the presence of $Ca^{2+}$–calmodulin-dependent phosphatases which are activated at higher $Ca^{2+}$ concentrations than are the $Ca^{2+}$–calmodulin-dependent protein kinases (Stewart et al., 1982). The slight increase in basal phosphorylation caused by trifluoperazine shown in Fig. 3 could also be due to the presence of a $Ca^{2+}$–calmodulin-dependent phosphatase that was inhibited by trifluoperazine.

Cyclic GMP and cyclic AMP stimulated phosphorylation of the same peptides, of $M_r$ 137000 and 85000. The effects of cyclic AMP and cyclic GMP in increasing phosphorylation of these two peptides were not additive. The concentration of cyclic AMP that caused a half-maximal increase in phosphorylation of the $M_r$-137000 peptide was much lower than that for cyclic GMP (0.3 $\mu$M compared with 4.6 $\mu$M), and the extent of stimulation was greater for cyclic AMP. In contrast, for the $M_r$-85000 peptide the half-maximal concentration for cyclic GMP-stimulated phosphorylation was much lower than that for cyclic AMP (0.05 $\mu$M compared with 3.9 $\mu$M), whereas cyclic AMP still caused a greater increase in phosphorylation. These data are consistent with the view that phosphorylation of the $M_r$-137000 protein is regulated by a cyclic AMP-dependent protein kinase, whereas phosphorylation of the $M_r$-85000 peptide is regulated by a cyclic GMP-dependent protein kinase. Activation of cyclic GMP kinase by relatively high concentrations of cyclic AMP, and of cyclic AMP kinase by high concentrations of cyclic GMP, previously has been shown for other tissues and also for the cyclic GMP-dependent protein kinase in intestinal microvilli (Schlatz et al., 1979; De Jonge, 1981; Hofmann & Gensheimer, 1983). The non-additivity of the phosphorylation in the presence of cyclic AMP and cyclic GMP is consistent with this hypothesis. This view is also consistent with previous suggestions that phosphorylation of the $M_r$-85000 peptide represents autophosphorylation of brush-border cyclic GMP protein kinase (De Jonge, 1981; De Jonge & von Dommelen, 1981). De Jonge (1981) reported that cyclic GMP increased phosphorylation of an $M_r$-86000 peptide in rat ileum. This was present in brush-border vesicles containing microvillus membrane and core protein, and in core protein prepared by treating the brush-border vesicles with detergent. It was hypothesized that the $M_r$-86000 peptide linked core proteins to membrane. Our finding of an $M_r$-85000 substrate for cyclic GMP-dependent protein kinase in purified microvillus membrane provides additional support for this hypothesis. In view of similarities in $M_r$ values and in the reported half-maximal concentrations for both cyclic AMP and cyclic GMP, it seems likely that we are describing the same peptide. One would also expect such a linking protein to be found in both purified membrane and core preparations. Schlatz et al. (1979) reported the presence of a $M_r$-103000 peptide phosphorylated by cyclic nucleotide-dependent protein kinase in microvillus membrane. We did not see such a peptide in the present work, and are uncertain about the relationship of this peptide to those observed here; however, the Coomassie Blue-staining pattern described by Schlatz et al. (1979) was also significantly different from that described here.

We attempted to test further the view that high concentrations of cyclic AMP and cyclic GMP activated both cyclic AMP- and cyclic GMP-dependent protein kinases by using cyclic AMP-dependent protein kinase inhibitor on both cyclic AMP- and cyclic GMP-dependent phosphorylation of the $M_r$-137000 and -85000 peptides. This was not successful, since neither the cyclic AMP nor the cyclic GMP effects were inhibited in the microvillus-membrane preparation, though the protein kinase inhibitor did inhibit cyclic AMP-induced phosphorylation of cytosol prepared from rabbit ileal mucosa (M. Donowitz, R. Gudewich, M. E. Cohen & G. W. G. Sharp, unpublished work). Furthermore, it remains difficult to define the relative physiological importance of cyclic AMP and cyclic GMP in regulation of phosphorylation of the $M_r$-85000 substrate in situ, since stimulated cyclic AMP concentrations in response to theophylline and cholera toxin are approx. 20–100 times tissue cyclic GMP concentrations seen after stimulation with heat-stable Escherichia coli enterotoxin, and in addition basal concentrations are also much higher for cyclic AMP than for cyclic GMP (Beavo et al., 1974; De Jonge, 1975).

In addition, we have not been able to determine whether $Ca^{2+}$–calmodulin and cyclic AMP are increasing phosphorylation of the same or different $M_r$-137000 peptides, since use of both 5–15% and 6% acrylamide gels did not show that different peptides were increased (results not shown).

Previous work has shown that $Ca^{2+}$–calmodulin and cyclic nucleotides alter ion transport across the microvillus membrane, and in this paper the presence of $Ca^{2+}$–calmodulin- and cyclic nucleotide-dependent protein kinases and substrates in this membrane are demonstrated. This suggests that

ion transport across the microvillus membrane may be regulated either by phosphorylation of ion-channel proteins in the membrane, or by phosphorylation of proteins which regulate the activity of channel proteins. Supporting this hypothesis, phosphorylation of a subunit of the $Na^+$ channel of rat brain by cyclic AMP-dependent protein kinase has been demonstrated (Costa et al., 1982). However, the $Na^+$- and $Cl^-$-channel proteins of intestinal epithelial cells still are not identified, and a direct relation between phosphorylation of channel proteins and ion transport remains to be demonstrated.

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