J. Membrane Biol. 107, 137–144 (1989)

The Journal of
Membrane Biology

Activation of Chloride Conductance in Pig Jejunal Brush Border Vesicles

G.W. Forsyth and S.E. Gabriel
Veterinary Physiological Sciences, University of Saskatchewan, Saskatoon, Saskatchewan, Canada S7N 0W0

Summary. Requirements for the activation of Cl conductance have been investigated in pig jejunal brush border vesicles. The stability of ATP as a substrate for protein kinase activity, the stability of the phosphoprotein product of protein kinase action, and the choice of buffer system used for vesicle preparation were studied as variables which affected the outcome of in vitro activation attempts. Arsenate was selected as the most effective agent in protecting ATP from hydrolysis by the phosphatase activity in this vesicle system. Brush border vesicle protein appeared to prevent the accumulation of phosphoprotein in a cAMP-dependent protein kinase reaction, and vesicle protein only had phosphate acceptor activity when KF was added as a presumptive inhibitor of phosphoprotein phosphatase.

A Cl^- conductance response to a potassium gradient and valinomycin was present in vesicles prepared in buffers containing tetramethylammonium. Cl^- conductance activity was not increased in this system by the addition of ATP, dibutyryl cyclic AMP, and cyclic AMP-dependent protein kinase.

There was no Cl conductance response to a potassium gradient in vesicles buffered with imidazolium-acetate. Incorporation of ATP, AsO_4^{3-}, and F^- into these nonconductive vesicles by homogenization, followed by addition of dibutyryl cAMP, produced substantial conductance activity. Maximal activation of Cl^- conductance was obtained with vesicles prepared in imidazolium-acetate buffering, using precautions to stabilize ATP and phosphoprotein prior to conductance measurements.

Key Words Cl conductance · conductance activation · intestinal secretion · cystic fibrosis

Introduction

Protein phosphorylation was assumed to control ion secretion by enterocytes from the time of early studies that showed a connection between intestinal fluid secretion and elevated levels of cAMP in intestinal epithelial cells. Lucid and Cox reported in 1972 that cholera toxin increased the phosphorylation of brush border membrane proteins [17]. The suggested target of this protein kinase activity was a process controlling Cl^- secretion across the intestinal mucosa in a serosal-to-mucosal direction [5]. Subsequently the process of active chloride secretion has been shown to be driven by the electrochemical potential of intracellular Cl ion, and limited by the permeability of the apical membrane for chloride [8, 10].

Current measurements of epithelial chloride channels by patch-clamp procedures indicate activation of these conductive channels by cyclic AMP and calcium [7, 9]. The demonstration of regulation of Cl^- channels contained in excised membrane patches suggested that it should also be possible to activate conductance in membrane vesicles where there would be sufficient membrane protein to permit identification of channel regulatory elements and isolation of channel proteins.

Chloride conductance has been demonstrated in enterocyte brush border membrane vesicles from several species [6, 14, 16]. However, direct evidence for activation of conductance in vesicle systems has not been reported in spite of intensive efforts to identify the relevant targets of protein kinase [2, 4, 12, 21–23].

Large amounts of material for vesicle preparation and identification of channel components are available from pig jejunum [18, 19]. However, there are specific buffer requirements for the demonstration of chloride conductance in pig jejunal brush border membrane vesicles [6]. There was no Cl^- conductance response to a transmembranous K^+ gradient in brush border vesicles prepared in imidazolium-acetate buffer according to Liedtke and Hopfer [16]. On the other hand, vesicles obtained from the same tissue showed a consistent conductance response when prepared in N-2-hydroxyethylpiperazine-N′-2-ethanesulfonate (HEPES)-tetramethylammonium buffer (TMA) [14]. We hypothesize that a demonstration of in vitro activation may require starting material which resembles the basal state in vivo; i.e., lacks Cl^- conductance activity. This study reports some requirements for activation of Cl^- conductance in vesicles isolated with the basal, or closed configuration of the Cl^- conductance carrier.

Materials and Methods

Vesicle Preparation

Brush border membrane vesicles were prepared from pig jejunal mucosal scrapings as described previously [18]. All steps in vesicle preparation were carried out at 0–4°C. Scrapings from 40-cm segments were suspended in 60 ml of either of the buffers described below, and homogenized with 4–30 sec bursts on a Brinkmann Polytron homogenizer. Cell debris was removed by centrifuging for 10 min at 4,500 × *g*. The supernatant was centrifuged for 60 min at 4,500 × *g* to collect a crude vesicle preparation. These vesicles were resuspended in 30 ml of homogenizing buffer using one pass of a Potter-Elvehjem homogenizer at 1,000 rpm. The suspended material was made 10 mM in Mg^{2+} by addition of Mg gluconate and left for 20 min to allow aggregation of nonmembranous material. The Mg^{2+}-aggregate was then removed by centrifuging for 15 min at 3,000 × *g*. The brush border fraction was collected as a pellet by centrifuging at 27,000 × *g* for 30 min. Sodium-potassium ATPase and alkaline phosphatase activity were assayed to determine purification of brush border material and contamination with basal-lateral membranes [18].

Vesicles were prepared in either of two homogenizing buffers. The first buffer contained 300 mM mannitol, 1.0 mM $CaCl_2$, and 70 mM imidazolium-acetate at pH 7.4. In the second buffer the imidazolium-acetate was replaced by 10 mM HEPES-Tris, pH 7.4. Minor variations included the addition of ATP and other components of a cyclic AMP-dependent protein kinase (A-kinase) system at various points in the preparation procedure as indicated below.

Preequilibration and Uptake Conditions

Vesicles prepared in HEPES-Tris buffer were pre-equilibrated with HEPES-tetramethylammonium (TMA) by resuspending in 300 mM mannitol, 10 mM HEPES-TMA, pH 7.4, followed by a second collection step at 27,000 × *g* for 30 min. ^{36}Cl uptake by vesicle suspensions was initiated by diluting the suspension with an equal volume of isotonic uptake media (replacements of mannitol with 100 mM K gluconate and 10 mM KCl plus 1.0 μCi/ml of ^{36}Cl, specific activity 1.0 μCi/μmol). For conductance conditions valinomycin (7.0 μg/mg vesicle protein) was added 5 min prior to starting the uptake. ^{36}Cl content of the vesicle suspensions was determined by collecting triplicate samples of 0.2 mg vesicle protein on cellulose acetate filters (0.45 μm pore size) followed by liquid scintillation counting.

ATP Persistence in Vesicle Suspensions

ATP was mixed with brush border vesicles and residual ATP assayed by a luciferin-luciferase kit (Sigma Chemical Co.) to determine the availability of ATP for incorporation into vesicles, and for action as a substrate for A-kinase. Alkaline phosphatase activity of pig jejunal vesicles was assayed according to Hirano et al. [12], using *p*-nitrophenylphosphate as a substrate. Inhibition of pig jejunal brush border alkaline phosphatase by arsenate was investigated under the same conditions. ATPase was assayed as the appearance of inorganic phosphate in a buffer system containing 2.5 mM ATP, 100 mM NaCl, 10 mM KCl, 50 mM Tris HCl at pH 7.5, 1.0 mM EDTA and 1.0 mM $MgCl_2$, with 50 μg of vesicle protein added. The assay was carried out in the presence and absence of 1.0×10^{-4} M ouabain.

A-Kinase Activity on Vesicle Protein

The activity of A-kinase with pig jejunal brush border vesicle protein as substrate was investigated using the conditions suggested by Roskoski [20]. A-kinase from beef heart (Sigma) was used at a level of 120 pM units per assay. Comparative phosphorylation of bovine serum albumin, type IIA calf thymus histone, and vesicle protein was determined using $\gamma^{32}P$ ATP as phosphate donor and trapping the reaction products on phosphocellulose paper to quantitate ^{32}P transfer to protein.

Conductance Activation Conditions

The standard activation system consisted of: Na_2HAsO_4, 5.0 mM; KF, 5.0 mM; ATP 5.0 mM; plus 20 μM dibutryl cAMP. These activators were introduced into the vesicles by a single pass of a Potter-Elvehjem homogenizer operating at 1,000 rpm. This homogenizing procedure produced a mannitol space of 1.6 μl/mg of vesicle protein when ^{3}H-mannitol (1.0 μCi/ml) was added immediately before homogenizing, and the mannitol space was completely lost on lysing the vesicles with 0.5% sodium dodecylsulfate. The time of addition of ATP and the inhibitors was varied to examine effects on conductance activation. Dibutryl cAMP was added 5 min prior to initiating ^{36}Cl uptake.

Results

Vesicle Properties and Buffer Effects

The procedures outlined above gave vesicles with an equilibrated chloride space of 1.4 to 1.6 μl per mg of protein. This space was independent of the buffer used during vesicle preparation. Alkaline phosphatase activity was enriched 12- to 15-fold during the purification, and Na-K ATPase activity increased ~1.2 times. The enrichment of the marker enzymes, and the vesicle protein profiles separated in the presence of sodium dodecyl sulfate on 7.5% polyacrylamide gels [15] did not differ with preparation in HEPES-Tris or in imidazolium-acetate buffers.

Stability of Exogenous ATP in Vesicle Suspensions

Relatively high concentrations of vesicle protein (13 mg protein per ml) used for ion transport studies contained correspondingly large amounts of phosphatase activity. ATP added to vesicle suspensions at 1 to 5 mM initial concentration was reduced to less than 10 μM within 1 min. Total ATPase activity of the vesicle suspensions was measured at 1.36 μmol/min/mg of vesicle protein, and this activity

Table 1. Phosphoprotein production in the presence of jejunal brush border vesicle protein

Protein substrate	pmol P_i attached to protein
Bovine serum albumin (BSA)	64
BSA + BBV protein	42
BSA + BBV protein + AsO_4^{3-}	60
BSA + histone	2030
BSA + histone + BBV protein	218
BSA + histone + boiled BBV protein	1540
BSA + histone + BBV protein + KF	400
BSA + BBV protein + KF	93

Reaction additions: 5.0 nmol γ^{32}P ATP, 120 pM units A-kinase, 0.2 nmol BSA, 50 μg (3.6 nmol) type IIA histone, 50 μg (~1 nmol) BBV protein.

was reduced by 0.15 μmol in the presence of ouabain. When a group of reported inhibitors including phenylalanine, ascorbate, bismuth and pyrophosphate were tested for inhibition of phosphatase activity toward *p*-nitrophenylphosphate the best inhibition was obtained using AsO_4^{3-} ion. Inhibition of *p*-nitrophenylphosphate phosphatase was maximal at 90% with 5.0 mM arsenate, so this condition was used during subsequent attempts at conductance activation.

Phosphorylation of Vesicular Protein

The dynamics of in vivo phosphorylation and dephosphorylation require that protein kinase activity be balanced by corresponding phosphoprotein phosphatase activity. With limited intravesicular supplies of ATP this phosphatase activity could present a problem for conductance activation if protein kinase activity is matched by an equally active phosphoprotein phosphatase. Pig jejunal brush border vesicle protein did not appear to be a good substrate for phosphorylation by A-kinase under in vitro assay conditions (Table 1). It was also noted that vesicle protein included with histone or bovine serum albumin significantly reduced the amount of ^{32}P associated with these proteins at the end of a 10-min kinase assay. Addition of arsenate ion to the assay did not prevent the inhibitory action of vesicle protein on histone and BSA phosphorylation. The inhibitory effect of the vesicles was heat labile. Fluoride ion is reported to be one of the more effective inhibitors of phosphoprotein phosphatase [11]. The labeling of the mixture of histone and vesicle protein was doubled by including 5.0 mM KF in the kinase assay system, and KF was required to show any net ^{32}P transfer to vesicle protein (*see* Table 1).

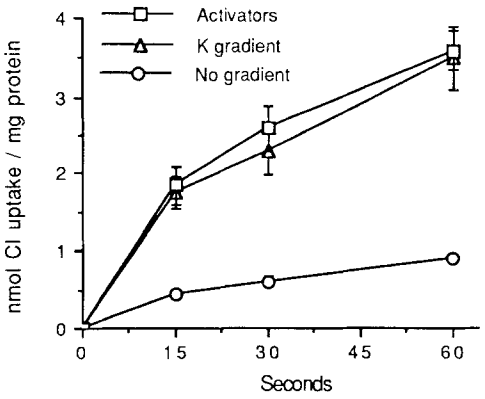

Fig. 1. Chloride conductance of brush border vesicles buffered with HEPES-TMA. Vesicles were prepared in medium containing 300 mM mannitol, 10 mM HEPES-Tris at pH 7.5, 5.0 mM ATP, 5.0 mM Na_2HAsO_4, 5.0 mM KF. Before measuring Cl uptake the vesicles were equilibrated with 100 mM mannitol, 100 mM TMA gluconate with 10 mM HEPES-TMA at pH 7.5. Valinomycin (7.0 μg/mg vesicle protein) was added to vesicle suspensions labeled *K gradient* (△), and *Activators* (□) 5 min before starting Cl uptake. 20 μM dibutyryl cAMP added with valinomycin for activation (□). The *No gradient* (○) condition lacked valinomycin. Cl uptake was started by diluting vesicle suspensions in equal volumes of media containing mannitol, 100 mM K gluconate, 10 mM KCl, 1.0 μCi ^{36}Cl per ml. Values are $\bar{x} \pm$ SEM (n = 12). Missing error bars are smaller than the symbol size

Absence of Activation in Vesicles Prepared with HEPES-Tris

A substantial conductance effect was present in vesicles prepared in the mannitol HEPES-Tris homogenizing buffer, and preequilibrated with HEPES-TMA before measuring Cl^- uptake. The extent of the conductance response is shown in Fig. 1. The initial rate of Cl uptake was increased approximately fourfold by imposing a K^+ gradient.

Activation of the conductance state in these vesicles was attempted by addition of ATP as substrate, substrate stabilizer, and protein phosphatase inhibitor. These additions were included in the initial homogenizing buffer to ensure internalization within the intravesicular space during the homogenization. Dibutryl cAMP was added as a kinase activator with the valinomycin at the beginning of the Cl^- uptake. Under conductance conditions there was no significant increase in the rate of Cl^- uptake relative to the uptake rate measured in the absence of activators (Fig. 1).

Several procedures were used to try to obtain activation of conductance. Conductance rates were not increased by addition of all activation reagents 10 min before initiating the Cl uptake conditions, or by using detergents to permeabilize vesicles for activator access to internal protein kinase substrate

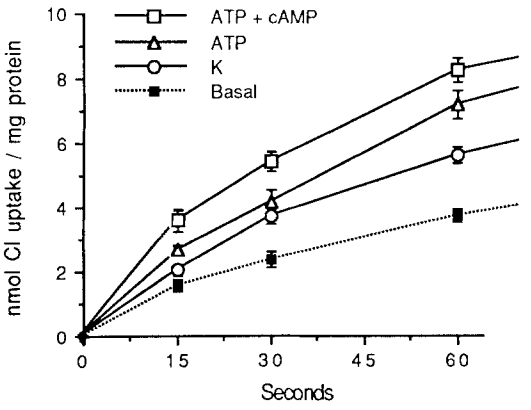

Fig. 2. Chloride conductance activation in brush border vesicles buffered with imidazolium-acetate. Vesicles were prepared in 263 mM mannitol, 1.0 mM $CaCl_2$, buffered to pH 7.5 with 50 mM imidazolium-acetate. ATP, AsO_4^{3-}, and F incorporation is indicated by *ATP*. Uptake media for measuring conductance contained 52.5 mM mannitol, 50 mM imidazolium-acetate pH 7.5, 100 mM K gluconate, 10 mM KCl, 1.0 mM $CaCl_2$, with 1.0 μCi ^{36}Cl per ml and 7.0 μg of valinomycin per mg vesicle protein. ATP and valinomycin were omitted in the basal condition (□). Valinomycin, but no activation components, were present in the K condition (○). Vesicles with incorporated ATP were used in the conductance condition, without (△), and with (□) added dibutyryl cAMP. Values are $\bar{x} \pm$ SEM (n = 12). Missing error bars were smaller than the symbol size

sites. Activation was also unsuccessful when exogenous A-kinase (50 to 500 pM units/mg of vesicle protein) was included in the activation mixture.

Absence of Conductance in Vesicles Prepared with Imidazolium-Acetate

The vesicles prepared in this buffer system had a normal permeability to Cl^- ion when measurements of uptake were carried out in the absence of a K^+ gradient. A range of conditions was employed to try to obtain increased rates of Cl uptake in response to a K^+ gradient imposed across vesicles treated with valinomycin. A conductance response could not be obtained by varying the osmolarity of the homogenizing and uptake media, the valinomycin concentration, or the magnitude of the potassium gradient. Effective valinomycin concentrations required for K^+ permeability have been determined for this system [6]. The control rate of uptake for 10 mM Cl^- was 7.7 ± 0.79 nmol $Cl^- \cdot min^{-1} \cdot$ mg $protein^{-1}$ (n = 27), compared to 7.0 ± 0.89 nmol $Cl^- \cdot min^{-1} \cdot$ mg $protein^{-1}$ (n = 29) with 7 μg of valinomycin per mg vesicle protein.

Conductance Activation

The activation system which was tried unsuccessfully with vesicles buffered in HEPES-TMA was also tested in vesicles prepared in imidazolium-acetate (I-A) buffer. Activators were added after the divalent cation precipitation step in vesicle preparation. The supernatant solution after divalent cation precipitation was made 5.0 mM in ATP, sodium arsenate, and KF. These ingredients were introduced into the intravesicular space by a single pass with a Potter-Elvejhem homogenizer at 1,000 rpm. The vesicles were harvested by a final centrifugation step, and dibutryl cAMP (20 μM) was added to the resuspended vesicles 5 min prior to starting Cl uptake measurements. The vesicles prepared freshly in imidazolium-acetate had slightly higher Cl permeability than similar preparations made in HEPES-TMA (*compare* Figs. 1 and 2).

Incorporation of ATP plus inhibitors increased the initial rate of Cl^- uptake observed with the K^+ gradient, and this rate was increased further by addition of dibutryl cAMP prior to starting the Cl^- uptake. The results show a significant increase in Cl^- conductance activity after the addition of protein kinase substrate and activator. The equilibrated Cl content of these vesicles was 15 ± 1.4 nmol Cl per mg vesicle protein, and this content was not changed by any of the additions used in the activation study.

Requirements for AsO_4 and F in the Activation System

Sodium arsenate was added to reduce the destruction of ATP by alkaline phosphatase, and KF was used for its inhibitory activity toward phosphoprotein phosphatase as outlined above. The effect of ommitting both inhibitors from the otherwise complete activation system is shown in Fig. 3. There was no detectable activation in comparison to basal conductance rates reported in Fig. 2 when the inhibitors were both ommitted. For purposes of clarity, the separate ommissions of arsenate and fluoride are not shown in Fig. 3, but the data indicate a synergistic effect of the two agents in promoting activation of Cl^- conductance.

Role of Ca^{2+} in Conductance Activation

The Ca^{2+} requirement of the activation system was examined by omitting Ca^{2+} from the homogenizing buffer. Ca^{2+} (1.0 mM) or 1.0 mM ethyleneglycol-

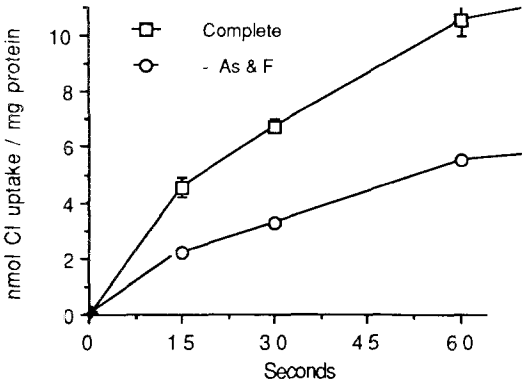

Fig. 3. The requirement for arsenate and fluoride in activation of chloride conductance. Vesicle preparation was carried out as described for Fig. 2. The effect of omitting 5.0 mM Na_2HAsO_4 and KF (○) from the ATP incorporation step were compared with conductance activity measured in the complete activation system containing arsenate, fluoride, and ATP (□). Dibutyryl cAMP and valinomycin were added prior to Cl uptake in both conditions. Values are $\bar{x}$ ± SEM (n = 6)

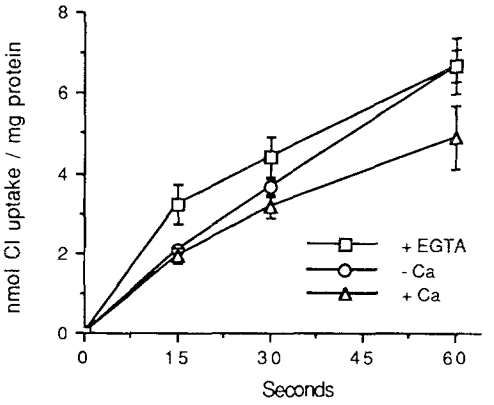

Fig. 4. Effect of delaying Ca^{2+} addition on the activation of chloride conductance. Vesicles were prepared as described for Fig. 2 except for ommission of $CaCl_2$ from the initial homogenizing buffer. At the time of ATP incorporation (after the precipitation of nonbrush border material by Mg^{2+}), $CaCl_2$ (1.0 mM) (△), EGTA (1.0 mM) (□), or neither (○) were added to the otherwise complete activation system. Values are $\bar{x}$ ± SEM (n = 6)

bis-[β-aminoethyl ether] N,N,N′,N′-tetraacetic acid (EGTA) were then added at the time of ATP incorporation. EGTA addition at this time caused some increase in conductance above the basal level, while Ca^{2+} addition at this stage in vesicle preparation did not permit the normal activation usually observed in the presence of ATP and dibutryl cAMP (Fig. 4). The characteristic activation response reported in Fig. 2 appeared to require the presence of 1.0 mM Ca^{2+} throughout the vesicle preparation.

Optimal Timing for ATP Addition

Both the ATP substrate and the phosphoprotein product of protein kinase activity may be labile in the brush border vesicle system. Thus the route and timing of ATP addition could be critical to producing conductance activation. Extravesicular ATP did not increase Cl conductance, so ATP addition had to be combined with a technique to insert ATP into the intravesicular space. Conductance activation in response to different times of ATP insertion is shown in Table 2. ATP insertion at the time of the initial homogenization of mucosal scrapings (approximately 3 hr prior to addition of dibutryl cAMP) gave an increase of about 20% over the basal conductance rate. ATP introduction by homogenizing after the divalent cation precipitation step (45 min prior to conductance measurements) gave the greatest activation response. Homogenizing vesicle suspensions with ATP within 10 min of starting the conductance assay gave relatively high rates of Cl conductance under both the activation condition and the basal state. The largest response to addition of ATP and dibutryl cAMP clearly occurred with the intermediate time of ATP addition.

Effect of Vesicle Buffer System on Activation

Differences in the potential for conductance activation were reassessed in vesicles prepared in the two different buffer systems used in this study. Ca^{2+} was present throughout the preparation, and ATP was incorporated after divalent cation precipitation of nonbrush border material. Under these optimal activation conditions for vesicles prepared with imidazolium-acetate buffering, there was no significant activation of conductance in vesicles prepared in HEPES-TMA buffer (Fig. 5).

Discussion

There may be a number of reasons why activation of Cl conductance in vesicle systems has proven difficult. Some of the more obvious problems include the techniques required to introduce soluble proteins, cofactors and substrates for the activation system into closed vesicles. There may also be difficulties in maintaining functional levels of labile substrates until vesicles can be resealed for ion transport measurements. Steps must be taken to ensure

Table 2. Effect of different modes of ATP addition on chloride conductance activity (as initial rates of ^{36}Cl uptake) in brush border vesicles

Concentration and mode of ATP addition	Basal rate	+ ATP rate	+ ATP increment
	(nmol Cl/min/mg vesicle protein)		
5.0 mM incorporated at mucosal scrapings homog	10.8 ± 3.0	12.9 ± 1.6	2.1
5.0 mM incorporated after Mg^{2+} ppct[a] step	8.4 ± 0.6	15.6 ± 1.2	7.2
5.0 mM added to vesicles before Cl uptake	10.8 ± 3.0	10.4 ± 1.0	—
1.0 mM incorporated before Cl uptake	14.4 ± 1.4	12.5 ± 0.7	—
5.0 mM incorporated before Cl uptake	14.4 ± 1.4	15.6 ± 1.6	1.2

$n = 6 \pm$ SEM except for condition 2 (incorporation after Mg^{2+} ppct[a]) where $n = 14$.

the persistence of phosphoprotein products after protein kinase activation. Finally, there is the question of the criteria to be met in a system in order to claim that Cl conductance has been increased in a similar manner, and to a similar extent, as in the activation process occurring in intact cells and tissues.

Consideration of these factors in the design of an in vitro activation system did not produce a detectable increase in the initial rates of Cl entry into brush border membrane vesicles prepared in HEPES-TMA buffer. The final requirement for conductance activation in pig jejunal brush border membrane vesicles was a source of vesicles isolated with a basal state of the Cl conductance channel. Vesicles isolated with imidazolium-acetate buffering had no demonstrable conductance activity [6]. The choice of these vesicles as starting material for the activation system appeared to be the key to the successful application of the conditions considered above.

Donowitz et al. have used freeze-thaw and high voltage discharge procedures to incorporate ATP and dextran into rabbit ileal brush border vesicles [3]. The homogenizing technique used in this study was faster and less complex than the freeze-thaw procedure. Measurements of the mannitol space in vesicles opened by this method indicate that it produces a good equilibration of the intravesicular space with the suspending buffer. Mannitol spaces of 1.6 μl per mg protein agree well with an equilibrated Cl space of approximately 1.5 μl per mg. The only apparent disadvantage of the technique was the time requirement of 20 to 30 min for completion of vesicle resealing as noted in Table 2.

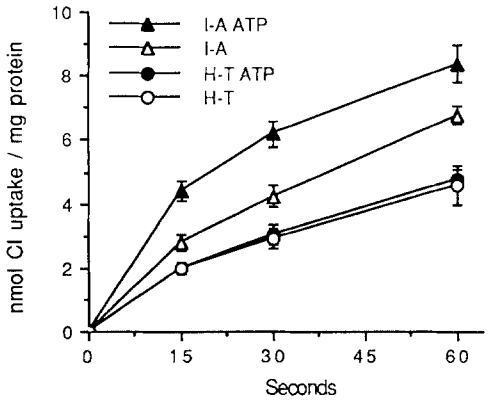

Fig. 5. The effect of buffer system on the capacity for conductance activation in vesicles. One batch of mucosal scrapings was used to prepare vesicles as described for Fig. 1 with HEPES-TMA buffering, or as for Fig. 2 with imidazolium-acetate buffering. Basal HEPES-TMA (○), and basal imidazolium-acetate (△) conductance was measured with 100 mM external K^+ ion and valinomycin, but no incorporated activators. Activation was attempted with 5.0 mM ATP, AsO_4^{3-}, and F^- incorporation into vesicles prepared in each buffer by homogenizing vesicle suspensions after divalent cation precipitation of nonbrush border material (ATP, filled symbols). 20 μM dibutyryl cAMP was added with valinomycin 5 min prior to initiating Cl uptakes. Values are $\bar{x} \pm$ SEM ($n = 6$)

Phosphatase enzymes are common markers for intestinal brush border membranes, and some of them were very effective in catalysing the dephosphorylation of ATP. The rapid breakdown of ATP that was observed in suspensions of vesicles could severely deplete added ATP even in the short time intervals between ATP addition to a vesicle suspension and homogenizing for insertion. It is not clear from these data if the arsenate contribution to conductance activation involved protection of extravesicular ATP prior to incorporation, or perhaps some protection of intravesicular ATP from phosphatase activity within the vesicles. Since the native location of the alkaline phosphatase is on the luminal surface of the brush border membrane, the arsenate should be acting primarily to stabilize ATP before incorporation.

Evidence relating to the stability of phosphoprotein was obtained from a study of protein kinase activity toward different substrates. Pig jejunal vesicle protein was a very poor substrate for A-kinase from beef heart. Mixing type IIA histone with vesicle protein caused a major suppression of ^{32}P protein product in comparison to the histone alone. The inhibitory effect could have arisen at several levels, including destruction of ATP, inhibition of A-kinase, or removal of P from phosphoprotein. ATP destruction may be partially ruled out since arsenate addition to the A-kinase assay did not reverse the inhibition of histone labeling by vesicle

protein. Phosphoprotein phosphatase is a normal enzyme activity in any tissue using protein kinases for intracellular signalling. Evidence of heat lability, and partial reversal of inhibition of histone phosphorylation when KF was added to the system containing vesicle protein, suggested that suppression of phosphoprotein phosphatase may be necessary to produce persistent phosphorylation, and activation of Cl conductance.

Application of the conditions developed for producing a phosphoprotein product in pig jejunal brush border vesicles did not lead to any changes in the Cl conductance measured in vesicles prepared in HEPES-TMA buffers. It was not clear why these vesicles should have been refractory to activation attempts. The "success" of activation attempts in vesicles prepared in I-A buffer could have resulted from more favorable conditions for phosphoprotein accumulation within the vesicles, but it also seems possible that conductance activation may work best in vesicles with basal conductance activity. If the level of conductance obtained after activation were close to the maximum to be expected in the system then it would not be surprizing that HEPES-TMA vesicles, which start with a substantial conductance activity, did not increase conductance under activation conditions.

Changes in short-circuit current (I) on induction of intestinal secretion usually lie in the range of 30 to 50 μA cm^{-2} of intestinal mucosa mounted in Ussing chambers [5, 8]. This change is assumed to consist largely of Cl efflux across the apical enterocyte membrane via Cl conductance channels. Giraldez et al. have recently used microelectrode techniques to calculate Cl flux rates of 1×10^{-9} mol cm^{-2} sec^{-1} in *Necturus* enterocytes [10]. This flux rate corresponds to a value of 97 μA cm^{-2} for I_{Cl}. The brush border vesicles used in this study had initial rates of Cl uptake which would produce saturation at 45 sec with 10 mM Cl ion. Spherical vesicles with a diameter of 200 nm would have a volume of 4×10^{-18} liter, and at saturation with 10 mM Cl ion would contain 4×10^{-20} mol of Cl ion. The average vesicle surface area would be 1.2×10^{-9} cm^2. The flux rate across the activated vesicles becomes 7.3×10^{-13} mol cm^{-2} sec^{-1}. This is equivalent to 7.1×10^{-8} A cm^{-2} sec^{-1} for the surface area of the membrane vesicles.

A correction factor of 10 for the villi, and 20 for the microvilli has been suggested to relate mucosal surface area to the area of cytoplasmic membrane [1, 24]. Multiplying the current density for Cl transport in vesicles by these correction factors for membrane area gives an I_{Cl} value of 14 μA cm^{-2}. These calculations suggest that rates of Cl transport into membrane vesicles with activated conductance may differ by a factor of two or three from measurements carried out across intact tissues. These small differences could arise entirely from differences in Cl concentration gradients between whole cell and vesicle systems. Membrane potentials, which are difficult to measure in vesicles or to control completely, could also be involved with the lower rate of Cl transport observed in the vesicle system. On the basis of these calculations we speculate that the Cl conductance channels measured after in vitro activation could represent the channels that are activated in vivo in response to intestinal secretogogues.

Activation increases apical membrane permeability to Cl some 15-fold in *Necturus* enterocytes [10], while it is only doubled in membrane vesicles. This discrepancy probably arises from differences in the Cl permeability in the basal state. Because vesicles are prepared by mechanical disruption of apical membranes they do not seal tightly enough to regain the impermeability of the native membranes to Cl ion. This can be seen in the Cl transport rates measured in short intervals after membrane disruption in Table 2. When the activation response in vesicles is compared to the relatively high basal Cl permeability it appears to be a small response, but would be much larger on a relative basis if the unactivated vesicles could be resealed as tightly as the plasma membranes of enterocytes.

If the activation response obtained in vesicles buffered in I-A represents the normal extent of Cl channel activation which is observed in vivo, then vesicles prepared in HEPES-TMA buffering have a somewhat lower Cl transport capacity, as well as being refractory to activation. The difference in buffer ions could alter membrane potential to reduce the rates of Cl uptake, but the nature of the resistance to activation is not clear. Speculation could range from buffer incompatability with production or maintenance of phosphoprotein, to a buffer-induced opening of Cl conductance channels which cannot be altered further by normal control mechanisms.

This demonstration of conditions for in vitro activation of Cl conductance raises the possibility of an enzymatic assay for the defective conductance activation system in cystic fibrosis. The resistance of vesicles buffered in HEPES-TMA to conductance activation raises the possibility of an in vitro model for manipulating the protein components required for conductance activation. Pursuit of these ideas may provide new information about the molecular nature of the cystic fibrosis syndrome.

This research was supported by the Medical Research Council of Canada, and the Saskatchewan Agriculture Development Fund. Technical support was ably provided by Eva Yonge.

References

1. Brown, A.L., Jr. 1962. Microvilli of the human jejunal cell. *J. Cell. Biol.* **12:**623–627
2. Dommelen, F.S. van, Jonge, H.R. de 1984. Cyclic-GMP- and cyclic-AMP-induced intestinal ion secretion: Analysis at the level of brush border membrane vesicles. *Adv. Cyclic Nucleotide Protein Phosphorylation Res.* **17:**303–313
3. Donowitz, M., Emmer, E., McCullen, J., Reinlib, L., Cohen, M.E., Rood, R.P., Madara, J., Sharp, G.W.G., Murer, H., Malmstrom, K. 1987. Freeze thaw and high voltage discharge allow macromolecule uptake into ileal brush border vesicles. *Am. J. Physiol.* **252:**G723–G735
4. Dubinski, W.P., Monti, L.B. 1986. Solubilization and reconstitution of a chloride transporter from tracheal apical membrane. *Am. J. Physiol.* **251:**C713–C720
5. Field, M., Fromm, D., Wallace, C.K., Greenough, W.B., III. 1969. Stimulation of active chloride secretion by cholera toxin. *J. Clin. Invest.* **48:**24*a*
6. Forsyth, G.W., Gabriel, S.G., 1988. Chloride uptake by pig jejunal brush border membrane vesicles: Absence of NaCl cotransport and evidence for chloride conductance. *J. Physiol. (London)* **402:**555–564
7. Frizzell, R.A., 1987. Cystic fibrosis: A disease of ion channels. *Trends Neuro. Sci.* **10:**190–193
8. Frizzell, R.A., Field, M., Schultz, S.G. 1979. Sodium-coupled chloride transport by epithelial tissues. *Am. J. Physiol.* **236:**F1–F8
9. Frizzell, R.A., Halm, D.R., Rechkemmer, G., Shoemaker, R.L. 1986. Chloride channel regulation in secretory epithelia. *Fed. Proc.* **45:**2727–2731
10. Giraldez, F., Sepulveda, F.V., Sheppard, D.N. 1988. A chloride conductance activated by cyclic AMP in the apical membrane of *Necturus* enterocytes. *J. Physiol. (London)* **395:**597–623
11. Goris, J., Pijnenborg-Vercruysse, L., Merlevede, W. 1972. Effect of fluoride on liver phosphorylase phosphatase. *Biochim. Biophys. Acta* **268:**158–165
12. Hirano, K., Illzumi, Y., Mori, Y., Toyoshi, K., Sugiura, M., Ilno, S. 1985. Role of alkaline phosphatase in phosphate uptake into brush border membrane vesicles from human intestinal mucosa. *J. Biochem.* **97:**1461–1466
13. Jonge, H.R. de 1976. Cyclic nucleotide-dependent phosphorylation of intestinal epithelium proteins. *Nature (London)* **262:**590–592
14. Knicklebein, R., Aronson, P.S., Schron, C.M., Seifter, J., Dobbins, J.W. 1985. Sodium and chloride transport across rabbit ileal brush border: II. Evidence for $Cl\text{-}HCO_3$ exchange and mechanism of coupling. *Am. J. Physiol.* **249:**G236–G245
15. Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227:**680–685
16. Liedtke, C.M., Hopfer, U. 1982. Mechanism of Cl^- translocation across the small intestinal brush-border membrane: II. Demonstration of Cl^-OH^- exchange and Cl^- conductance. *Am. J. Physiol.* **242:**G272–280
17. Lucid, A.C., Cox, A.C. 1972. The effect of cholera toxin on the phosphorylation of protein in epithelial cells and their brush borders. *Biochem. Biophys. Res. Commun.* **49:**1183–1186
18. Maenz, D.D., Forsyth, G.W. 1982. Ricinoleate and deoxycholate are calcium ionophores in jejunal brush border vesicles. *J. Membrane Biol.* **70:**125–133
19. Moe, A.J., Jackson, M.J. 1987. Isolation and characterization of brush border membrane vesicles from pig small intestine. *Comp. Biochem. Physiol.* **88A:**511–517
20. Roskoski, R. 1983. Assay of cyclic-AMP dependent protein kinase. *Methods Enzymol.* **99:**3–7
21. Scalera, V., Biber, J., Murer, H. 1983. The effect of cyclic nucleotides and cholera toxin on in vivo and in vitro phosphorylation of small intestinal brush border membranes. *Biochim. Biophys. Acta* **730:**321–326
22. Sharp, G.W.G., Cohen, M.E., Gudewich, R., Taylor, L., Button, D., Albrewczynski, D., Donowitz, M. 1984. Protein phosphorylation and the control of intestinal electrolyte transport. *In:* Mechanisms of Intestinal Electrolyte Transport and Regulation by Calcium. M. Donowitz and G.W.G. Sharp, editors. pp. 242–261. Alan R. Liss, New York
23. Taylor, L., Guerina, V.J., Donowitz, M., Cohen, M., Sharp, G.W.G. 1981. Calcium and calmodulin-dependent phosphorylation in rabbit ileum. *FEBS Lett.* **131:**322–324
24. Verzar, F., McDougall, E.J. 1936. Absorption from the intestine. Logmans, Green, London

Received 10 May 1988; revised 26 August 1988