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Rapid Regulation of Electrolyte Absorption in Sweat Duct

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Abstract. Even though the same Cl channel (CFTR) is common to certain fluid transport functions that are oppositely directed, i.e., secretion and absorption, only fluid secretion has clearly been shown to be acutely regulated. It is now clear that fluid secretion activated by β-adrenergic stimulation is controlled by cAMP-mediated opening and closing of CFTR-Cl channels. Since the conductance of the human sweat duct is almost wholly due to CFTR-Cl conductance (CFTR-G_{Cl}), we sought to determine whether salt absorption via CFTR-Cl channels could also be subject to acute regulation in this purely absorptive epithelium. After α-toxin permeabilization, we found that addition of cAMP resulted in a large increase in Cl diffusion potentials across the apical membrane and a more than twofold increase in the average membrane conductance. Since the cAMP effects were dependent on Cl alone, not on Na, and since apical Cl conductance appears to be almost exclusively comprised of CFTR-G_{Cl}, we surmise that this form of electrolyte absorption like secretion is also subject to acute control through CFTR-G_{Cl}. Acute regulation of absorption involves both activation by phosphorylation (PKA) and inactivation by dephosphorylation (unknown endogenous phosphatase) of CFTR. Phosphorylation of CFTR was shown by the facts that CFTR-G_{Cl} could be activated by cAMP and inhibited by the kinase antagonist staurosporine, or by removal of either substrate ATP or Mg^{2+} cofactor. Inactivation of CFTR-G_{Cl} by endogenous phosphatase(s) was indicated by a spontaneous but reversible loss of CFTR-G_{Cl} upon removal of cAMP. Such loss of CFTR-G_{Cl} activity could be prevented either by application of phosphatase inhibitors or by using phosphatase-resistant ATP-γ-S as substrate to phosphorylate CFTR. We surmise that absorptive function is subject to rapid regulation which can be switched "on" and "off" acutely by a control system that is common to both absorptive and secretory processes and that this control is crucial to switching between conductive and nonconductive transport mechanisms during salt absorption.

Key words: Sweat duct — Acute regulation — Absorption — cAMP — Phosphatases — CFTR

Introduction

Higher animals closely regulate the volume and ionic composition of body fluids by exerting tight control over absorption and secretion of electrolytes into and out of body compartments via epithelial cells. Cl transport is an intrinsic component of both of these reversed transport functions [20, 35]. It is well established that fluid secretion by epithelial cells is acutely regulated by intracellular messenger cAMP [1, 3, 4, 20, 35] which rapidly activates apical membrane Cl channels. In contrast, electrolyte absorption generally has been regarded as a constitutively active process, modulated relatively slowly by humoral agents e.g., aldosterone and arginine vasopressin which require from several minutes to hours or even days to exert maximal effect [10, 29].

However, the defect in Cl conductance expressed in the genetic disease cystic fibrosis (CF; [18, 35]) along with recent direct evidence that the CF gene product is indeed a Cl channel [6, 28, 35] show that the same ion channel is crucial to absorptive as well as secretory processes. The CFTR-Cl channel is opened by cAMP-dependent protein kinase (PKA) phosphorylation in native secretory cells and transfected nonsecretory cells induced to express CFTR-Cl channels [5, 6, 28, 32, 35]. Even though the CFTR protein is abundantly expressed in the apical membranes of human reabsorptive sweat

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duct [13, 16, 18, 21, 26], there has been no direct evidence that electrolyte absorption involving CFTR-G_{Cl} in this purely absorptive epithelium can be acutely (moment to moment) regulated.

We used the human reabsorptive sweat duct to address the issue of whether absorption is acutely regulated because it is an almost ideal model system for absorption. That is, (i) there is only one cell type in the duct epithelium, (ii) the principal function is NaCl absorption, (iii) Cl conductance and CFTR expression are very high in the duct, and (iv) Cl conductance as shown by the sweat anomaly in CF can limit electrolyte absorption very effectively [18, 20]. By selectively permeabilizing the basolateral membrane of the sweat duct to medium-sized molecules (~5,000 MW), we show clear evidence of acute regulation of absorptive function.

Materials and Methods

Tissue Acquisition

Sweat glands were obtained as previously described [18, 24] from adult male volunteers without medical history who gave informed consent. Briefly, 3 mm diameter full thickness biopsies were taken over the scapula and stored overnight in Ringer solution at room temperature. Individual sweat glands were isolated from the skin in chilled Ringer solution by dissection with fine-tipped tweezers visualized at a magnification of 80×. The isolated glands were transferred to a dissection cuvette with Ringer solution cooled to 4°C where the segments of reabsorptive duct (~1 mm in length) were separated from the secretory coil of the sweat gland. The sweat duct was transferred to a perfusion chamber containing Ringer solution at 35 ± 2°C.

Selective Permeabilization of the Basolateral Membrane

The basolateral membrane of the sweat duct was selectively permeabilized with 1,000 U/ml of the pore-forming agent α-toxin derived from *Staphylococcus aureus* in cytoplasmic Ringer solution containing 140 mM KGlu (potassium gluconate) and 5 mM ATP which was applied to the basolateral surface of the microperfused sweat duct for 15 to 30 min. α-Toxin forms pores which are permanent to larger molecules (~5,000 MW), so that the intracellular messenger (cAMP) and the substrate for PKA phosphorylation (ATP) could be freely manipulated [4, 8, 17, 22].

Electrical Measurements

Electrical Setup

After cannulating the lumen of the sweat duct with a double lumen cannula made from theta glass (1.5 mm diameter, Clark Electromedical Instruments, Reading, UK), a constant current pulse of 50–100 nA for a duration of 0.5 sec was injected through one barrel of the cannulating pipette containing NaCl Ringer solution. The other barrel of the cannulating pipette served as an electrode for measuring transepithelial potential (V_t) with respect to the contraluminal bath and as a cannula for perfusing the lumen of the duct with selected solutions. V_t was monitored continuously using one channel of a WPI-700 dual electrometer referenced to the contraluminal bath. Transepithelial conductance (G_t) was measured as described earlier, applying the cable equation to the amplitude of transepithelial voltage deflections in response to the constant current pulse [18, 26].

Apical Cl Conductance (G_{Cl})

Cl diffusion potentials (V_{Cl}) and G_{Cl} were monitored to indicate the level of activation of G_{Cl} following α-toxin permeabilization of the basolateral membrane. In this preparation, the epithelium is simplified to a single (apical) membrane with parallel Na and Cl conductances [18, 25, 26]. Application of amiloride further simplified the system into a predominantly Cl-selective membrane. The composition of Ringer solution in bath and lumen was designed to set up a single ion gradient, i.e., exclusively for Cl [140 mM K gluconate (bath)/150 mM NaCl (lumen)]. Under these conditions, V_t and G_t can be essentially regarded as V_{Cl} and G_{Cl}, respectively. In a few experiments, the Cl gradient was reversed [140 mM KCl (bath)/150 NaCl + Amiloride (lumen)] to show that cAMP-induced V_t follows the direction of the Cl gradient.

Solutions

The luminal perfusion solutions contained (in mM): NaCl (150), K (5.0), PO_4 (3.5), $MgSO_4$ (1.2), Ca (1.0), and amiloride (0.01), pH 7.4. The cytoplasmic/bath solution contained K (145), gluconate (140), PO_4 (3.5), $MgSO_4$ (1.2), and 260 μM Ca buffered with EGTA (2.0) to 80 nM free Ca, pH 6.8. Mg^{2+}-free Ringer solution was prepared by complete replacement of Mg^{2+} with EDTA (2.0–5.0). cAMP (0.1) and ATP (5.0) or ATP-γ-S (5.0) were added as required. Protein kinase inhibitor staurosporine (0.001), phosphatase inhibitors: fluoride (5.0), vanadate (0.001) and okadaic acid (0.001) were added to the cytoplasmic bath either in the presence or absence of cAMP as required.

Data Analysis

The data are presented as the mean ± SE (where n = number of ducts from a minimum of four human subjects). Statistical significance was determined on the basis of Student's t-test for paired samples. A P value of <0.05 was taken to be significantly different. Whenever appropriate, representative traces are presented to reflect at least five experiments from as many subjects.

Results

Permeabilization

The primary objective behind the selective permeabilization of the basolateral membrane of the sweat duct is: (i) to effectively control the cytoplasmic composition of medium-sized molecules (such as cAMP and ATP) without affecting the functional integrity of the apical membrane or the cytosolic macromolecular machinery (such as kinases and phosphatases) essential for regulation of absorptive function; (ii) to isolate CFTR-G_{Cl} function in the apical membrane, and (iii) to develop a model system in which the basal level of CFTR-G_{Cl} in the apical membrane is initially low in order to demonstrate subsequent activation. Application of 1,000 U/ml

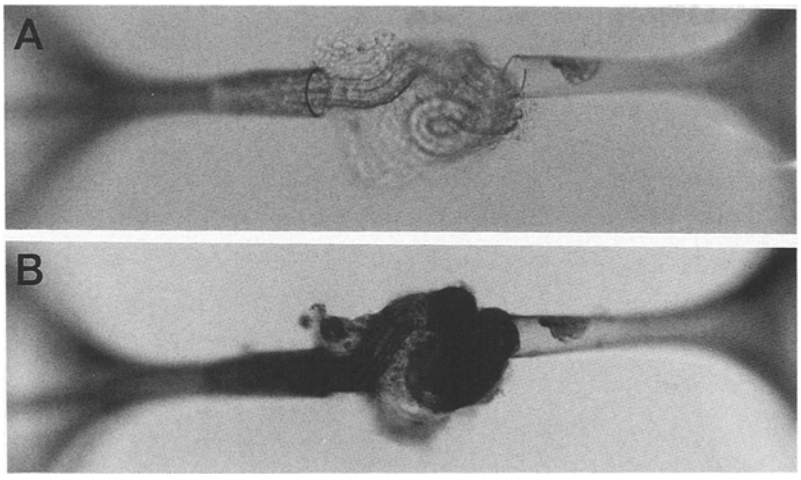

Fig. 1. Trypan blue uptake following asymmetric permeabilization of a sweat duct with α-toxin. (*A*) Prior to permeabilization, sweat duct cells excluded vital dye trypan blue as evidenced by virtual lack of staining. (*B*) α-Toxin treatment of the basolateral membrane resulted in the intense staining of cells by trypan blue, indicating permeabilization.

of α-toxin permeabilized the basolateral membrane of the sweat duct as evidenced by morphological staining and electrophysiological changes.

Morphological Staining

Intact, nonpermeabilized sweat ducts effectively excluded trypan blue as no staining of the duct was observed even after exposing the intact tissue to the stain for several minutes. In contrast, sweat ducts exposed to α-toxin for about 15 min stained intensely, indicating successful permeabilization (Fig. 1).

Electrophysiological Indications

α-Toxin induced a biphasic response in G_t. We observed an initial increase in conductance within the first 2 min, indicating initiation of pore formation. This was followed by a subsequent decrease in conductance with a concomitant abolition of V_t, indicating a decrease in G_{Cl} likely due to the loss of cAMP and ATP from the cell via α-toxin pores (Fig. 2). As shown in Fig. 3, cAMP did not have any detectable electrophysiological effect on the duct prior to the application of α-toxin. However, the same duct, after α-toxin permeabilization, responded to cAMP and ATP with large increases in G_t and V_t. These results showed that α-toxin treatment creates pores in the basolateral membrane without affecting the functional integrity of the apical membrane (Fig. 3).

Luminal α-Toxin

Direct application of α-toxin to the apical membrane from the lumen neither permeabilized nor affected the functional integrity of this membrane as shown by the absence of electrophysiological changes seen in ducts

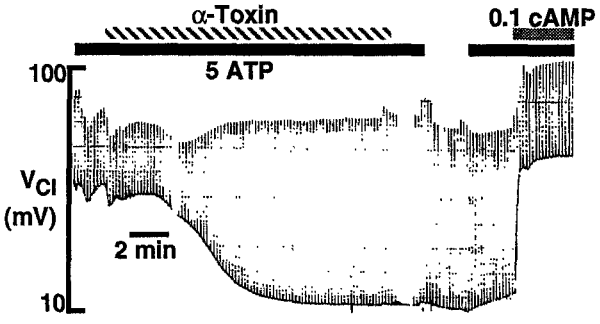

Fig. 2. Effect of α-toxin on epithelial potential and conductance. After permeabilization of the basolateral membrane, the transepithelial potential is a function of the ion selectivity and the ionic gradients across the apical membrane. Inhibition of Na conductance with amiloride turns the apical membrane into an exclusively Cl-selective membrane. An anion diffusion gradient exclusively for Cl was set up by complete Cl substitution in the cytoplasmic compartment. Under these conditions, changes in transepithelial potential (due to Cl gradient) and conductance (in the absence of any other parallel conductance) essentially reflect Cl diffusion potential (V_{Cl}) and conductance (G_{Cl}), respectively. Note that permeabilization of the basolateral membrane depletes the required intracellular crystalloides; i.e., cAMP and ATP required for activation of CFTR-G_{Cl} such that V_{Cl} and G_{Cl} both fall markedly and V_{Cl} approaches the measured junction potential (*ca.* +11 mV). Addition of cAMP (0.1 mM) and ATP (5 mM) to the cytosolic perfusate (bath) activates CFTR-G_{Cl} as indicated by large increases in V_{Cl} and G_{Cl}. Upward voltage deflections are in response to 50 nA/500 msec constant current pulses (lumen to bath). The magnitude of voltage deflections are related inversely to G_{Cl}.

perfused luminally with the same levels of α-toxin (*results not shown*).

EFFECT OF cAMP

General

Application of cAMP in the presence of ATP almost instantaneously increased both apical membrane potential

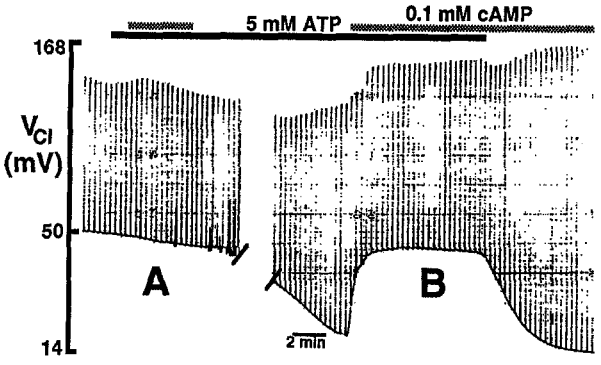

Fig. 3. Effect of physiological cAMP on the electrical properties of sweat duct before (*A*) and after (*B*) α-toxin permeabilization. Note that cAMP (0.1 mM) has no effect on the electrical properties of the sweat duct before permeabilization. However, in the same duct, cAMP significantly increased the Cl diffusion potential and Cl conductance after permeabilization indicating that these compounds which were otherwise impermeable were able to permeate through α-toxin-induced pores.

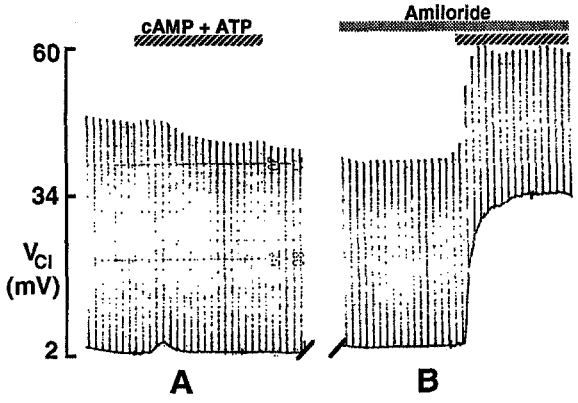

Fig. 4. Effect of cAMP in the absence (*A*) and presence (*B*) of Cl in the medium. Normally, Cl is present either in the lumen or cytoplasmic bath. After complete removal of Cl from both sides of the epithelium (*A*), cAMP (0.1 mM) had no effect on the electrical properties of duct even though Na conductance was intact (no amiloride in the lumen). In contrast, cAMP had significant effect on the potential and conductance after introduction of Cl into the lumen even though Na conductance was inhibited by amiloride (*B*). These results indicated that cAMP exclusively activated a G_{Cl} without affecting any other conductance in the apical membrane.

and conductance (Figs. 2, 3). In contrast to nonpermeabilized ducts (where the effect of cAMP was not detected, Fig. 3) cAMP consistently increased conductance and potential in virtually all permeabilized ducts studied.

Ion Specific Conductance

The following experiments were performed to determine which ion-specific conductance is affected by cAMP.

Cl Removal. Complete Cl substitution from the perfusate solutions, both in the cytoplasmic bath and lumen by the impermeant anion gluconate (Glu) completely abolished the electrical effects of cAMP (Fig. 4). In these experiments, we left Na conductance intact (i.e., with no amiloride in the lumen, significant sodium conductance resides in the apical membrane of the α-toxin permeabilized duct) while Cl was completely removed from both sides of the apical membrane (150 Na Glu in lumen/140 K Glu in bath). Under these conditions, cAMP had no effect on either the potential or conductance. However, in the same ducts, cAMP significantly increased both apical membrane conductance and potential following the reintroduction of Cl into the medium even in the absence of Na conductance (amiloride in the lumen, Fig. 4).

Reversed Cl Gradient. Usually the Cl gradient was set up in the direction of lumen to cytoplasmic bath (150 mM Cl in lumen/140 Glu in cytoplasmic bath). Under these conditions, addition of cAMP depolarized the apical membrane potential with a concomitant increase in conductance (Figs. 2, 3 and 4). However, when the Cl gradient was reversed such that the gradient was established from cytoplasmic bath to the lumen (140 mM Cl in bath/150 mM Glu in lumen), the shift in the apical membrane potential caused by cAMP was also reversed i.e., the potential hyperpolarized (Fig. 5). These results establish that cAMP activates G_{Cl} exclusively which is

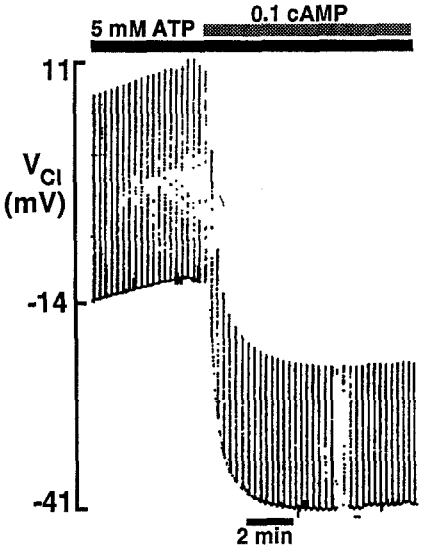

Fig. 5. Effect of reversed Cl gradient on cAMP-activated potential and conductance. Normally, Cl gradient was set up from lumen to bath (Fig. 2 and 3). Under these conditions, cAMP (0.1 mM) depolarized the potential in the expected direction for Cl gradient. However, when the Cl gradient was reversed from cytoplasmic bath to lumen (as in the present experiment), cAMP hyperpolarized following the direction of reversed Cl gradient suggesting that cAMP in fact activates a G_{Cl}.

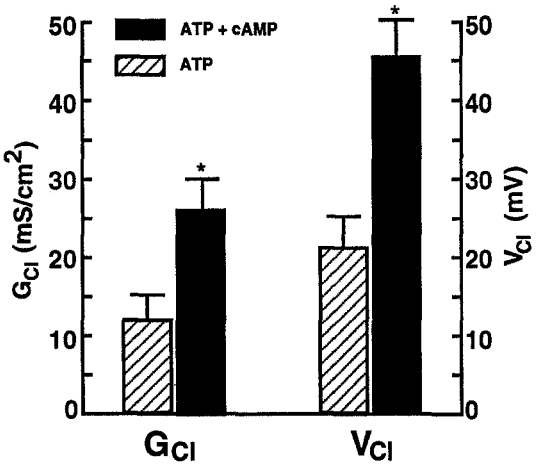

Fig. 6. Effect of cAMP on the G_{Cl} and V_{Cl}. Note that in the presence of ATP (5 mM), cAMP (0.1 mM) significantly increased G_{Cl} and V_{Cl} (in the presence of 140 mM KGlu, bath/150 mM NaCl, lumen) of every preparation studied (n = number of ducts = 17, from human subjects. *Significantly different from their respective controls, $P < 0.05$.

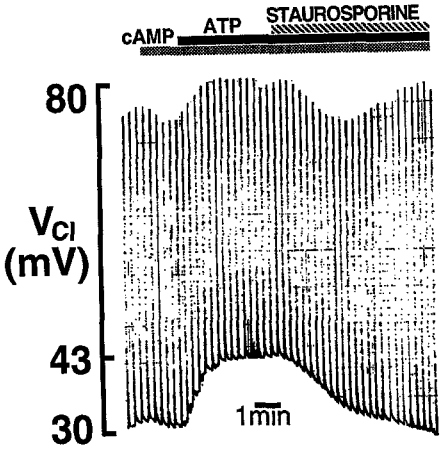

Fig. 7. Effect of ATP (PKA substrate) and staurosporine (PKA inhibitor) on cAMP-activated G_{Cl}: In the absence of ATP (5 mM), cAMP (0.1 mM) failed to activate G_{Cl} as indicated by a lack of effect on G_{Cl} and V_{Cl}. In contrast, addition of ATP in the presence of cAMP increased G_{Cl} and V_{Cl} which was reversed by staurosporine (10^{-6} M) showing that phosphorylation is required for the activation of G_{Cl}. Further, complete reversal of cAMP effect by staurosporine also indicates that cAMP almost exclusively acts via kinase phosphorylation.

almost entirely, if not exclusively, comprised of CFTR-Cl conductance [16, 26]. Therefore, we refer to the cAMP-activated apical membrane potential as the Cl diffusion potential (V_{Cl}) and its conductance as the Cl conductance (G_{Cl} or CFTR-G_{Cl} as appropriate).

Quantitative Effects of cAMP

Cl Diffusion Potentials. cAMP increased V_{Cl} by a minimum of 5 mV to a maximum of 49 mV with a mean value of 24 ± 2.4 mV (n = 17 = number of ducts from 11 human subjects, Fig. 6). The increase in the V_{Cl} began almost instantaneously with the addition of cAMP, and peaked in less than one minute in most cases (Figs. 2, 3, 4 and 12).

Cl Conductance. The cAMP-induced increase in V_{Cl} was correlated with a significant increase in G_{Cl}. Application of cAMP increased G_{Cl} by a minimum of 3 mS/cm^2 to a maximum of 51 mS/cm^2 with a mean value of 13.2 ± 2.7 mS/cm^2 (n = 17, from 11 human subjects, Fig. 6).

Evidence of Phosphorylation

Effect of Staurosporine

cAMP had no effect on CFTR-G_{Cl} after inhibition of protein kinases with nonspecific kinase inhibitor, staurosporine (10^{-6} M). Addition of staurosporine resulted in an almost complete reversal of previously activated CFTR-G_{Cl} with cAMP in the continued presence of ATP (Fig. 7).

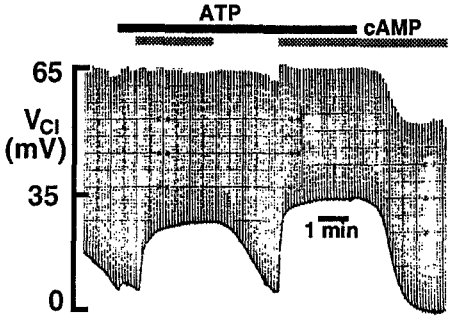

Fig. 8. Effect of either ATP, or cAMP or both on G_{Cl}. Note that G_{Cl} could not be sustained by either ATP or cAMP alone but both are required for activation of G_{Cl}. Also note that removal of either cAMP (0.1 mM) or ATP (5 mM) returned G_{Cl} and V_{Cl} to baseline values.

Effect of ATP

ATP was absolutely required for cAMP activation of CFTR-G_{Cl} (Fig. 8, 9). Addition of cAMP in the absence of ATP had no effect on CFTR-G_{Cl} (Fig. 8). Further, removal of ATP after activation of CFTR-G_{Cl} resulted in a rapid inactivation of CFTR-G_{Cl} as indicated by an almost instantaneous decline in V_{Cl} and G_{Cl} to baseline values (Fig. 8).

Effect of Mg^{2+}

In the absence of Mg^{2+}, cAMP was ineffective in the activation of CFTR-G_{Cl} as indicated by a small, transient

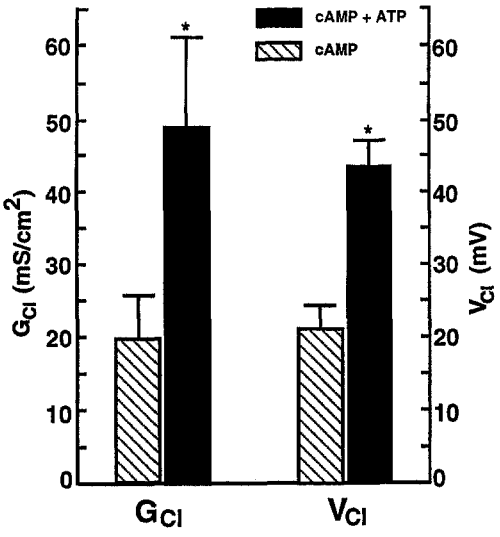

Fig. 9. Effect of ATP on the G_{Cl} and V_{Cl}. Notice that ATP (5 mM) is required for cAMP activation of G_{Cl}. cAMP (0.1 mM) activation of G_{Cl} does not occur without ATP as shown in Figs. 8 an 10. Similarly, previously activated G_{Cl} is acutely inhibited following the removal of ATP. *Significantly different from their respective controls, $P < 0.05$.

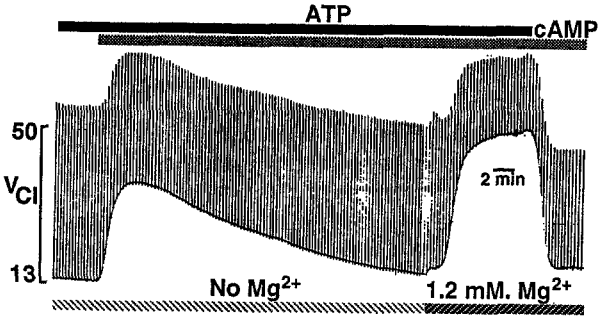

Fig. 10. Effect of Mg^{2+} on cAMP activation of Cl conductance. A representative example of the effect of Mg^{2+} on the cAMP (0.1 mM)-activated G_{Cl} and V_{Cl}. In the absence of Mg^{2+}, cAMP had only a small transient effect on G_{Cl}. Addition of Mg^{2+} fully activated G_{Cl}.

effect on V_{Cl} and G_{Cl} (Figs. 10 and 11). However, both were fully activated almost immediately upon restoration of normal levels of Mg^{2+} (1.2 mM).

Evidence of Dephosphorylation

Inactivation Following cAMP Removal

cAMP-activated CFTR-G_{Cl} returned rapidly to baseline almost immediately after washout of cAMP even in the continued presence of ATP. Rundown of CFTR-G_{Cl} following withdrawal of cAMP was observed in every

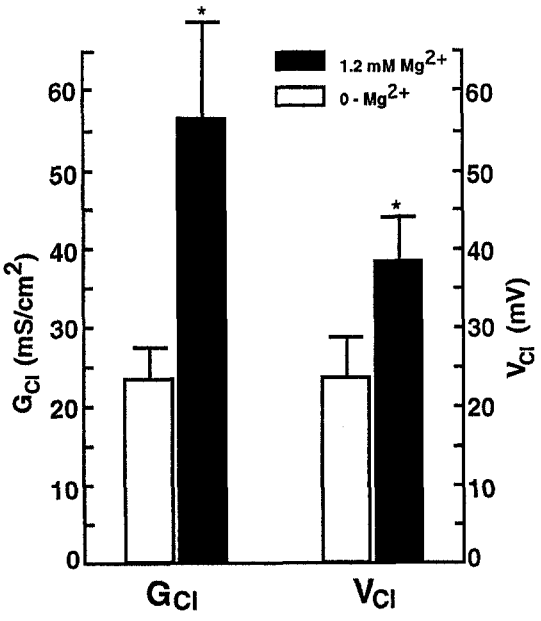

Fig. 11. Effect of Mg^{2+} on cAMP activation of G_{Cl}. Note that in the absence of Mg^{2+}, G_{Cl} and V_{Cl} are significantly lower than in the presence of Mg^{2+}. The effect of Mg^{2+} on cAMP (0.1 mM) activation of G_{Cl} was tested either by the addition of cAMP in the complete absence of Mg^{2+} as in Fig. 10 or by removing Mg^{2+} after activation of G_{Cl} with cAMP in regular Mg^{2+}-containing medium. In either case cAMP failed to induce sustained activation of G_{Cl}, showing that Mg^{2+} is essential for cAMP activation of G_{Cl}. *Significantly different from their respective controls, $P < 0.05$.

preparation even when a physiological concentration of ATP was present (Figs. 3 and 8).

Phosphatase Antagonists

We had examined the effect of several protein phosphatase (PrP) antagonists [30] such as fluoride (PrP-1, PrP-2A, and PrP-2C), vanadate (PrP-1, PrP-2A and PrP-2B inhibitor) and okadaic acid (PrP-1 and PrP-2A inhibitor) on the electrical properties of permeabilized sweat duct before and after activation of CFTR-G_{Cl}. Phosphatase inhibitors had no detectable effect on the electrical properties of sweat duct in the absence of cAMP. Likewise, the cocktail of phosphatase inhibitors (vanadate, fluoride and okadaic acid) had little additional effect on cAMP-activated G_{Cl}. However, phosphatase inhibitor cocktail prevented the spontaneous inactivation of G_{Cl} that normally occurred after removing cAMP (Fig. 12). After inhibition of phosphatases, the G_{Cl} remained activated as long as ATP was present in the medium. The effect of phosphatase inhibitor cocktail lasted several minutes following washout of the cocktail as indicated by the fact that ATP alone could activate G_{Cl} even in the absence of cAMP (Fig. 12)

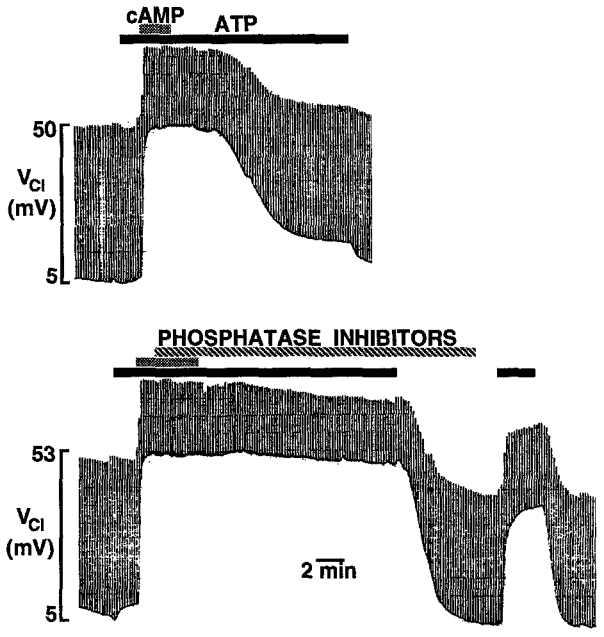

Fig. 12. Effect of phosphatase inhibition cocktail on the inactivation of G_{Cl} following removal of cAMP. Normally, removal of cAMP (0.1 mM) results in the rapid inactivation of G_{Cl} (upper trace). However, in the presence of the phosphatase inhibitor cocktail (okadaic acid, fluoride and vanadate), G_{Cl} remains activated after cAMP washout as long as ATP (5 mM) was present. After inhibition of phosphatase(s), ATP alone could reactivate G_{Cl}, indicating that CFTR remained phosphorylated after treatment with phosphatase antagonists (lower trace).

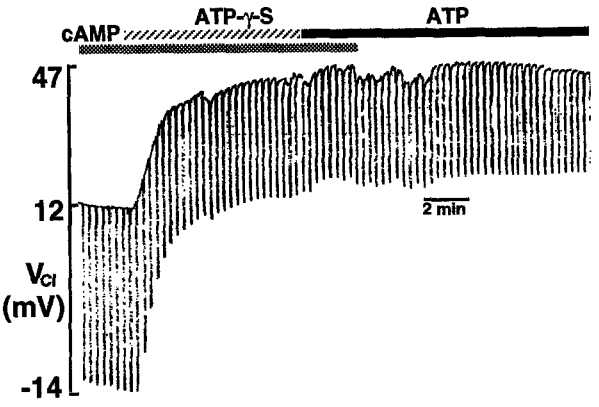

Fig. 13. Effect of ATP-γ-S on the inactivation of G_{Cl} following removal of cAMP. Notice that G_{Cl} remains activated even after washout of cAMP (0.1 mM) when ATP-γ-S (5 mM) is used as substrate to phosphorylate CFTR which is in contrast to the rapid reversal of G_{Cl} when ATP was used as substrate (Fig. 8). These results indicated that rundown of G_{Cl} following the removal of cAMP is at least partly due to dephosphorylation of Cl channel proteins by endogenous phosphatase(s).

(once phosphorylated, CFTR-G_{Cl} can be activated by ATP alone; [35]).

ATP-γ-S as Substrate

Rundown of CFTR-G_{Cl} following removal of cAMP (in the continued presence of ATP) could be greatly retarded by using phosphatase-resistant ATP-γ-S as a substrate [2, 7, 22] for phosphorylating the CFTR protein (Fig. 13). In a separate set of experiments, we examined the ability of ATP alone (without cAMP) to activate CFTR-G_{Cl} after phosphorylating CFTR using either ATP or ATP-γ-S as substrate. We found that ATP alone could activate CFTR-G_{Cl} in the absence of cAMP in ducts whose CFTR was previously phosphorylated using ATP-γ-S, but not ATP, as substrate (Fig. 14).

Discussion

Is Absorption Regulated?

Almost all fluid secretory processes that have been characterized in a variety of epithelial cells (e.g., rectal glands, cornea, intestine, trachea and pancreatic ducts, etc.) are subject to acute regulation (moment to moment). In contrast, acute regulation (as opposed to long-term modulation) of electrolyte absorptive processes is not well documented. Even though there is some recent speculation of acute regulation of absorptive function [11], the underlying molecular and electrophysiological mechanisms are poorly understood. A clue for possible rapid regulation of absorptive function was provided by the fact that the same CFTR-Cl channel which is acutely regulated in secretion is also present in the apical membrane of the purely absorptive duct [13, 20, 27, 35].

Consequently, we first examined acute regulation of G_{Cl} in the intact microperfused sweat duct as a function of pharmacological agonists known to activate CFTR-Cl channels (such as chlorophenylthio-adenosine 3′-5′ cyclic monophosphate, forskolin, isoproterenol, 3-isobutyl-1-methylxanthine and PGE_2), but we did not find consistent evidence of acute regulation of absorptive function in this preparation [21, 26]. In those few ducts where cAMP-elevating agents elicited detectable electrophysiological responses (changes in electrical potentials and conductance), there was a small effect on the apical membrane, raising the question of whether acute regulation might occur at the critical step of electrolyte entry into the cell across the apical membrane. These initial studies also suggested that the G_{Cl} in sweat duct may have been maximally activated by endogenous cAMP so that the agents elevating this intracellular messenger had little effect [26]. In this study, we took advantage of a system simplified by permeabilizing the basolateral membrane to directly alter the cytoplasmic concentrations of cAMP and ATP. Here, we find good evidence that absorption in the duct, like many forms of

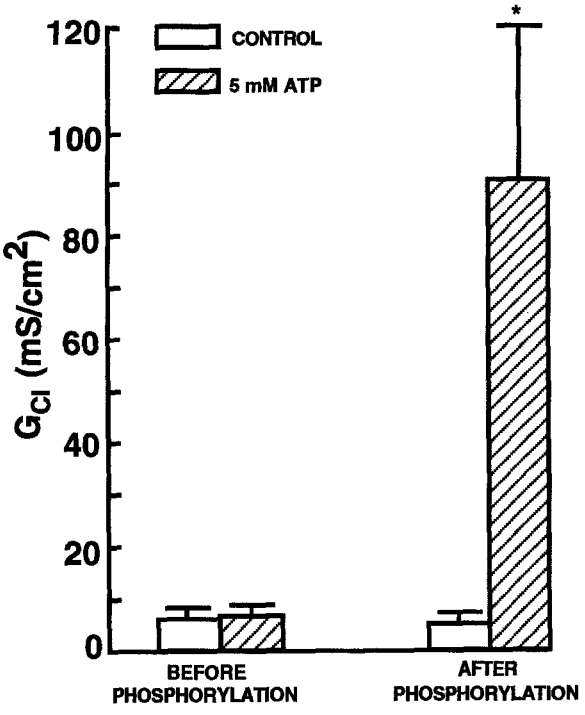

Fig. 14. Effect of phosphorylation of CFTR with either ATP or ATP-γ-S on the nucleotide regulation of CFTR-G_{Cl}. Notice that ATP (5 mM) could activate CFTR-G_{Cl} only after being phosphorylated with ATP-γ-S (5 mM) but not with regular ATP. These results also suggested that the CFTR phosphorylated with physiological ATP could be rapidly dephosphorylated by endogenous phosphatases.

secretion, can be acutely regulated by activating and deactivating a cAMP/ATP sensitive G_{Cl} in the apical membrane of the sweat duct.

In marked contrast to our observations on intact sweat duct [21, 26], we found that electrolyte absorption across the apical membrane of permeabilized sweat duct is, clearly, subject to acute regulation. Addition of cAMP in the presence of physiological concentration of ATP increased transepithelial potential and conductance in every preparation, and cAMP activation of G_{Cl} was rapid and completely reversible (Figs. 3 and 8). It is clear that the cAMP regulation of absorptive function is specifically through the effect on Cl conductance because, in the absence of Cl, cAMP had no effect on the electrical properties of the apical membrane of the sweat duct even when Na conductance was intact (Fig. 4) and because reversing the Cl gradient also reversed the polarity of the cAMP-induced potential shift (Figs. 2, 3, 4 and 5). These results show that cAMP specifically affects G_{Cl}, but not Na, K or any other conductance in the apical membrane. Accordingly, since Na absorption cannot occur without the absorption of an anion—in this tissue, Cl—the activity of G_{Cl} can function as an intrinsic regulatory component of the absorptive transport system.

How is G_{Cl} Turned On?

Since the G_{Cl} in the sweat duct is comprised almost exclusively of CFTR-G_{Cl} [13, 16, 18, 25], cAMP must activate CFTR-G_{Cl}. At least ten consensus sites for phosphorylation occur in CFTR and the molecule can be phosphorylated directly with PKA in vitro [6, 28, 35]. PKA-dependent phosphorylation of CFTR has also been demonstrated to be a mechanism for activating CFTR-Cl channels in secretory cells and in nonsecretory cells induced to express CFTR [5, 32, 35]. Similarly, we found that as in secretion the activation of CFTR-G_{Cl} in the sweat duct is apparently PKA phosphorylation dependent. Application of the protein kinase inhibitor staurosporine completely blocked cAMP activation of G_{Cl} (Fig. 7). In addition, removal of essential elements of the protein phosphorylation reaction such as ATP substrate and Mg^{2+} cofactor (Figs. 8, 9, 10 and 11) inhibited cAMP activation of G_{Cl}. We have shown that ATP is required for activation of G_{Cl}, most probably as an allosteric modulator ([22]; Figs. 8, 9 and 12), but ATP is also required for the phosphorylation of CFTR because G_{Cl} cannot be activated with ATP alone (without prior application of cAMP, Fig. 12). These results not only establish that cAMP effects are mediated by protein phosphorylation but also suggest that relatively higher concentrations of cAMP used in our experiments had no direct or nonspecific effects on these membrane functions. Taken with the fact that the kinase reaction is activated by cAMP, it seems clear that, as in secretion, PKA-dependent phosphorylation can determine the level of absorptive activity through rapid regulation of G_{Cl} as a function of cytosolic cAMP levels. But, for relevance, this regulatory system must also be capable of turning off absorptive activity physiologically.

How is G_{Cl} Turned Off?

Dephosphorylation by phosphatases is an integral component of systems regulated by phosphorylation [5, 7, 15, 30, 32]. Previously, a number of reports suggested that dephosphorylation inhibits CFTR-G_{Cl} expressed in NIH 3T3 fibroblasts and Chinese hamster ovary cells [5, 32]. However, that the intact native sweat duct CFTR-G_{Cl} appeared constitutively active [21, 26] raised the question as to the role of endogenous phosphatases in acute regulation of CFTR-G_{Cl} in the native absorptive membrane.

Closing down G_{Cl} and hence the absorptive process appears to be directly linked to dephosphorylation. In every duct, CFTR-G_{Cl} returned almost to junction potential levels when cAMP was removed (Figs. 3 and 8). Furthermore, this inactivation of G_{Cl} was prevented either by inhibiting phosphatase(s) or by using phos-

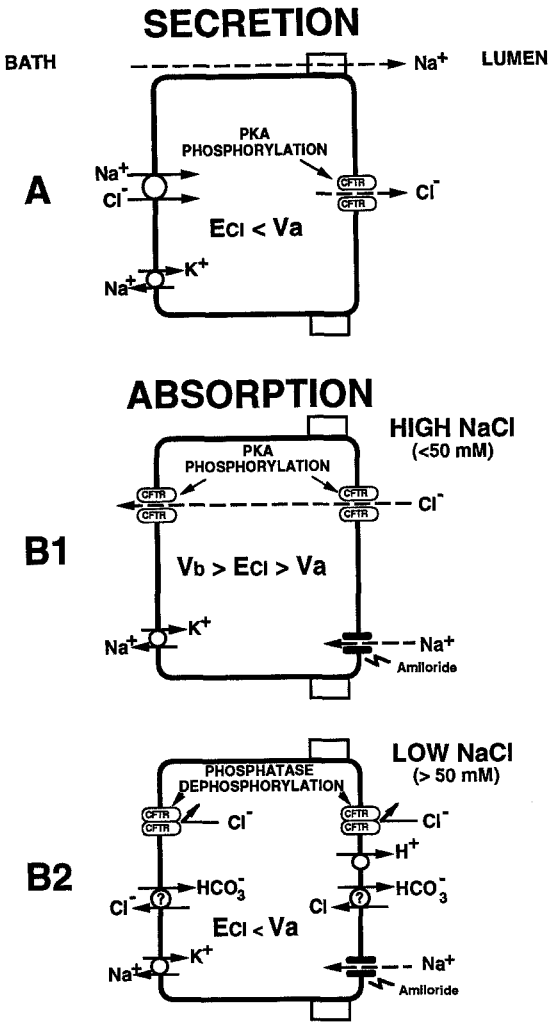

Fig. 15. Models of fluid secretion and absorption. Models *A* and *B* describe the mechanism by which activation of a common control system could acutely regulate secretion in secretory epithelium (e.g., shark rectal gland, tracheal and intestinal epithelium, [12, 31, 34]) or absorption (e.g., sweat duct) depending upon the direction of the driving force for Cl movement. Secretion (*A*)—the chemical gradient for sodium created by the Na/K pump powers a NaCl cotransporter in the basolateral membrane which raises $[Cl]_i$ "above equilibrium" across the apical membrane (i.e., $E_{Cl} < V_a$) [9, 34]. Opening of CFTR-Cl channels in the apical membrane leads to Cl secretion which increases luminal negativity, pulling Na into the lumen via the paracellular shunt. Absorption (*B;* in high luminal Cl concentration, *ca.* > 50 mM and *C* in low luminal Cl concentration, *ca.* < 50 mM)—Na ions enter the cell across the amiloride-sensitive Na channel down a gradient created by the Na/K pump in the basolateral membrane. Cl may enter the cell by either of two different mechanisms depending on the luminal Cl concentration (*B-1* and *B-2*). When the luminal salt concentration is high (*B-1*), Cl enters the cell from the lumen via phosphorylation-activated CFTR-Cl channels until the Cl equilibrium potential, $E_{Cl} \geq V_a$ [26]. The relatively higher basolateral membrane potential (V_b) provides a downhill driving force for Cl exit ($E_{Cl} < V_b$) into the basolateral compartment via phosphorylation-activated CFTR-Cl channels located in that membrane [26]. Therefore, in both secretion and absorption (at high luminal Cl concentration as in *B-1*) transport activity is limited by the rate at which Cl can cross the apical membrane (G_{Cl}) so that CFTR-Cl conductance is a common control point for rapid regulation of both processes. However, when the luminal Cl concentration drops to <50 mM (*B-2*), the driving force for Cl reverses such that if the CFTR-Cl channels remain open, Cl could leak back into the lumen instead of being absorbed. Under these conditions, dephosphorylation of CFTR would inhibit CFTR-Cl channels such that Cl could be absorbed through a carrier transporter, such as a Cl^-/HCO_3^- exchanger driven by a HCO_3^- chemical gradient (created by luminal acidification due to proton secretion, [19, 23]). Net Cl transport against its electrochemical gradient would then occur even when the luminal Cl concentration is very low [19, 23, 27].

phatase-resistant ATP-γ-S as a substrate for phosphorylation [2, 7, 22]. Once phosphatases were inhibited (Fig. 12) or CFTR was stably phosphorylated with ATP-γ-S (Fig. 13, 14), G_{Cl} remained activated even after removing cAMP. Under these conditions, ATP alone reversibly activated CFTR-G_{Cl} (Figs. 12, 13, 14). These results show that endogenous phosphatases are active in the sweat duct and that dephosphorylation of CFTR is an integral component of a rapid regulatory mechanism contributing to the acute deactivation of absorptive function in the native epithelial membrane.

Thus, it now seems certain that, as in secretory tissues, the sweat duct has all the essential elements required for acute regulation including the presence of (i) adrenergic nerve terminals [33]; (ii) β-adrenergic receptors on the cell membranes [21, 26]; (iii) an intracellular messenger cAMP, whose concentration is elevated in response to physiological stimulation [14]; (iv) key enzymes in the metabolism of cAMP such as adenylate cyclase and phosphodiesterase [14, 26] and finally (v) the kinases and phosphatase required for activation and inactivation of CFTR-Cl channels ([14], present results). In short, all the necessary cAMP regulatory components for acute control appear to be present endogenously in absorptive duct.

Physiological Significance

These observations present clear evidence that regardless of the differences in the physiologic function, CFTR-Cl channels in absorption are intrinsically related by the same cascade of physiological events as in β-adrenergically stimulated secretion; that is, an increase in the concentration of intracellular messenger, cAMP, activation of PKA, phosphorylation activation of CFTR-Cl channels, and dephosphorylation deactivation of CFTR-Cl channels in the apical membranes. Considering that salt moves across the apical membrane in completely opposite directions in absorption and secretion, it is striking that the same Cl channel, activated by the same regulatory system controls the activity

of electrolyte transport in each direction. Since CFTR-G_{Cl} is nonrectifying, the single determinant of Cl secretion or absorption across the apical membrane is the electrochemical driving force for Cl. We noted that (as mentioned earlier, [23, 26]) the intracellular Cl activity in sweat duct cells (with an isotonic luminal solution), is below electrochemical equilibrium levels across the apical membrane and above electrochemical equilibrium across the basolateral membrane. Under these conditions, Cl can be passively absorbed from luminal to serosal compartment upon cAMP activation of CFTR-Cl channels present in both membranes [26]. In contrast, the intracellular Cl activity in secretory epithelium is above equilibrium across the apical membrane due to the activity of the Cl cotransporter in the basolateral membrane such that activation of CFTR-G_{Cl} allows Cl secretion into the lumen (Figs. 15*A* and *B*) [9, 27, 31, 34].

In view of the present evidence of acute regulation of G_{Cl} in absorptive duct, we are intrigued with the question of why acute regulation of G_{Cl} is needed when a constitutively open G_{Cl} could accomplish salt absorption subject only to the availability of Cl in the lumen? That is, ductal salt absorption would seem to be regulated by glandular salt secretion. When secretion is inactivated, salt does not appear in the lumen and absorption cannot proceed. When secretion is active, the duct lumen is filled with salt solution and absorption proceeds. Simplistically, with a constitutively open Cl channel, absorptive activation would be the slave of secretory activity and the need for cAMP activation of CFTR seems superfluous. But a closer inspection reveals that acute regulation of G_{Cl} is probably a physiological necessity to accommodate distinct transport processes (electrodiffusive *vs.* carrier mediated) depending upon the luminal salt concentration. We have previously shown [19, 23, 27] that when the luminal salt concentration is high (~isotonic), the electrochemical driving force supports diffusion of Cl from lumen to cell. Under these conditions, G_{Cl} must be "open" such that Cl can be transported passively (Fig. 15*B*). However, as the luminal salt concentration falls to less than about 50 mM [19, 23, 27], the direction of the electrochemical driving force for Cl reverses and intracellular Cl would leak back through the same Cl channel if it remained open. Physiologically, Cl can be absorbed to luminal Cl concentrations as low as 15 mM [19, 23]. This fact demands another Cl absorptive mechanism in the sweat duct [19, 23]. It seems likely that a nonconductive mechanism must become the basis for Cl absorption. If an apical membrane anion exchanger becomes active, G_{Cl} must be "shut off" to prevent backflux of Cl into the lumen (Fig. 15*C*). Since secreted sweat begins in the proximal part of the absorptive duct as an isotonic precursor and arrives at the skin surface as a Cl-depleted hypotonic solution, an axial Cl gradient exists along the duct length. Changes in secretory rate, obligatorily displace this axial gradient, with the forced consequence of changing the Cl gradients across the apical membranes of absorptive cells along the duct. The degree of the change is largely a function of luminal fluid flow rate. Therefore, the transition from electrodiffusive to carrier-mediated transport must occur within short time periods, and rapid activation/inactivation of G_{Cl} through interaction of kinase and phosphatase with CFTR-Cl channels appears to be essential for sustained Cl absorption against steep electrochemical gradients. Of course, such control of G_{Cl} would also benefit the cell by providing tight regulation of the amount of salt that enters the cell at any given moment to avoid cytoplasmic ionic turbulence.

In conclusion, our results offer clear evidence of acute control of electrolyte absorptive activity. Even though the fluid transport is reversed, the molecular machinery responsible for acute regulation of electrolyte transport is similar in absorption and secretion. Acute regulation of absorptive activity seems to be essential for sustained electrolyte absorption in the face of rapidly changing electrochemical gradients for Cl.

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