

Digitonin-permeabilized Colonic Cell Layers

Demonstration of Calcium-activated Basolateral K^+ and Cl^- Conductances

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ABSTRACT Sheets of isolated turtle colon were exposed to digitonin on the mucosal side to chemically remove the apical membrane as a permeability barrier. Increases in the mucosal uptake of ^{86}Rb , [3H]mannitol, and ^{45}Ca -EGTA, and the appearance of the cytosolic marker enzyme lactate dehydrogenase in the mucosal bath confirmed the permeabilizing effect of the detergent. Basolateral K^+ and Cl^- currents were generated by imposing transmural ion gradients, and cytosolic free Ca^{2+} was manipulated by means of a Ca^{2+} -EGTA buffer system in the mucosal bathing solution. Raising the cytosolic free Ca^{2+} concentration from the nanomolar to the micromolar range activated basolateral conductances for K^+ and Cl^- . Differences in ion selectivity, blocker specificity, calcium activation kinetics, and divalent cation activation selectivity indicated that the Ca^{2+} -induced increases in the K^+ and Cl^- conductances were due to separate populations of channels. The results are consistent with the notion that the apical membranes of turtle colon epithelial cells can be functionally removed under conditions that preserve some of the conductive properties of the basolateral membrane, specifically Ca^{2+} -activated conductive pathways for K^+ and Cl^- . This permeabilized preparation should offer a means for the identification of macroscopic currents that are due to presumed Ca^{2+} -activated channels, and may also provide a model system for the functional reconstitution of channel regulatory mechanisms.

INTRODUCTION

Application of single-channel recording techniques to a variety of cell types has revealed the widespread occurrence of channels that are activated by increases in cytoplasmic Ca^{2+} (Barrett et al., 1982; Latorre and Miller, 1983). The basolateral membranes of secretory (Petersen and Maruyama, 1984) and absorptive (Richards, N. W., and D. C. Dawson, 1987) epithelial cells appear to contain at least two types of Ca^{2+} -activated K^+ channels and, in addition, Ca^{2+} -activated Cl^- channels (Marty

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et al., 1984; Evans and Marty, 1986). Establishing the effects of Ca^{2+} on the gating of these channels has been facilitated enormously by the fact that channel activity persists in patches of membrane that have been detached from the cell so that the cytoplasmic aspect of the membrane faces the bath (inside-out patch). In this configuration it is possible to determine quantitatively the relation between Ca^{2+} activity and the open probability of the channel.

The study of the Ca^{2+} -dependent macroscopic membrane currents, which would presumably result from the presence of such channels in cell membranes, is a more difficult problem because it is generally not possible to effect well-controlled changes in Ca^{2+} activity while macroscopic currents are being measured. Intracellular Ca^{2+} activity can be perturbed by the use of ionophores or permeable buffers, but it would be desirable to clamp the intracellular Ca^{2+} activity at fixed values under conditions that would permit macroscopic currents to be continuously monitored.

In previous studies from this laboratory, we employed the pore-forming antibiotic amphotericin-B to functionally remove the apical membrane of the turtle colon as a barrier to monovalent cation flow. The polyene pores, however, are virtually impermeable to calcium, so amphotericin-treated tissues are not useful for manipulating cellular Ca^{2+} activity. Furthermore, to clamp intracellular Ca^{2+} activity it is necessary to introduce a suitable Ca^{2+} buffer into the cell. In this article, we report the results of our attempts to achieve this goal by using digitonin to permeabilize the apical membranes of colonic cells.

Digitonin has been widely employed to permeabilize the plasma membranes of nonpolar cells, particularly in studies of the metabolism of intracellular Ca^{2+} (Blaustein et al., 1978*a, b*; Fiskum et al., 1980; Murphy et al., 1980; Burgess et al., 1983). The purpose of the experiments described herein was twofold: (*a*) we wished to examine the possibility that basolateral ion currents could be determined in cells that were permeabilized on the apical side with digitonin, and (*b*) we attempted to manipulate intracellular Ca^{2+} activity by means of a Ca^{2+} -EGTA buffer system that could enter the cell through the permeabilized apical membrane. The results of these experiments led us to conclude that: (*a*) it is possible to permeabilize apical membranes of colonic cell layers with digitonin while maintaining the functional integrity of the basolateral membrane, (*b*) this permeabilized preparation is a useful tool for studying the effects of Ca^{2+} or other putative cellular regulators on ion channels, and (*c*) the basolateral membranes of turtle colon cells exhibit two distinct Ca^{2+} -activated conductances, one relatively specific for K^+ , and another specific for Cl^- and other anions.

MATERIALS AND METHODS

Tissue Preparation and Measurement of Ion Currents

Colons were excised from freshwater turtles (*Pseudemys scripta*; Kons Scientific Co., Inc., Germantown, WI) and stripped of outer longitudinal and circular musculature, as previously described (Dawson, 1977). Isolated tissue sections were mounted in Lucite Ussing chambers (area = 5.2 cm²) and bathed on both sides by vigorously aerated Ringer's solution at room temperature (22–25°C). The transmural potential difference (PD) was clamped to 0 mV by an automatic voltage clamp adjusted to compensate for the fluid resistance. When asymmetric

bathing solutions were used, the fluid resistance was determined for each and the average value was employed as a correction. Measurement of the transmural PD was achieved with calomel half-cells, which were connected by 0.5 M KCl–agar bridges to both chamber halves. Current was passed across the epithelium with Ag/AgCl electrodes, which were connected to both chamber halves by 1.0 M KCl–agar bridges. The short-circuit current (I_{sc}) was continuously recorded on a stripchart recorder. Tissue conductance was measured every 200 s by measuring the change in current brought about by a brief (1 s) 10-mV change in clamping potential. The preincubation Ringer's solution (pH 8.2) contained (in millimolar): 112 Na⁺, 2.5 K⁺, 1 Ca²⁺, 114 Cl⁻, 2.5 HCO₃⁻, and 10 glucose.

After a steady state I_{sc} was attained during preincubation (30–60 min), the mucosal and serosal baths were washed five times and replaced by their respective test solutions. To study basolateral K⁺ currents, tissues were bathed in Cl⁻-free solutions. A K⁺ gradient was imposed by bathing the mucosal or luminal side with a K-aspartate Ringer's solution of the following composition (in millimolar): 120 K⁺, 80 aspartate, 20 PIPES (1,4-piperazinediethanesulfonic acid), 5 EGTA, and 10 glucose. The serosal bath contained a Na-aspartate Ringer's solution that was identical except for the addition of 6 mM Ca²⁺ gluconate (1 mM free Ca²⁺), 0.1 mM ouabain (to abolish active transport), and 2 μM atropine (to block the effects of endogenous acetylcholine; Venglarik and Dawson, 1986). To study currents due to Cl⁻, the cell layer was bathed by K⁺-free solutions. A Cl⁻ gradient was imposed by placing a NaCl Na-aspartate Ringer's solution in the serosal bath and a Ca²⁺-free Na-aspartate Ringer's solution in the mucosal bath. The NaCl Na-aspartate Ringer's solution had the following composition (in millimolar): 120 Na⁺, 60 Cl⁻, 20 aspartate, 20 PIPES, 5 EGTA, 6 Ca²⁺ gluconate, 0.1 ouabain, 10 glucose, and 0.002 atropine. The pH of all test solutions was buffered to 6.6. Concentrations of Na⁺ and K⁺ were verified by flame photometry.

The Ca²⁺-EGTA Buffer System

The mucosal solutions containing the Ca²⁺-EGTA buffer system were buffered to a pH of 6.6. This relatively acidic pH had two advantages. First, at this pH the slope of the titration curve (Chang et al., 1988) is such that it is relatively easy to obtain free Ca²⁺ concentrations in the 10–10,000 nM range by altering the total Ca²⁺ concentration. Second, at this pH the conductance of the basolateral membrane in the absence of Ca²⁺ was relatively low. This may be a result of the fact that the resting conductance of the basolateral membrane, which is due primarily to K⁺ (Germann et al., 1986b; Venglarik and Dawson, 1986) and Cl⁻ (Venglarik et al., 1986), is blocked or inactivated at an acidic pH. A reduction in basolateral conductance can be produced in amphotericin-treated cell layers by lowering mucosal pH (D. J. LeGault and D. C. Dawson, unpublished observations).

To clamp the cytosolic free Ca²⁺ concentration at values ranging from 1 to 10,000 nM, it was necessary to compute the amounts of EGTA and total Ca²⁺ required in each case. As discussed previously by Fabiato and Fabiato (1979) and Tsien (1980), this calculation is complicated by the fact that the four acetate moieties and two nitrogen atoms that comprise the Ca²⁺ binding sites on the EGTA molecule can also be protonated. Thus, in the absence of Ca²⁺, several different forms of EGTA can exist in solution, and two of these, the unprotonated and singly protonated forms, can bind one Ca²⁺ ion. The apparent equilibrium constant relating the concentrations of free Ca²⁺, bound Ca²⁺, and EGTA is thus highly pH dependent. In addition, other divalent ions of importance in this study (Ba²⁺, Sr²⁺, Mn²⁺, and Cd²⁺) can compete for binding to the various forms of EGTA. To facilitate these calculations, we developed an algorithm for computing the multiple equilibria of EGTA, Ca²⁺, and protons in conjunction with published values for the appropriate stability constants (Chang et al., 1988). The program is sufficiently general to incorporate at least two divalent species, i.e., Ca²⁺ and Ba²⁺ for instance, and a second ligand (e.g., ATP). The use of this scheme yielded calculated

values for free Ca^{2+} concentrations that differed by only 3–4% (in the range of 10 nM–1 μM free Ca^{2+} concentration) from those described by Tsien (1980) under similar conditions. The presence of the EGTA buffer system in the mucosal bath enabled us to vary the free concentration of divalent cations accurately by adding acetate salts of the divalent cation.

Digitonin Permeabilization

Permeabilization of the apical membrane was achieved by the addition of 10–20 μl of a concentrated (20 mM) solution of digitonin to the mucosal bathing solution. Because digitonin is poorly soluble in water the stock solution was warmed before its addition to dissolve the detergent. As judged from increases in tissue conductance, the onset of permeabilization varied from 1 to 5 min after the addition of the detergent and was complete after 15 min. The tissues displayed remarkable stability; the tissue conductance remained constant for up to 2 h after digitonin permeabilization. The permeabilizing effect of digitonin appeared to be, for practical purposes, irreversible. Repeated replacement of the mucosal solution with detergent-free solutions, for up to 20 min after the initial incubation, did not reduce the permeabilizing effect as judged from the activation of currents brought about by added Ca^{2+} . Dunn and Holz (1983) demonstrated that, in cultured adrenal chromaffin cells after a 5-min preincubation with 20 μM digitonin, the continued presence of the detergent in the bathing solution was not necessary for the maintenance of the permeabilizing effect.

Measurement of Unidirectional Tracer Influx

The unidirectional influx of ^{45}Ca and ^{86}Rb from the mucosal bathing solution into the mucosal cell layer was estimated by measuring the uptake of tracer according to the method described by Thompson and Dawson (1978). The test solutions in the mucosal bath contained 5 μCi of ^{45}Ca or 7.5 μCi of ^{86}Rb along with 60 μCi of [^3H]mannitol in the K-aspartate Ringer's solution. Because of the extreme permeabilization of the apical membrane, the true initial rates were not determined. An uptake time of 30 s was chosen to provide a qualitative index of the permeabilizing effect.

Measurement of Lactate Dehydrogenase (LDH) Release

The mucosal and serosal bathing solutions were assayed for the release of LDH in the presence or absence of digitonin (with or without Ca^{2+}) in paired tissues 30–60 min after digitonin addition. The procedure employed to measure LDH activity was that described by Kornberg (1955). Total protein was determined by the microassay method of Bradford (1976).

Measurement of Total Ca^{2+}

To estimate levels of Ca^{2+} contamination, we measured the total Ca^{2+} present in the nominally Ca^{2+} -free mucosal bathing solutions (with no EGTA and added Ca^{2+}) after digitonin permeabilization by means of atomic absorption spectrophotometry. The mucosal bathing solution was assayed as undiluted samples using an atomic absorption spectrophotometer (model 2380; Perkin-Elmer Corp., Norwalk, CT) at 422.7 nm with a high-spectral-output hollow cathode lamp (Fisher Scientific Co., Pittsburgh, PA) for Ca^{2+} and Mg^{2+} .

Measurement of Free Ca^{2+}

The level of contaminating amounts of free Ca^{2+} present in mucosal bathing solutions (to which no Ca^{2+} or EGTA were added) after digitonin permeabilization was assessed with a Ca^{2+} -selective electrode equipped with the neutral Ca^{2+} ionophore ETH 1001 (Ammann et al., 1979). The Ca^{2+} -selective electrode was calibrated with Ca^{2+} -EGTA buffer solutions

described by Chang et al. (1988). The electrode response was stable, rapid (response time <2 s), and linear, in the 1–10 μM Ca^{2+} concentration range (slope = 30.9 ± 0.9 mV at 25°C , $n = 8$).

RESULTS

Digitonin Removes the Apical Membrane as a Permeability Barrier

The representative traces shown in Fig. 1 illustrate the experimental paradigm employed to measure Ca^{2+} -activated currents due to K^+ and Cl^- under short-circuit conditions. Before the addition of digitonin, the I_{sc} was typically <10 $\mu\text{A}/\text{cm}^2$

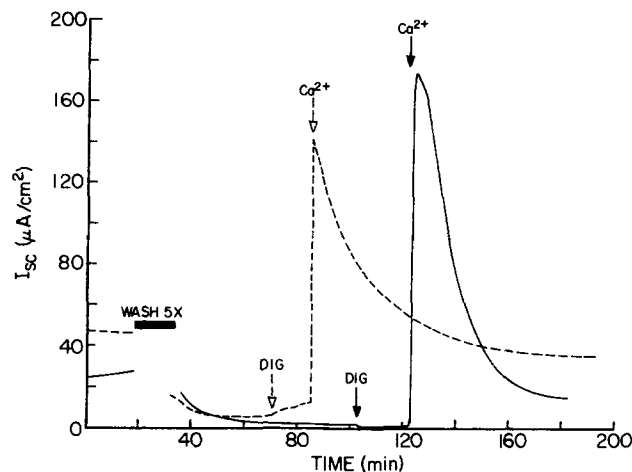


FIGURE 1. Effect of raising mucosal free $[\text{Ca}^{2+}]$ to $1 \mu\text{M}$ on basolateral Cl^- current (solid trace) and K^+ current (dashed trace) in digitonin-permeabilized colonic cell layers. Representative paired experiment comparing the long-term response of basolateral ion currents to activation by Ca^{2+} . Initially, tissues were bathed on both sides by NaCl Ringer's. Tissues were then washed five times on both sides by their respective test solutions as described in Materials and Methods. In the presence of an M-to-S K^+ gradient (dashed trace) or an S-to-M Cl^- gradient (solid trace), digitonin was added to the mucosal bath to a final concentration of $20 \mu\text{M}$. After digitonin permeabilization of the apical membrane, the I_{sc} for both tissues was quite low. Raising the free $[\text{Ca}^{2+}]$ to $1 \mu\text{M}$ by the addition of 1.47 mM total $[\text{Ca}^{2+}]$ as Ca^{2+} acetate to the EGTA buffer system in the mucosal bath activated basolateral ion currents due to K^+ (dashed trace) and Cl^- (solid trace).

despite the imposition of a mucosal-to-serosal K^+ gradient (mucosal [M], 120 mM ; serosal [S], $\sim 0 \text{ mM}$) or an S-to-M Cl^- gradient (M, $\sim 0 \text{ mM}$; S, $60\text{--}80 \text{ mM}$). This result is in accord with the notion that, under these conditions, the native K^+ and Cl^- conductances of the apical membranes of the colonic cells are negligible. In addition, the resistance and relative nonselectivity of the paracellular path precluded any significant "shunt" currents under these conditions (Halm and Dawson, 1984). Active Na^+ transport is electrogenic (Kirk et al., 1980) but was absent due to the inhibition of the pump by ouabain. Upon the addition of digitonin ($20\text{--}40 \mu\text{M}$) to the mucosal bath in the presence of a 1 nM free Ca^{2+} concentration and a trans-

transmural ion gradient, a small increase or no change in I_{sc} was observed. The absence of a significant current under these conditions suggested that the conductance of the basolateral membrane in the absence of Ca^{2+} was quite low. As indicated in Methods, this appears to be due, at least in part, to the acidic cellular pH.

In the presence of mucosal digitonin, adding an amount of calcium acetate to the mucosal bath sufficient to raise the free concentration of Ca^{2+} to $1 \mu M$, led to the prompt development of an M-to-S current in both tissues (Fig. 1). The ionic basis for these currents was determined by ion substitution and blocker specificity (also see below). When the mucosal and serosal bathing solutions were Cl^- -free (K-aspartate and Na-aspartate, respectively), the direction of Ca^{2+} -activated currents was determined by the orientation of the K^+ gradient. An M-to-S K^+ gradient resulted in an M-to-S current, and reversing the orientation of the gradient gave rise to Ca^{2+} -activated currents in the S-to-M direction but with the same magnitude and time course (not shown). In contrast, Na^+ gradients produced by simply diluting Na-

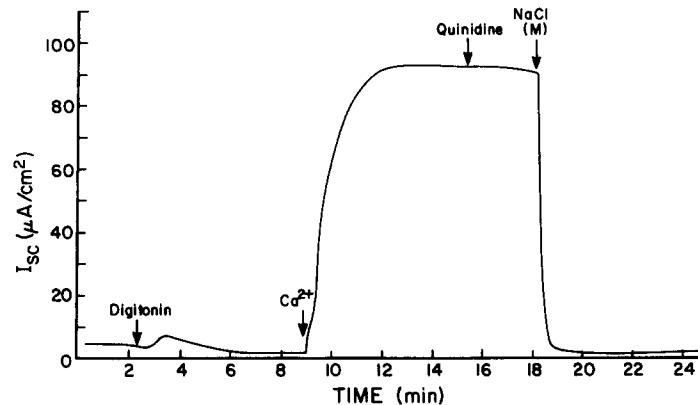


FIGURE 2. The effect of quinidine on Ca^{2+} -activated Cl^- currents. The addition of $200 \mu M$ quinidine to the mucosal bath did not inhibit the I_{sc} . The I_{Cl} was promptly abolished by nonisotonic addition of 60–80 mM NaCl to the mucosal bath to reduce E_{Cl} to zero.

aspartate Ringer's isosmotically with sucrose produced no currents. Finally, currents activated in the presence of a K^+ gradient were abolished by quinidine or Ba^{2+} , both of which are specific blockers of K^+ channels (see below). In the absence of K^+ , the direction of Ca^{2+} -activated Cl^- currents was determined by the orientation of the transmural Cl^- gradient. S-to-M and M-to-S Cl^- gradients (produced by appropriate mixtures of NaCl and Na-aspartate Ringer's) gave rise to M-to-S and S-to-M currents, respectively. The currents were insensitive to quinidine and could only be activated in the presence of a permeant anion (see below). The Cl^- current was abolished, however, if the Cl^- concentration gradient was eliminated by adding sufficient Cl^- (as NaCl) to the mucosal bathing solution (Fig. 2).

In the absence of digitonin, the addition of Ca^{2+} to the mucosal bath had no effect on the I_{sc} . If, under identical conditions, amphotericin-B was added to the mucosal bath rather than digitonin, Ca^{2+} addition was without effect. Because acute addition of Ca^{2+} to the EGTA-containing solutions lowered the pH by ≤ 0.2 units

(owing to the displacement of protons from the EGTA), we tested the effect of a similar decrease in pH in the absence of a change in Ca^{2+} concentration. This maneuver was also without effect on I_{sc} (data not shown).

After Ca^{2+} addition, both K^+ and Cl^- currents exhibited a characteristic time course: a (1–5 s) lag period, a rapid (30–60 s) activation, and a slower (~1 h) spontaneous inactivation. The inactivation process was not altered by further additions of Ca^{2+} . As shown in Fig. 3, currents due to K^+ or Cl^- were rapidly inactivated if Ca^{2+} activity was reduced by the addition of excess (20 mM) EDTA to the mucosal bathing solution.

The activation of ionic currents by Ca^{2+} in digitonin-treated cell layers suggested that this treatment permeabilized the apical membranes to small anions and cations

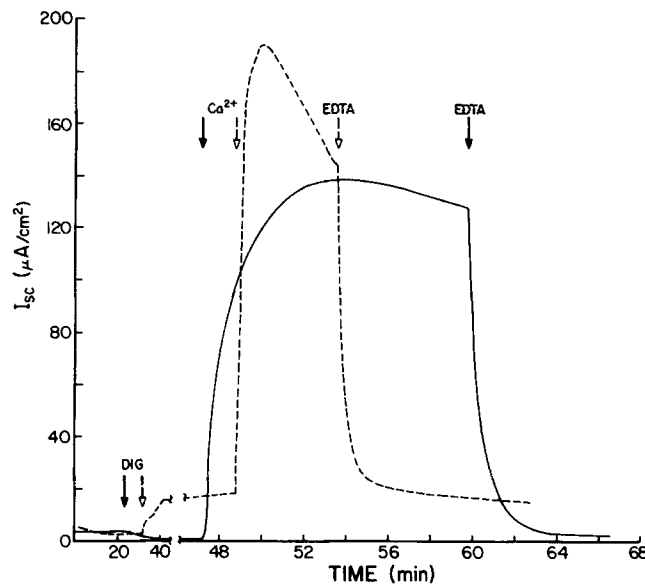


FIGURE 3. The short-term response of basolateral K^+ (dashed trace) and Cl^- (solid trace) conductances to activation by Ca^{2+} ($1 \mu\text{M}$ free $[\text{Ca}^{2+}]$) after digitonin permeabilization in an experiment similar to the one discussed in Fig. 1. The addition of 20 mM EDTA to the mucosal bathing solutions promptly abolished both conductances by chelating the Ca^{2+} .

as well as EGTA, while the basolateral membranes remained intact. Table I summarizes the results of experiments designed to provide an independent test of the extent of permeabilization caused by digitonin. Mucosal uptakes of ^{45}Ca -EGTA and $[^3\text{H}]$ mannitol, as well as ^{86}Rb , were measured in the presence of an M-to-S K^+ gradient under conditions identical to those employed for the activation of K^+ currents (see Methods). Because of the extreme degree of permeabilization caused by the detergent, no attempt was made to determine extracellular space during the brief uptake period. The uptakes of $[^3\text{H}]$ mannitol, ^{45}Ca -EGTA, and ^{86}Rb are presented in the form of apparent rate coefficients for a 30-s uptake period. These represent a minimum estimate because, as discussed in Methods, a 30-s uptake is not likely to be a measure of the initial rate in the presence of digitonin. Nevertheless, the effective

TABLE I
Effect of Digitonin on Tracer Uptake from the Mucosal Bath

Condition	Apparent rate coefficient for uptake (λ)*		
	[³ H]Mannitol	⁴⁵ Ca	⁸⁶ Rb
		(cm/h) × 1,000	
Without digitonin (10 ⁻⁹ M Ca ²⁺) (n = 6)	47 ± 8	36 ± 6	133 ± 50
Without digitonin (10 ⁻⁶ M Ca ²⁺) (n = 6)	29 ± 1	23 ± 2	95 ± 30
With digitonin (10 ⁻⁹ M Ca ²⁺) (n = 7)	324 ± 108	276 ± 84	512 ± 159
With digitonin (10 ⁻⁶ M Ca ²⁺) (n = 7)	300 ± 216	612 ± 144	509 ± 50

*Presented as the mean ± SEM.

rate of uptake for all three isotopes was increased by five- to tenfold in the presence of digitonin regardless of the free Ca²⁺ concentration.

Dunn and Holz (1983) assessed the permeabilizing effect of digitonin on chromaffin cells by monitoring the release of the cytoplasmic enzyme LDH. Table II contains a summary of the results of experiments in which we measured LDH release from colonic cells into the mucosal and serosal baths. In paired tissues, LDH activity and protein levels were measured in the absence or presence of digitonin (20-min incubation) and Ca²⁺. Regardless of the mucosal Ca²⁺ concentration, activity of this cytosolic enzyme (134,000 mol wt) and protein was detectable in the mucosal bath only in the presence of digitonin. Digitonin-induced release of LDH in the presence of either activating or nonactivating concentrations of mucosal Ca²⁺ confirmed the notion that the permeabilization process is not Ca²⁺ dependent. In contrast, LDH activity was barely detectable in the serosal bath in the presence or in the absence of digitonin. Other investigators (Streb and Schulz, 1983; Streb et al., 1983) have reported that simply washing cells in a "Ca²⁺-free solution" containing micromolar amounts of free Ca²⁺ rendered the plasma membrane permeable to LDH. In contrast, we found that simply exposing the cell membrane to low Ca²⁺ media (10⁻⁹–10⁻⁶ M) did not release appreciable amounts of LDH.

Ca²⁺-activated K⁺ Currents: Ion Selectivity and Blockade

Ion selectivity and blocker specificity are primary criteria for identifying macroscopic ion conductances with a particular population of ion channels. We attempted

TABLE II
Effect of Digitonin on LDH Release into the Mucosal and Serosal Baths*

Condition	LDH Activity‡	Protein‡
Mucosal		
	U/ml	
Without digitonin (10 ⁻⁹ or 10 ⁻⁶ M Ca ²⁺) (n = 4 tissues)	1.4 ± 0.8	0.6 ± 0.3
With digitonin (10 ⁻⁹ M Ca ²⁺) (n = 3 tissues)	71.4 ± 12.7	14.3 ± 2.7
With digitonin (10 ⁻⁶ M Ca ²⁺) (n = 4 tissues)	81.0 ± 16.9	15.3 ± 2.1
Serosal		
	U/ml	
Without digitonin (10 ⁻⁹ M Ca ²⁺) (n = 10 tissues)	1.2 ± 0.3	
With digitonin (10 ⁻⁶ M Ca ²⁺) (n = 9 tissues)	1.8 ± 1.1	

Samples were taken 30–60 min after the addition of digitonin to the mucosal bath. *Presented as the mean ± SEM. ‡Mean ± SEM for 3 samples per tissue.

to characterize the Ca^{2+} -activated currents by as many criteria as possible so as to improve our chances of identifying these currents under more physiological conditions and to provide a "fingerprint" that could be used to identify the single channels responsible for these currents. Fig. 4 shows a representative experiment comparing Ca^{2+} -activated currents in the presence of M-to-S gradients of either K^+ or Rb^+ . In seven paired experiments, the ratio of the peak Ca^{2+} -activated currents was $I_{\text{Rb}}/I_{\text{K}} = 0.65 \pm 0.13$ ($\bar{x} \pm \text{SEM}$). Ca^{2+} did not activate currents in the presence of identical Na^+ or Cs^+ gradients (not shown).

We were particularly interested in the efficacy of quinidine, lidocaine, and Ba^{2+} as blockers of the Ca^{2+} -activated K^+ conductance because we had previously identified these agents as blockers of a particular basolateral K^+ conductance that was associated with cell swelling (Germann et al., 1986*a, b*). Fig. 5 compares the blockade of Ca^{2+} -activated K^+ currents by quinidine and lidocaine. 200 μM quinidine completely inhibited the Ca^{2+} -activated I_{K} . The effect of lidocaine was variable, but significant inhibition was obtained only with concentrations in the millimolar range.

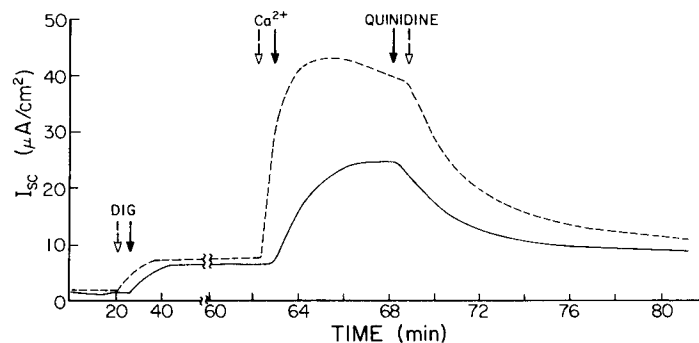


FIGURE 4. A representative experiment comparing basolateral Ca^{2+} -activated currents in the presence of identical 120:~0 mM M-to-S gradients of Rb^+ (solid trace) or K^+ (dashed trace). 200 μM quinidine added to the mucosal bath completely inhibited both I_{K} and I_{Rb} .

Inhibition of ion currents due to K^+ or Cl^- (see below) could, in principle, result either from the blockade of ionic channels or from interference with the Ca^{2+} activation process. The experiments reported here did not distinguish between these alternatives. However, most of the compounds tested have been identified in studies of single ion channels as blockers of the channel conduction process.

Ba^{2+} blocks K^+ conductances in a variety of cell types and blocks both the resting and osmotically activated basolateral K^+ conductances in colonic cells (Germann et al., 1986*a*). The results of a representative experiment with Ba^{2+} are shown in Fig. 6. Ba^{2+} was a potent blocker of the Ca^{2+} -activated K^+ currents when added to the mucosal (cellular) bath; maximal inhibition was achieved at 0.5 mM total Ba^{2+} . Under these conditions, the free concentrations of Ca^{2+} and Ba^{2+} were 2 and 75 μM , respectively. In marked contrast, Ba^{2+} added to the serosal bath produced no discernible reduction in I_{K} even after prolonged preincubation (20 min) at concentrations up to 5 mM total Ba^{2+} (free $[\text{Ba}^{2+}] = 4.85$ mM).

Fig. 7 illustrates the blockade produced by tetraethylammonium (TEA) when applied on the mucosal side. Half-maximal inhibition and complete inhibition were obtained with 10 and 50 mM TEA, respectively. TEA on the serosal side was without effect, however, even after prolonged preincubation (20 min).

Ca²⁺-activated Cl⁻ Currents: Ion Selectivity and Blockade

To characterize the anion selectivity of the Ca²⁺-activated Cl⁻ conductance, we measured Ca²⁺-activated currents in the presence of gradients of Br⁻, NO₃⁻, I⁻, SCN⁻, and acetate using mixtures of the sodium salts of these anions and Na-aspartate as

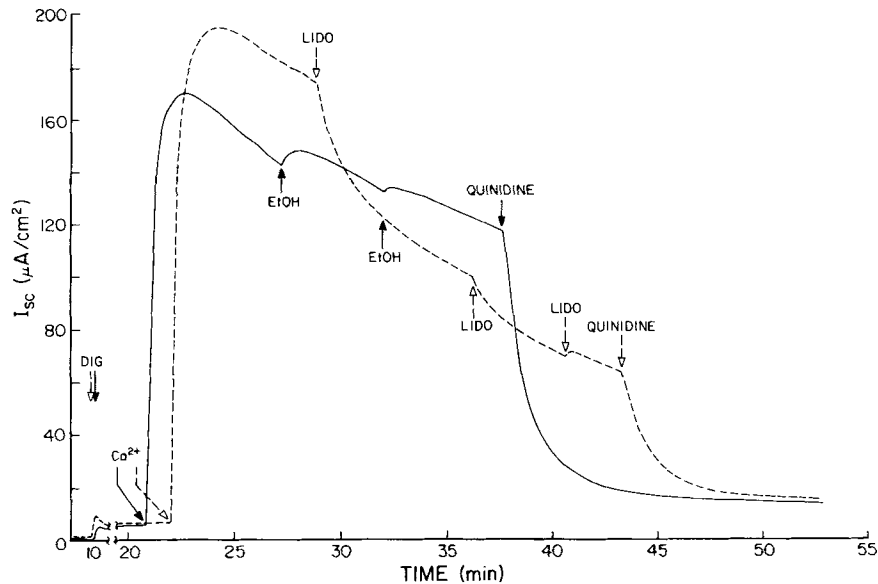


FIGURE 5. Comparison of the effects of quinidine and lidocaine on basolateral K⁺ currents. Transmural K⁺ currents were activated by Ca²⁺ as described previously. Quinidine (solid trace) was added only to the mucosal bath as a concentrated solution until it was brought to a final concentration of 200 μM. Lidocaine (dashed trace) was applied to both mucosal and serosal baths as a concentrated solution dissolved in ethanol (EtOH) to bring about final concentrations of 0.2, 1, and 4 mM. 200 μM quinidine was added to the mucosal bath to abolish the remaining I_K (dashed trace). As a control, 100 μl of ethanol was applied twice to both mucosal and serosal baths (in the solid trace) to determine its effect on I_K.

described in Methods. As shown in Fig. 8, substantial Ca²⁺-activated currents were obtained with gradients of Br⁻, NO₃⁻, SCN⁻, and I⁻ that appeared to be indistinguishable, with respect to peak current and time course, from those seen with Cl⁻ gradients. Acetate, however, appeared to be impermeant. On the basis of a comparison of ionic currents, the Ca²⁺-activated conductance appeared not to discriminate appreciably among the permeant anions tested.

We were particularly interested in finding a blocker for this conductance that might be employed to implicate its function in a more physiological setting. Derivatives of anthranilic acid have been suggested as blockers of Cl⁻ channels in renal

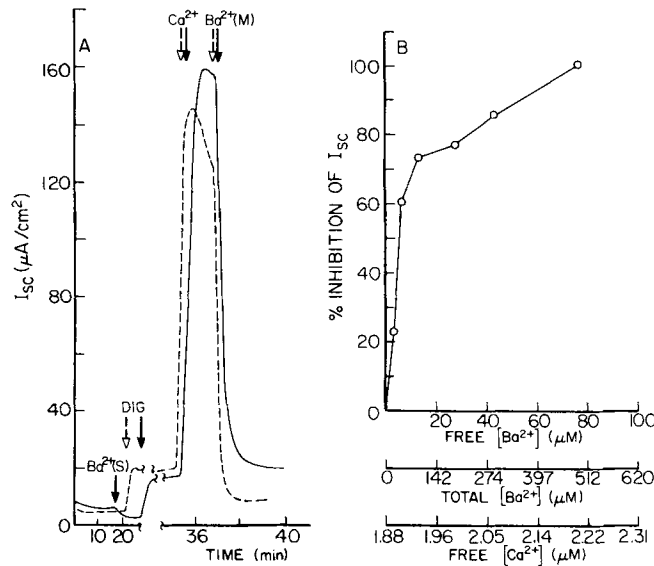


FIGURE 6. Inhibition of Ca^{2+} -activated basolateral K^+ currents by Ba^{2+} . (A) Effect of mucosal vs. serosal Ba^{2+} on Ca^{2+} -activated K^+ currents. Preincubation with 5 mM Ba^{2+} (as barium acetate) in the serosal bath (solid trace) for >15 min did not prevent the activation of I_K by Ca^{2+} . The addition of 0.5 mM Ba^{2+} to the mucosal bath (solid and dashed traces) promptly abolished I_K . (B) The response of I_K to increasing concentrations of free Ba^{2+} . The total $[Ba^{2+}]$ needed to give the specified free $[Ba^{2+}]$ is given on the abscissa. Changes in the free $[Ca^{2+}]$ resulting from increasing total $[Ba^{2+}]$ are also shown. Calculations for free $[Ca^{2+}]$, free $[Ba^{2+}]$, and total $[Ba^{2+}]$ are described in Materials and Methods.

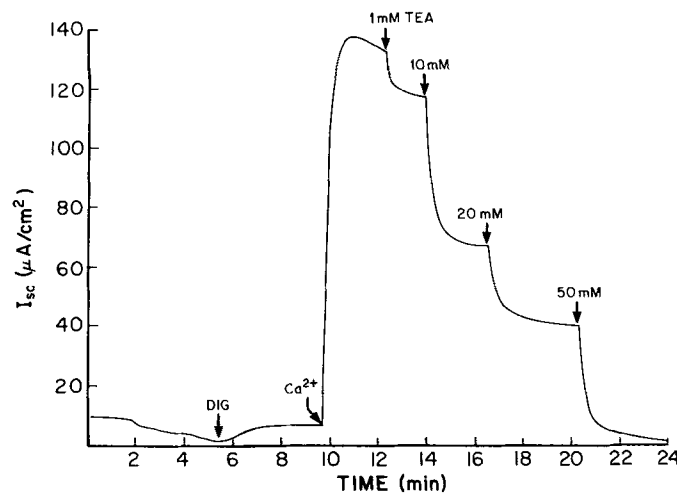


FIGURE 7. The effect of mucosal TEA on Ca^{2+} -activated K^+ currents. Final concentrations of TEA in the mucosal bath are indicated above the arrows. The application of TEA on the serosal bath was without effect, even after prolonged (20 min) preincubation.

tubules (Wangemann et al., 1986) and tracheal epithelium (Welsh, 1984), so we tested two of these: 9-anthracene carboxylic acid (9-AC) and *N*-phenyl anthranilic acid (*n*-phen). The results of a representative experiment are shown in Fig. 9, which compares the effect of 9-AC on Ca^{2+} -activated K^+ and Cl^- currents. It is clear from the figure that the dose of 9-AC that produced maximal inhibition of I_{Cl} (5 mM) also produced maximal inhibition of I_{K} . Experiments with 2 mM *n*-phen produced identical results (not shown). In the present experimental setting, the 9-AC-derived class

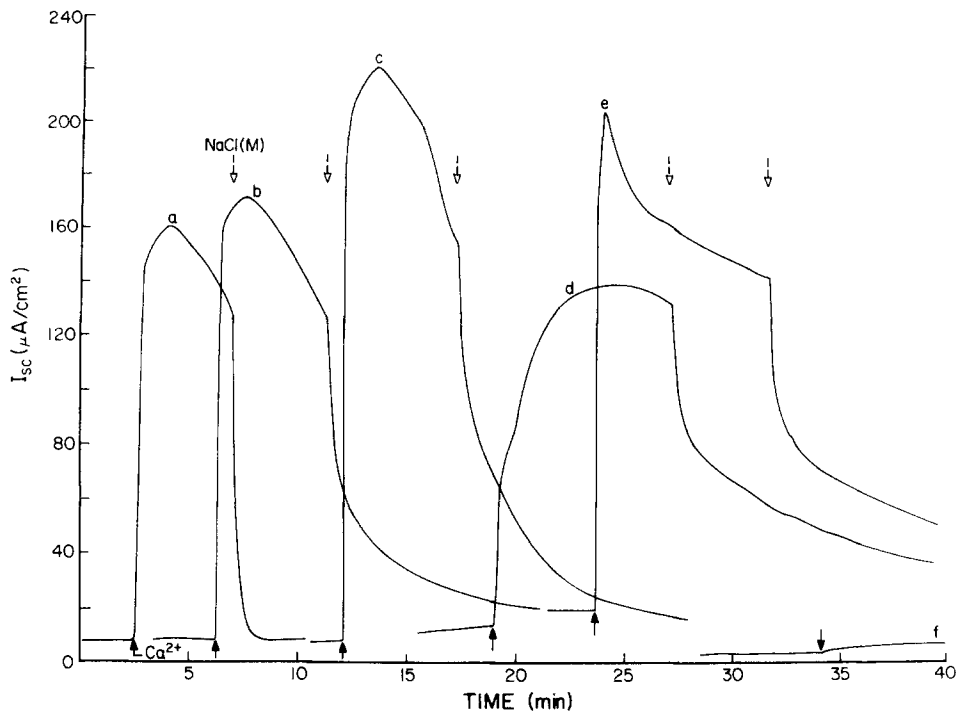


FIGURE 8. The selectivity of the Ca^{2+} -activated Cl^- conductance for other anions. Identical transmural S-to-M gradients of (a) Cl^- , (b) Br^- , (c) I^- , (d) NO_3^- , (e) SCN^- , and (f) acetate were imposed by bathing the S side with 60 mM NaX Ringer's (where X = Cl^- , Br^- , etc.) and the mucosal side with X-free Na-aspartate Ringer's. Addition of $1 \mu\text{M}$ free $[\text{Ca}^{2+}]$ to the mucosal bath is indicated by the solid upward arrows and the addition of 60 mM NaCl is depicted by the dashed downward arrows.

of compounds may be useful as Cl^- channel blockers but they are not specific for Cl^- channels.

Independence of Ca^{2+} -activated K^+ and Cl^- Conductances

We used digitonin-treated cell layers exposed to opposing gradients of K^+ and Cl^- to determine if Ca^{2+} -activated conductances for K^+ and Cl^- could be manipulated independently. Fig. 10 shows the results of a typical experiment. An M-to-S K^+ current was activated (under Cl^- -free conditions) by raising mucosal Ca^{2+} from 10^{-9} to

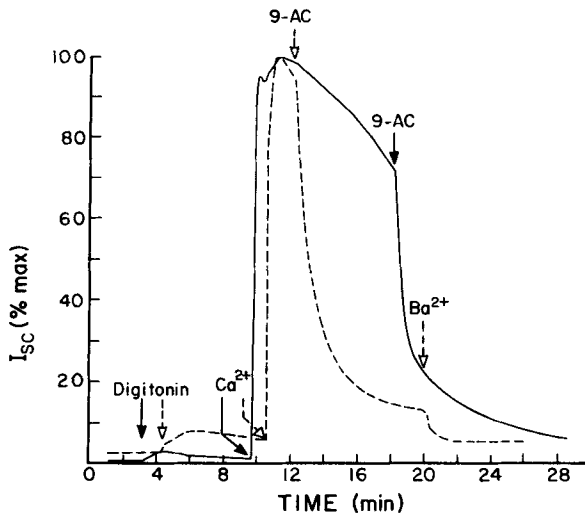


FIGURE 9. The effect of 9-AC on Ca^{2+} -activated K^+ and Cl^- currents. Transmural M-to-S K^+ currents (dashed trace) and S-to-M Cl^- currents (solid trace) were generated as described previously. 5 mM 9-AC was added to both tissues on M. 0.5 mM total Ba^{2+} was applied to the mucosal bath (dashed trace) to reduce the remaining I_K .

10^{-6} M. Subsequent addition of Cl^- to the serosal bath (as NaCl) gave rise to an additional M-to-S current. The M-to-S current due to K^+ was abolished by quinidine and the remaining (Cl^-) current was eliminated by adding an equimolar amount of NaCl to the mucosal bath. Similar experiments, illustrated by Fig. 11, showed that activation of a K^+ current in Cl-free solutions, followed by quinidine inhibition, did not prevent the production of an I_{Cl} by imposition of a Cl^- gradient. Similarly, pre-treating with quinidine did not prevent the activation of I_{Cl} by Ca^{2+} .

Ca²⁺ Activation: Kinetics

Using solutions designed as described in Methods, we investigated the dependence of the magnitude of the peak Ca^{2+} -activated I_K and I_{Cl} on the calculated concentra-

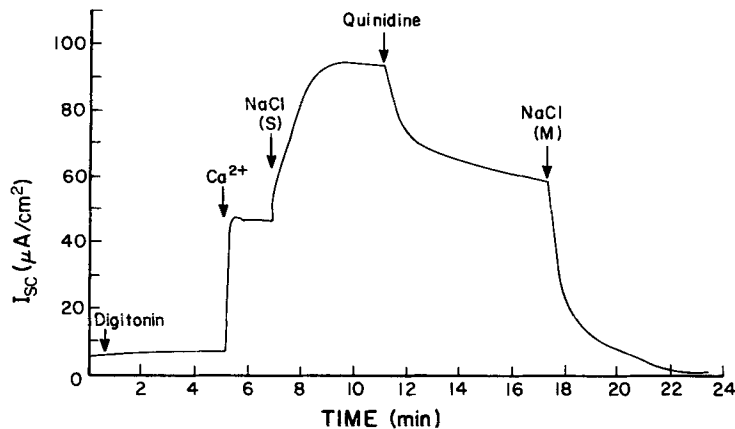


FIGURE 10. Activation of opposing M-to-S K^+ and S-to-M Cl^- currents by Ca^{2+} . Before Ca^{2+} activation, only an M-to-S K^+ gradient was present. 60 mM NaCl was used on both mucosal and serosal baths. Quinidine was added to the mucosal bath to a final concentration of 200 μM .

tion of free Ca^{2+} . Basolateral Ca^{2+} -activated K^+ and Cl^- conductances could be activated in a stepwise manner (Fig. 12 A) by progressively increasing the level of free Ca^{2+} in the mucosal bath. In paired experiments, there was no appreciable difference in the magnitude of the maximal response when activation was accomplished by stepwise addition of Ca^{2+} or by a single dose. The activation of both ionic conductances by Ca^{2+} was rapid, commencing within 5 s and reaching its peak 30–60 s after Ca^{2+} addition. Fig. 12 B shows the log dose-response relationship for both currents. The K^+ conductance displayed slightly greater sensitivity to Ca^{2+} activation

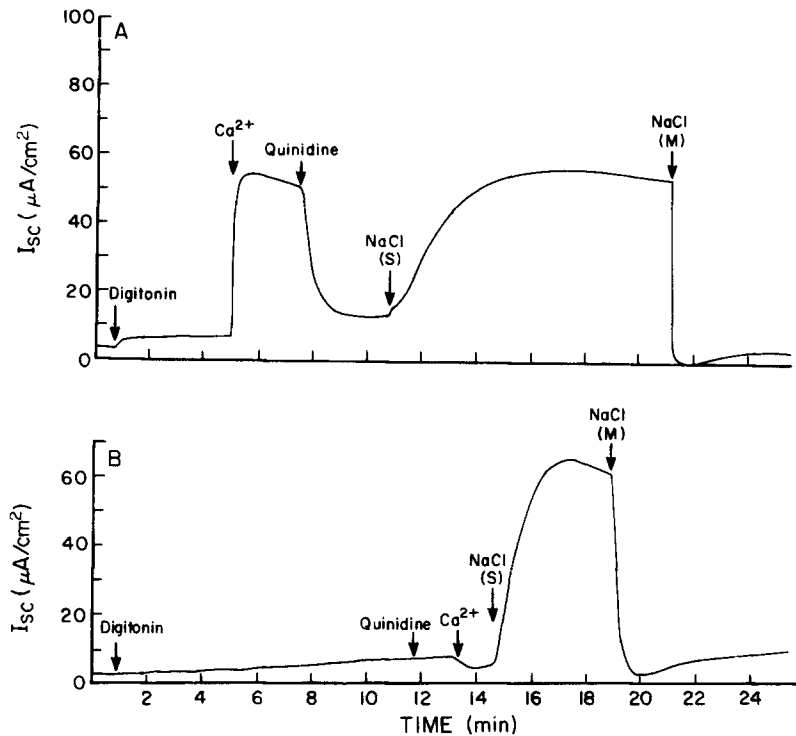


FIGURE 11. Demonstration of the independence of the Ca^{2+} -activated K^+ and Cl^- conductances. Initially, the tissues in both A and B were bathed with identical M-to-S K^+ gradients. Activation was accomplished with $1 \mu\text{M}$ free Ca^{2+} . $200 \mu\text{M}$ quinidine on the mucosal bath and 60 mM NaCl on mucosal and serosal baths were used as indicated.

(apparent $K_{1/2} = 364 \pm 65 \text{ nM}$, $n = 6$) than the Cl^- conductance (apparent $K_{1/2} = 546 \pm 33 \text{ nM}$, $n = 9$). The amount of mucosal (cytosolic) EGTA needed to adequately buffer cell Ca^{2+} was tested in a separate set of paired experiments that were conducted in the presence of different concentrations of EGTA buffer. The Ca^{2+} activation of basolateral K^+ currents with mucosal EGTA concentrations of 5 and 10 mM yielded apparent $K_{1/2}$ values of $368 \pm 5 \text{ nM}$ ($n = 3$) and $346 \pm 27 \text{ nM}$ ($n = 6$), respectively. The dependence of the currents on the activity of free Ca^{2+} appeared to be steeper than that predicted by a simple unimolecular binding isotherm, yielding a slope of 3–4 in a Hill plot over the 0.1–1.0 μM range (Fig. 13).

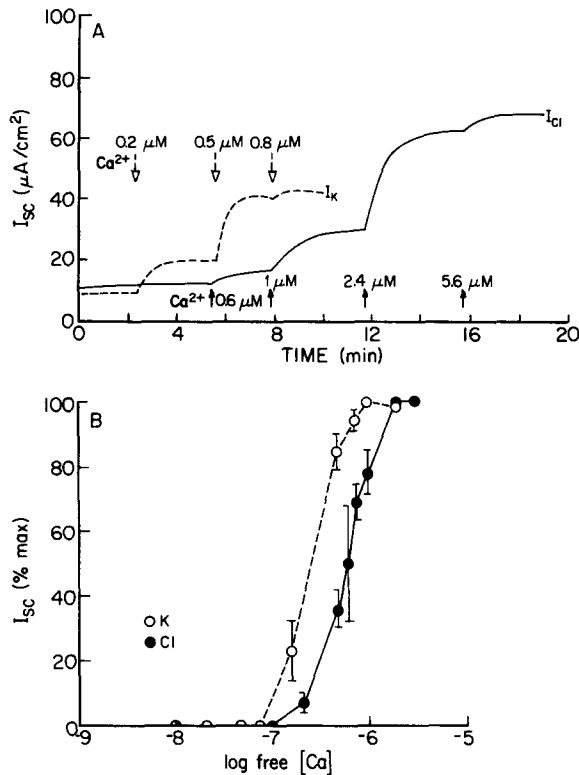


FIGURE 12. Ca^{2+} dependence of basolateral K^+ and Cl^- conductances. (A) Stepwise activation of I_K and I_{Cl} by increasing free Ca^{2+} concentrations. Solid trace, activation of I_{Cl} by final concentrations of free Ca^{2+} of 0.6, 1, 2.4, and 5.6 μM ; dashed trace, activation of I_K by final concentrations of free Ca^{2+} of 0.2, 0.5, and 0.8 μM . The values above the arrows indicate the concentration of free Ca^{2+} achieved in the mucosal bathing solution by the addition of specific amounts of Ca^{2+} . The concentration of total Ca^{2+} needed to obtain these free Ca^{2+} levels were calculated as described in Materials and Methods. (B) Log dose-response curve of the Ca^{2+} -activated basolateral K^+ (open circles) and Cl^- (filled circles) conductances to free $[Ca^{2+}]$.

Specificity of Divalent Cation Effects

The selectivity of divalent cation activation of basolateral conductances was of interest for two reasons. First, we wished to determine if the activation process exhibited an absolute specificity for Ca^{2+} or if, instead, other divalent cations could evoke

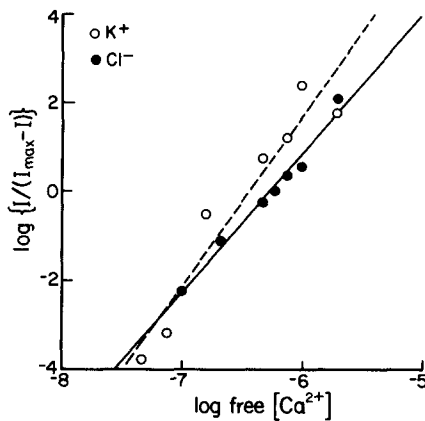


FIGURE 13. Hill plot of Ca^{2+} activation of basolateral K^+ and Cl^- conductances. The data in Fig. 12 B are graphically presented as a Hill plot with the Ca^{2+} -activated K^+ conductance shown as open circles and fitted by linear regression to the dashed line. The Ca^{2+} -activated Cl^- conductance is represented by the filled circles and the solid line. The slope of the Hill plot provides an estimate of the minimum number of Ca^{2+} binding sites required for activation in the limit of infinite positive cooperativity. The Hill plot yielded slopes of 3.80 for the K^+ conductance and 3.12 for the Cl^- conductance.

basolateral conductance increases. Second, we sought to identify in the selectivity of activation another possible difference in the conductances for K^+ and Cl^- . We employed Cd^{2+} , Mn^{2+} , Mg^{2+} , Sr^{2+} , and Ba^{2+} as substitute divalent cations. The strategy of the experiments was to use published metal-ligand stability constants to design EGTA-buffered solutions with well-defined free concentrations of each substitute ion in the nominal absence of Ca^{2+} (see Methods). A potential problem with this approach was the presence in solutions (to which no Ca^{2+} had been added) of contaminating Ca^{2+} that could originate in the deionized water and salts used to prepare the solutions, and from the cells of the epithelium, after permeabilization. Contaminating Ca^{2+} could become bound to EGTA and, if displaced from the chelator by a substitute divalent cation, could give the appearance of direct activation by the substitute divalent ion. The magnitude of the possible "displacement effect" was calculated for each substitute divalent cation. With the exception of Cd^{2+} , which exhibits a particularly high affinity for EGTA (Chang et al., 1988), displacement effects could not account for the activation of basolateral ionic currents unless the

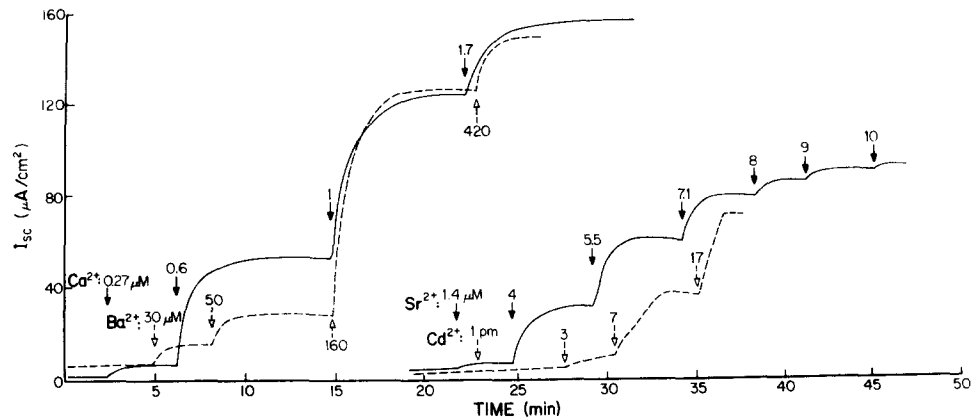


FIGURE 14. Activation of basolateral Cl^- currents by substitute divalent cations. Increasing doses of Ca^{2+} , Ba^{2+} , Sr^{2+} , and Cd^{2+} caused stepwise activation of Cl^- currents. The free concentration of divalent cation achieved with each dose is indicated by the values above the arrows. Free divalent cation concentrations were calculated as described in Materials and Methods.

total Ca^{2+} due to contaminants exceeded $100 \mu M$. With Cd^{2+} as a substitute ion, as little as $10 \mu M$ total contaminating Ca^{2+} could produce a significant displacement effect. We estimated the actual level of contaminating Ca^{2+} in two ways. In one we used the response of the basolateral conductance as a "bioassay" for the free Ca^{2+} concentration in a mucosal bathing solution that did not contain EGTA or added Ca^{2+} . A comparison of the peak currents obtained using these solutions with those generated under EGTA-buffered conditions indicated that the level of free contaminating Ca^{2+} was $\sim 1 \mu M$. In addition, we calibrated a Ca^{2+} -selective electrode using EGTA-buffered solutions (see Methods and Chang, et al., 1988). The electrode yielded an estimate of contaminating free Ca^{2+} of $1.7 \pm 0.3 \mu M$ ($\bar{x} \pm SEM$, $n = 8$). Total Ca^{2+} in these same solutions was determined by atomic absorption spectrophotometry to be $60 \pm 3 \mu M$ ($\bar{x} \pm SEM$, $n = 5$), which indicates that, even in the

absence of added EGTA, 97% of the total Ca^{2+} is bound, presumably to protein. These observations support the presumption that, with the possible exception of Cd^{2+} , activation by substitute divalent cations can be attributed to the substitute cations and not to displaced Ca^{2+} .

Fig. 14 shows examples of stepwise activation of I_{Cl} by Cd^{2+} , Sr^{2+} , Ba^{2+} , and Ca^{2+} . In such experiments, the tissue exposed to the substitute divalent ion was paired with one exposed to Ca^{2+} . The activation produced by Ca^{2+} was defined as 100%, and activation due to the substitute ions was normalized to this value. Of the substitute ions examined, only Mg^{2+} did not activate either I_{K} or I_{Cl} . Fig. 15 illustrates the log dose-response relationships for the activation of I_{K} and I_{Cl} by Mn^{2+} , Ca^{2+} , Sr^{2+} , and Ba^{2+} . Activation by Cd^{2+} is shown in the bottom panel because activation

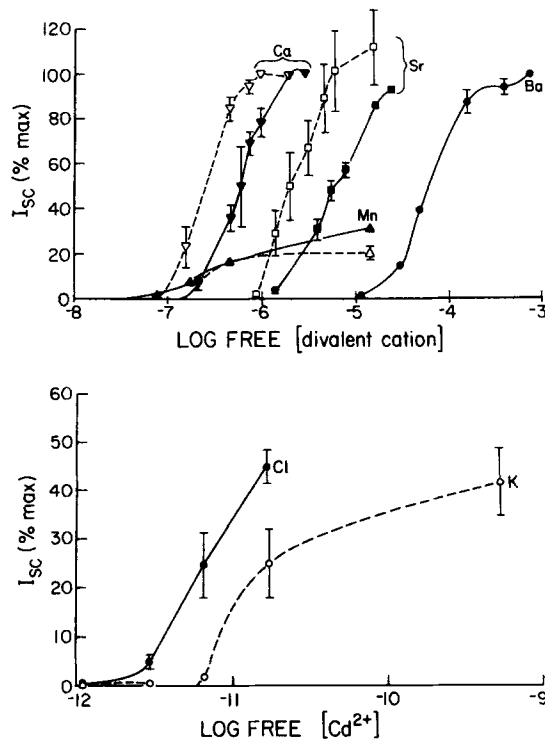


FIGURE 15. (Top) Log dose-response relationship of basolateral K^+ (open symbols and dashed lines) and Cl^- (filled symbols and solid lines) currents activated by divalent cations. (Bottom) Activation of I_{K} and I_{Cl} by Cd^{2+} . In paired experiments maximal activation produced by Ca^{2+} was defined as 100%, with activation produced by each substitute divalent ion normalized to this value.

occurred at concentrations in the picomolar-to-nanomolar range of free ion concentration. Sr^{2+} produced nearly 100% activation of I_{K} and I_{Cl} , but the ion was ~ 10 times less potent than Ca^{2+} . As noted previously for Ca^{2+} , the activation curve for I_{Cl} is shifted to the right, which demonstrates that the anion conductance is less sensitive to activation by both of these ions. Only one curve is shown for Ba^{2+} because this ion activated I_{Cl} but not I_{K} . In view of the fact that Ba^{2+} is a potent blocker of I_{K} on the cytoplasmic side, the question of possible activation of I_{K} by Ba^{2+} is moot. Mn^{2+} activated I_{K} and I_{Cl} to a similar extent, but neither current approached maximal activation. A similar response was obtained with Cd^{2+} but at lower concentrations of the free ion.

This limited comparison suggested that the selectivity of activation was nearly identical for I_K and I_{Cl} , although there were, as noted earlier, quantitative differences in sensitivity to Ca^{2+} and Sr^{2+} . The single major difference that emerged from this comparison was the activation by Ba^{2+} of I_{Cl} but not of I_K . This differential effect could permit Ba^{2+} to serve as a selective activator of I_{Cl} in a variety of experimental situations.

DISCUSSION

The objective of the experiments described here was twofold. First, we wished to determine the feasibility of using digitonin to functionally remove the apical membranes of the epithelial cells of turtle colon under conditions where the basolateral membranes retained their functional integrity and the basolateral ion conductance could be measured simply as a transmural current due to an applied ionic emf. Second, we wished to explore the possibility that, by using digitonin-permeabilized cells in conjunction with a mucosal Ca^{2+} -EGTA buffer system, it would be possible to control the concentration of free ionized Ca^{2+} at the cytoplasmic face of the basolateral membrane. The results indicate that mucosally applied digitonin can be used to selectively "remove" the apical membrane of turtle colon epithelial cells, and that this technique can be employed to provide access to the cytoplasmic face of the basolateral membrane and to "clamp" cytosolic Ca^{2+} at desired levels.

The Action of Digitonin on Cell Membranes

Digitonin is a member of a family of compounds referred to as saponins, all of which possess detergent properties and the ability to lyse cells. These compounds have been employed to "permeabilize" cells to Ca^{2+} and proteins (Becker et al., 1980; Murphy et al., 1980; Burgess et al., 1983). The permeabilizing effect appears to be due to the formation of insoluble, pore-like complexes of digitonin and membrane cholesterol (Bangham and Horne, 1962; Glauert et al., 1962; Blaustein et al., 1978*a, b*), which results in a disordering of lipid bilayer structure to the extent that it can be visualized by means of electron microscopy (Seeman et al., 1973; Fiskum et al., 1980). The permeabilizing effects of digitonin are thought to be relatively specific for plasma membranes because of the higher cholesterol/phospholipid ratio in these structures. Mitochondrial and endoplasmic reticulum membranes, having a lower cholesterol to phospholipid ratio, are less sensitive to the detergent. We found that 20–40 μM digitonin was sufficient to selectively remove the apical membrane of turtle colon cells. Fiskum (1985) reported that the minimum concentration needed for digitonin to permeabilize plasma membranes of a variety of cell types ranges from 40 to 800 μM . A 10–50- μM concentration of digitonin released soluble components of the cytosol (LDH) from cultured chromaffin cells (Dunn and Holz, 1983).

On the basis of the results presented here, there seems to be little doubt that exposure to digitonin caused a striking permeabilization of the apical membranes of colonic cells, to the extent that the cytoplasmic enzyme, LDH, was released into the mucosal bath. In the presence of mucosal digitonin and a nonactivating (nanomolar) free Ca^{2+} concentration, the apical membranes were highly permeable to ^{45}Ca -

EGTA, ^{86}Rb , and LDH. Thus, the lack of a significant I_{sc} under these conditions must be attributed to the low ionic conductance of the basolateral membranes. It seems likely that at least one reason for the low K^+ conductance was the relatively acidic pH (6.6) employed in these experiments (see above and Discussion). It follows that the activation of I_{K} must occur at the basolateral membranes of the epithelial cells. These results eliminate the alternative hypothesis that the effect of Ca^{2+} addition was to activate the digitonin-permeabilization process. The efficacy of digitonin in permeabilizing the apical membrane raises the question of what factors lead to the preservation of the basolateral membrane in the presence of the detergent. A portion of the digitonin in the mucosal solution is expected to be depleted because of reactions with apical membrane cholesterol and, to a lesser extent, with the cholesterol of apically directed, mucous-containing secretory vesicles. In addition, the long, thin, columnar shape of the colonic epithelial cells may function as a barrier that prevents access of the digitonin to the basolateral membrane.

The digitonin-treated cell appears to be a useful intermediate between the intact cell and a preparation of isolated membranes (vesicles) or organelles. It is certain that treatment with digitonin, which facilitated entry of Ca^{2+} -EGTA, also resulted in the release of a significant percentage of the cytoplasmic constituents, including soluble proteins, amino acids, nucleotides, etc., all of which could be important for the integrated function of the cell. On the other hand, it is equally true that the remaining components, in this case the basolateral membrane and the intracellular membranes, are probably present in a context that is structurally more realistic than that encountered, for instance, in a membrane vesicle.

Ca²⁺ Activity in Digitonin-treated Cells

Digitonin-treated cells of various kinds have been widely used to study the effects and the metabolism of cellular Ca^{2+} (Blaustein et al., 1978*a, b*; Becker et al., 1980; Burgess et al., 1983; Streb and Schulz, 1983). The general strategy of such experiments has been to use the detergent to bring cellular Ca^{2+} into equilibrium with the solutions bathing the cells. Several observations led us to conclude that, in digitonin-treated colonic sheets, cytoplasmic Ca^{2+} activity was identical to that of the mucosal bathing solution. First, the degree of permeabilization of the apical membrane is such that there should be little or no barrier to the diffusional entry of Ca^{2+} or EGTA. Second, the lag time between the introduction of the Ca^{2+} into the mucosal bath and the activation of current was on the order of several seconds. If the cytoplasmic diffusion coefficient for Ca^{2+} is of the order of 10^{-7} cm^2/s , as suggested by Kushmerick and Podolsky (1969), then this allows for a diffusion distance of ~ 10 μm , which is the approximate height of the cell layer. Finally, the dose-response curve for Ca^{2+} activation of I_{K} is slightly more sensitive than, but similar to, that determined for single K^+ channels in detached membrane patches (Barrett et al., 1982) from cultured rat skeletal muscle. The dose-response curve for Ca^{2+} activation of I_{Cl} is similar to that reported by Evans and Marty for single Cl^- channels (1986).

Although it seems reasonable to suppose that the average cytoplasmic Ca^{2+} activity in the digitonin-treated cells is in equilibrium with that of the mucosal bath, we cannot predict with certainty the activity at the inner surface of the basolateral

membrane. Specifically, we cannot exclude the possibility that Ca^{2+} exchange between the cytosol and cellular organelles might produce local variations in cytoplasmic Ca^{2+} . Available evidence, however, suggests that the magnitude of such effects should be small. As shown in Results, doubling the concentration of EGTA did not appreciably alter the relation between K^+ current activation and mucosal free Ca^{2+} , even though the increase in buffer capacity can be shown to reduce the sensitivity to contaminating Ca^{2+} by a factor of 2. Furthermore, the absence of ATP from the mucosal buffer solution should reduce energy-dependent Ca^{2+} transport processes. The possible effects of cellular sequestration can be estimated using rates of Ca^{2+} uptake by digitonin-permeabilized hepatocytes (Burgess et al., 1983) and enterocytes (van Corven et al., 1987). In the presence of 5 mM EGTA, a cell would require 500 min to change the free Ca^{2+} concentration by 100 nM in the 0.1–1.0- μM range of free Ca^{2+} concentration.

Ca^{2+} Activation of Basolateral Conductance

The observation that acute increases in the activity of free Ca^{2+} in the solution bathing the permeabilized apical membrane of colonic cells leads to the activation of ion conductances was important for two reasons. First, as discussed in more detail below, these observations provide evidence that basolateral conductances for both K^+ and Cl^- can be modulated by intracellular Ca^{2+} concentrations that we presume to be in the physiological range. In addition, however, the activation of basolateral conductances provided an important "bioassay," which indicated that perturbations in mucosal Ca^{2+} were rapidly sensed at the basolateral aspect of the cells. This observation suggested that the "site of action" of the acute changes in cellular Ca^{2+} was at the basolateral cell membrane. The steep relationship between ion conductance and the intracellular free Ca^{2+} concentration (Fig. 13) suggests that a positively cooperative process is involved in activating the ion conductances. Ca^{2+} -activated conductances are typically characterized by a steep power relationship between conductance or open probability and Ca^{2+} activity (Meech and Thomas, 1980; Barrett et al., 1982; Latorre and Miller, 1983), although a linear relation between the intracellular Ca^{2+} concentration and the magnitude of a Ca^{2+} -activated K^+ conductance was reported by Gorman and Thomas (1980) in *Aplysia* neurons.

Several observations suggested that the effect of the Ca^{2+} must be on plasma membrane rather than tight junctions. First, the paracellular path in turtle colon is characterized by ion selectivity that parallels that of free solution (Dawson, 1977; Halm and Dawson, 1984), whereas the Ca^{2+} -activated currents imply highly selective paths, which are atypical of tight junctions. Furthermore, the currents were blocked by agents (quinidine, Ba^{2+} , and anthranilic acid derivatives) that are thought to act on cell membranes. Quinidine, for instance, blocks single K^+ channels recorded in isolated cells using the patch-clamp technique (Richards and Dawson, 1986).

Studies on the tight junctions of the MDCK (Madin-Darby canine kidney) cell culture monolayers by Martinez-Palomo et al. (1980) demonstrated that the ability of occluding junctions to serve as permeability barriers specifically depended on millimolar concentrations of extracellular free Ca^{2+} . Replacement of extracellular Ca^{2+} with Mg^{2+} or Ba^{2+} did not suffice. In addition, the maintenance of lower intracellular Ca^{2+} concentrations (<1 mM) is required to stabilize the resistance of the occlud-

ing junctions. Under the permeabilized conditions in our experiments, an external free Ca^{2+} concentration of 1 mM on the serosal side and very low internal free Ca^{2+} concentrations on the mucosal side satisfy the Ca^{2+} requirements that are necessary for the tight junctions to remain effective as permeability barriers.

These experiments did not address the issue of possible cellular heterogeneity in the response, i.e., the possibility that Ca^{2+} -activated currents occur only in a fraction of the cells. Nor do we know whether the apical permeabilization effect was uniform across the epithelial area. The turtle colon is a relatively homogeneous cell layer, however, which lacks the crypt regions that characterize the mammalian colon (Germann et al., 1986a).

The experiments presented here also did not address the issue of the nature of the activation process. The rapid activation of currents caused by added Ca^{2+} and the inactivation caused by the addition of EDTA are both consistent with the gating properties expected for Ca^{2+} -activated channels, but could represent, in principle, the rapid, Ca^{2+} -dependent insertion and retrieval of channels.

Comparison with Ca^{2+} -activated Channels

If we assume, provisionally, that the Ca^{2+} -activated currents measured in this study reflect the properties of a population of Ca^{2+} -activated channels, it is then of interest to compare the behavior of the basolateral currents with the properties of the Ca^{2+} -activated channels that have been studied using single-channel recording techniques. Of these, the K^+ conductances are the best characterized, and it is possible to make comparisons on the basis of Ca^{2+} activation kinetics, blocker specificity, and ion selectivity. Our measurements yielded an apparent $K_{1/2}$ for activation of $\sim 0.4 \mu\text{M}$ free Ca^{2+} (at 0 mV). This value compares with values such as $5 \mu\text{M}$ (0 mV) obtained for cultured rat muscle (Barrett et al., 1982) and $30 \mu\text{M}$ (0 mV) for rabbit skeletal muscle (Vergara et al., 1984). The relatively acidic cellular pH employed in our studies would, if anything, be expected to shift the activation curve to the right, i.e., to reduce Ca^{2+} sensitivity by competing for binding sites. The Ca^{2+} -activated K^+ basolateral conductance was blocked by quinidine, Ba^{2+} , and TEA, all of which have been reported to block Ca^{2+} -activated K^+ channels. Despite such general similarities, however, there are differences in detail. For instance, Ba^{2+} was an effective blocker of Ca^{2+} -activated basolateral currents only from the cytosolic side. This extreme sidedness has not been reported in single-channel studies, although Ba^{2+} blockade is generally less potent at the extracellular face of the membranes. TEA has been identified as a blocker of K^+ channels in a variety of excitable cells and is thought to be a relatively "specific" blocker for some Ca^{2+} -activated K^+ channels (Latorre and Miller, 1983; Blatz and Magleby, 1984; Yellen, 1984). Unlike most Ca^{2+} -activated K^+ channels, which show greater sensitivity to external TEA (Latorre and Miller, 1983), basolateral K^+ currents exhibited a much greater sensitivity to internal TEA. To date, only Ca^{2+} -activated K^+ channels in cloned anterior pituitary cells exhibited a similar sidedness of TEA sensitivity (Wong and Adler, 1986). In addition, unlike the small conductance (10–14 pS) of Ca^{2+} -activated K^+ channels (SK channels) in cultured rat skeletal muscle (Blatz and Magleby, 1986), $100 \mu\text{M}$ apamin had no effect on either the cytoplasmic or external side.

We determined the selectivity of the Ca^{2+} -induced basolateral cation conductance

by comparing currents induced in the presence of gradients of K^+ , Rb^+ , Cs^+ , and Na^+ . Such comparisons are complicated by the fact that differences in currents may reflect differences in gating or efficacy of activation due to the substitute cation as well as simply to differences in the conduction properties of the channels. It was noteworthy, however, that the Ca^{2+} -activated Rb^+ currents were ~65% of the magnitude of the corresponding K^+ currents. This is in contrast to the observation that is typical for Ca^{2+} -activated K^+ channels, namely that although reversal potentials indicate that $P_{Rb} = P_K$, single-channel Rb^+ currents are greatly reduced or undetectable (Petersen and Maruyama, 1984). The comparatively high Rb^+ conductance observed in the present study should serve as an important tag for identifying the channels that are the basis for these currents.

Detailed comparisons for the Cl^- conductance are more difficult because fewer of these have been described in the literature. Ca^{2+} -activated Cl^- conductances have been identified in tracheal epithelial cells (Frizzell et al., 1986) and in rat lacrimal cells (Findlay and Petersen, 1985; Evans and Marty, 1986). Of the Ca^{2+} -dependent Cl^- conductances that have so far been described, detailed information about ion selectivity is available for only one of them, that in rat lacrimal gland membranes (Evans and Marty, 1986). Based on the measurements of macroscopic currents, we concluded that the Ca^{2+} -activated anion conductance did not discriminate among Cl^- , Br^- , I^- , NO_3^- , and SCN^- , whereas acetate could not carry current. Evans and Marty (1986) report a similar lack of selectivity (based on reversal potential measurements) for a Ca^{2+} -activated Cl^- channel in the lacrimal gland. Furthermore, our Ca^{2+} activation dose-response curve is in almost exact agreement with their findings (Evans and Marty [1986] reported Ca^{2+} activation in the 0.5–2.0- μM range). However, unlike the findings of Evans et al. (1986) we were unable to block the Ca^{2+} -activated Cl^- conductance in turtle colonic cells with 1 mM furosemide on either the mucosal or serosal side.

The Ca^{2+} -activated K^+ and Cl^- currents measured in this study exhibited another property not generally detected in single channel studies: spontaneous inactivation. We did not study the details of this process except to show that it was not altered by supplying additional Ca^{2+} .

Physiological Significance of the Ca^{2+} -activated Basolateral Channels

Ultimately, the objective of these studies is to use permeabilized cell layers to study the regulation of basolateral ion conductances by intracellular mediators. To achieve this goal, it was necessary to make a compromise experimentally that is not unlike that which must be made in experiments using whole-cell clamping or reconstitution methodology; to achieve the desired resolution and control of intracellular composition, it is necessary to examine the properties of the basolateral membrane under conditions that are different from that normally encountered in the functioning cell. This limitation, common to all such experiments, can only be overcome by using a detailed characterization of the properties of ion conductances as a "fingerprint" (Diamond and Wright, 1969) by which the channel can be identified in a more integrated setting. In previous studies, we have explored the properties of basolateral conductances in turtle colon in a variety of experimental conditions including normal in vitro transport (Halm and Dawson, 1984), amphotericin-perme-

abilized sheets (Germann et al., 1986*b*; Venglarik and Dawson, 1986), and single-channel recording from isolated cells (Richards and Dawson, 1986), so that we have some notion of what we expect to find in the basolateral membrane of digitonin-permeabilized cells. Briefly, previous studies provided evidence for at least two (and perhaps three) types of basolateral K^+ channels and a basolateral Cl^- channel (Germann et al., 1986*a, b*; Venglarik et al., 1986). Germann et al. (1986*a, b*) distinguished two types of basolateral K^+ channels, one associated with normal transport and another that was activated by cell swelling. The latter conductance was blocked by quinidine and lidocaine and appears to be due to a population of 17-pS K^+ channels identified by Richards and Dawson (1986). The Ca^{2+} -activated K^+ conductance measured in digitonin-permeabilized cells was blocked by quinidine, but it was relatively insensitive to lidocaine under the conditions of this study. Furthermore, the osmotically induced conductance studied by Germann et al. (1986*a, b*) and the K^+ channel reported by Richards and Dawson (1986), exhibited a high selectivity for K^+ over Rb^+ , a characteristic not shared by the conductance examined in the present study.

Venglarik and Dawson (1986) studied the effects of muscarinic agonists on the resting basolateral K^+ conductance. The resting K^+ conductance, which is insensitive to quinidine and lidocaine, is relatively nonselective for K^+ over Rb^+ , and is inactivated by carbachol. This study disclosed an additional conductance, however, which is transiently activated after the application of muscarinic agonist. Venglarik and Dawson (1986) speculated that this conductance could be due to a population of Ca^{2+} -activated channels that were responding to a transient increase in cellular Ca^{2+} after agonist binding. This conductance can support currents due to K^+ or Rb^+ but does not appear to be blocked by quinidine.

Venglarik et al. (1986) identified a Cl^- conductance in the basolateral membrane of polyene-treated colonic cells, but, in the absence of a specific blocker, the comparison of the properties of this channel with that activated by Ca^{2+} in permeabilized cells is difficult.

At this point, it is not possible to assign either of the Ca^{2+} -activated conductances measured in digitonin-treated cells a definite role in the function of the normal cell. In addition, we must await single-channel studies to determine if the two Ca^{2+} -activated conductances reside in the same cells or in separate cells. However, several features of the preparation are worth reiterating. First, we made no attempt to "enrich" the mucosal bath with any presumed cytoplasmic constituents (other than Ca^{2+}), nor did we include, for instance, protease inhibitors. This approach was motivated by our desire to make the experimental situation as simple as possible. Unquestionably, the major complicating factor with regards to the significance of the observed conductance was the relatively acidic pH used in these studies. After preliminary experiments at a variety of mucosal pHs, we chose the value of 6.6 for a variety of reasons. The overriding consideration in our early experiments was the behavior of the Ca^{2+} -EGTA buffer system and our desire to maintain control over free Ca^{2+} concentration over a wide range. As discussed in Materials and Methods, this situation is most easily achieved at an acidic pH where the intrinsic Ca^{2+} buffering capacity of EGTA is enhanced due to protonation of some of its sites. This allowed us to explore a wide range of Ca^{2+} activities by simply adding different

amounts of Ca^{2+} to the buffer system. A second reason was that the acidic cellular pH, while it complicated the interpretation of the results, simplified the experimental protocol enormously by reducing the background conductance of the basolateral membrane. Previous experiments, as discussed above, showed that this conductance is complex, consisting of as many as three K^+ conductances and at least one anion conductance, plus a small Na^+ conductance (Kirk and Dawson, 1985). The acidic pH reduced the basal conductance and facilitated the identification of the Ca^{2+} -activated components of the basolateral ion current. Protons are expected to exert a variety of effects on ion channels and it would not be surprising to find that activation kinetics, drug action, and ion selectivity all exhibited sensitivity to pH.

We are indebted to Melinda E. Lowy for her help with these experiments, to Dr. Ronald W. Holz for stimulating discussions and for the use of his Ca^{2+} -selective electrode, and to Dr. Joel M. Weinberg for the use of his atomic absorption spectrophotometer. We are grateful to E. R. Squibb & Son, Inc. (Princeton, NJ) for the gift of amphotericin-B.

This work was supported by National Institutes of Health grants AM-32901, AM-29786, and AM-27559 to D. C. Dawson.

Original version received 26 October 1987 and accepted version received 26 February 1988.

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