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Pathways for K^+ Efflux in Isolated Surface and Crypt Colonic Cells. Activation by Calcium

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Abstract. K^+-conductive pathways were evaluated in isolated surface and crypt colonic cells, by measuring ^{86}Rb efflux. In crypt cells, basal K^+ efflux (rate constant: 0.24 ± 0.044 min^{-1}, span: 24 ± 1.3%) was inhibited by 30 mM TEA and 5 mM Ba^{2+} in an additive way, suggesting the existence of two different conductive pathways. Basal efflux was insensitive to apamin, iberiotoxin, charybdotoxin and clotrimazole. Ionomycin (5 μM) stimulated K^+ efflux, increasing the rate constant to 0.65 ± 0.007 min^{-1} and the span to 83 ± 3.2%. Ionomycin-induced K^+ efflux was inhibited by clotrimazole (IC_{50} of 25 ± 0.4 μM) and charybdotoxin (IC_{50} of 65 ± 5.0 nM) and was insensitive to TEA, Ba^{2+}, apamin and iberiotoxin, suggesting that this conductive pathway is related to the Ca^{2+}-activated intermediate-conductance K^+ channels (IK_{ca}). Absence of extracellular Ca^{2+} did neither affect basal nor ionomycin-induced K^+ efflux. However, intracellular Ca^{2+} depletion totally inhibited the ionomycin-induced K^+ efflux, indicating that the activation of these K^+ channels mainly depends on intracellular calcium liberation. K^+ efflux was stimulated by intracellular Ca^{2+} with an EC_{50} of 1.1 ± 0.04 μM. In surface cells, K^+ efflux (rate constant: 0.17 ± 0.027 min^{-1}; span: 25 ± 3.4%) was insensitive to TEA and Ba^{2+}. However, ionomycin induced K^+ efflux with characteristics identical to that observed in crypt cells. In conclusion, both surface and crypt cells present IK_{Ca} channels but only crypt cells have TEA- and Ba^{2+}-sensitive conductive pathways, which would determine their participation in colonic K^+ secretion.

Key words: Colonic crypt and surface cells — K^+ efflux — Ca^{2+}-dependent K^+ channels

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Introduction

K^+ secretion in the colon is supported by K^+ uptake across the basolateral membrane of the epithelial cell, mediated by the $Na^+/K^+/2Cl^-$ cotransporter and the Na^+/K^+ pump, producing an intracellular K^+ activity greater than that determined by electrochemical driving forces. The relative conductance of the apical and basolateral membranes for K^+ determines the movement of this ion into the luminal and serosal side of the epithelium, respectively. In the absence of an apical K^+ conductance, active K^+ secretion is absent; in contrast, when the K^+ conductance of the apical membrane is increased, active K^+ secretion may be observed. Thus, mucosal addition of K^+ channel blockers (e.g., TEA and Ba^{2+}) inhibit active K^+ secretion [1, 10, 30, 31]. Therefore, colonic cells involved in the K^+ secretion may have apical K^+ channels sensitive to TEA and barium. In addition to the secretory process, active K^+ absorption is also present in distal colon. K^+ enters the cell across the apical membrane in a process mediated by a K^+/H^+ ATPase [1, 6] and exits the cell through conductive pathways. In general, it is accepted that the secretory processes are located at the crypt level while the absorptive mechanisms are placed at the surface of the colonic epithelium [15, 20, 26].

Activation of basolateral K^+ channels is believed to play an important role in maintaining the driving force for the secretory process by allowing K^+, entering the cell through the $Na^+/K^+/2Cl^-$ cotransporter and the Na^+/K^+ ATPase, to be recycled back across the basolateral plasma membrane. The resulting cell hyperpolarization counteracts the depolarizing effects of apical Cl^- secretion, thus promoting further secretion. An increase in intracellular free calcium appears to be an important regulator of K^+ channels in intestinal epithelia [21, 26, 33] and Ca^{2+}-dependent K^+ conductances have been identified in different species including rat, rabbit and

human cultured cells [2, 4, 14, 16, 24, 32]. Moreover, Ca^{2+} has been reported to stimulate K^+ and Cl^- secretion [11, 23, 29]. The mechanisms by which an increase in intracellular Ca^{2+} stimulates K^+ secretion are poorly defined and it is not clear whether the effects of Ca^{2+} are direct or mediated through other Ca^{2+}-dependent modulators. There is evidence for direct ion-channel gating by Ca^{2+} in several systems [2, 4, 14, 16, 24, 32]. However, in others, Ca^{2+} mediates its effects indirectly through the activation of specific protein kinases (protein kinase C or Ca^{2+}/calmodulin protein kinase) [19, 24, 27].

Due to the importance of K^+ channels in the transepithelial ion movement, the study of their characteristics and their regulatory processes gains a particular relevance in colonic physiology. K^+ channels have been well characterized using electrophysiological techniques and pharmacological approaches. However, knowledge of the participation of these channels in the overall of K^+ transport in colonic cells is limited.

The aim of this study was to identify, measuring ^{86}Rb efflux, the K^+-conductive pathways present in surface and crypt cells isolated from guinea-pig distal colon and to evaluate the role of calcium as a potential regulator of these conductive pathways. The efflux technique permits the simultaneous evaluation of different K^+ channels in a particular cell type and the determination of the contribution of each conductive pathway in the overall K^+ movement in these cells. Additionally, the available pharmacological arsenal allows the characterization of each conductive pathway under physiological conditions and the study of their regulatory mechanisms.

Materials and Methods

Materials

^{86}Rb and [^{3}H]-inulin were purchased from Amersham (Little Chalfont, Buckinghamshire, England). All other chemicals of analytical grade were obtained from Sigma Chemicals (St. Louis, MO) or from Merck (Rahway, NJ). BAPTA (1,2-bis(2-aminophenoxy)ethane N,N,N′-tetraacetic acid), thapsigargin and fura-2/AM were supplied by Molecular Probes (Eugene, OR). Charybdotoxin was supplied by Alomone Labs (Jerusalem, Israel). Clotrimazole was purchased from Sigma.

Animals

Male guinea pigs (weight range of 300–350 g) were used. The animals were maintained on a regular laboratory diet.

Cell Isolation

Colonic surface and crypt cells were isolated by the procedure previously described [7]. Briefly, the distal colon was excised from the colonic flexure to the rectum and then rinsed, filled and incubated for 10 min at 37°C with Solution 1 [in mM: 7 K_2SO_4, 44 K_2HPO_4, 9 $NaHCO_3$, 10 sodium citrate, 10 HEPES-Tris (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid-tris (hydroxymethyl) aminomethane) and 180 glucose at pH 7.4 and a total osmolarity of 340 mosM]. The luminal content was discarded and the intestinal segment was refilled and incubated for 3 min at 37°C with Solution 2, which contained 0.5 mM dithiothreitol and 0.25 mM EDTA in substitution of the sodium citrate of Solution 1. The intestine was then gently palpated and the luminal content, containing isolated cells, was collected in 100 ml Dulbecco's medium at 4°C, filtered through a nylon mesh (60-μm pore diameter) and centrifuged twice at $100 \times g$ for 5 min. This step was repeated six times to obtain six different fractions.

Surface and crypt cells were obtained by sequential palpation of the intestinal segment, as indicated. As shown before [7], cells isolated in the first two palpations (fractions 1–2) were surface cells, and those isolated from the fifth and sixth palpation (fractions 5–6) were crypt cells. The isolated cells were resuspended in Dulbecco's medium and stored at 4°C in plastic tubes without agitation. Dulbecco's medium was modified to contain (in mM) 116 NaCl, 5 KCl, 10 $NaHCO_3$, 1 NaH_2PO_4, 10 K_2SO_4 and 10 HEPES-Tris, at pH 7.4 and a total osmolarity of 320 mOsm. All solutions were oxygenated with 100% O_2 for 20 min before use. Cell viability was evaluated by trypan blue exclusion; oxygen consumption and intracellular ion concentrations were determined as described earlier [5]. Cells were used only when viability was higher than 95%.

Cell Transport Experiments

For efflux experiments, cells were preincubated in Dulbecco's medium for 30 min at 25°C in the modified Dulbecco's medium containing 2 μCi/ml of ^{86}Rb to load cells. At the end of the loading period, cells were washed by centrifugation at $200 \times g$ for 1 min with fresh efflux buffer at 4°C. This washing procedure removes most of the extracellular ^{86}Rb. Efflux was initiated by resuspending the pre-incubated cells (pellet of approximately 30 μl) in 3 ml potassium-free incubation medium, without isotopic tracer, at 25°C, containing 1 mM ouabain and 50 μM bumetanide to block any ^{86}Rb uptake. One 250 μl sample was obtained at the desired time, diluted in 800 μl of ice-cold incubation medium and immediately centrifuged, through an oil layer of dibutylphthalate:dinonylphthalate (3:2), at $13{,}000 \times g$ for 20 s, as previously described [6]. The intracellular radioactivity was determined after correction for the radioactivity present in the trapped volume, measured using [^{3}H]-inulin as an extracellular space marker. ^{86}Rb was counted in a β-scintillation counter (Packard) following addition of Aquasol® scintillation fluid. Isotope effluxes were expressed as percentage of the total intracellular counts present at the beginning of the efflux period, after correction of each cell sample by total cell protein. Experimental data were fitted to a single-exponential decay equation [$y = y_0 + a\ e^{-kt}$] by nonlinear regression (Marquandt-Levenberg algorithm) using commercial programs (Origin 5.0, Microcal Software, Inc.; Prism 2.01, GraphPad Software Inc.), where a is the efflux span, k is the rate constant and y_0 is the plateau.

Measurement of intracellular Calcium

Intracellular calcium was determined in cells loaded with the Ca^{2+}-sensitive dye fura-2. Cells were incubated with 10 μM fura-2/AM at room temperature for 30 min and then washed by centrifugation at $100 \times g$ for 2 min in efflux buffer and resuspended in the same solution. Fluorescence emitted at 510 nm was alternately determined at 350 nm and 380 nm as excitation wavelengths in a Shimatsu spectrofluorometer. Fluorescence was corrected for background and cell autofluorescence before calculation of the 350/380 emission ratio.

Protein Determination

Sample protein content was determined by a modified Coomassie blue method [12]. Cell protein was determined for each batch of cells, using 0.5–0.6 mg of cell protein per experimental point.

Statistics

Results are expressed as mean ± SEM unless otherwise indicated. Difference between means was evaluated by analysis of variance (SigmaStat 2.0, Jandel Corporation) and considered significant at $P < 0.05$.

Results

K^+ Efflux

K^+ efflux was evaluated in isolated colonic crypt cells under basal conditions and the effect of 30 mM tetraethylammonium (TEA) and 5 mM barium, inhibitors of colonic K^+ secretion, was tested (Fig. 1). Experimental data were fitted to a single exponential equation, $y = a \cdot e^{-kt} + y_0$, where a corresponds to the span of the curve, t to incubation time and k to the efflux rate constant, expressed in min^{-1}. As shown in Fig. 1*A*, basal efflux fit to the exponential equation (R^2: 0.998) with a rate constant of 0.24 ± 0.016 min^{-1} and a span of 24 ± 0.8%. TEA and Ba^{2+} inhibited basal K^+ efflux, modifying both rate constant (Fig. 1*B*) and span (Fig. 1*A*) with an IC_{50} of 0.88 ± 0.15 mM and 0.22 ± 0.03 mM, respectively. Their effects were additive, suggesting the existence of at least two different conductive pathways.

In addition, the effect of ionomycin, a Ca^{2+} ionophore, was evaluated (Fig. 2). Ionomycin largely stimulated K^+ efflux, approximately doubling the rate constant and increasing the span to 83% (Fig. 2*A*). This effect of ionomycin on K^+ efflux was neither affected by 5 mM Ba^{2+} (Fig. 2*B*) nor by 30 mM TEA (Fig. 2*C*). These results indicate that colonic crypt cells, under basal conditions, have TEA- and Ba^{2+}-sensitive K^+-conductive pathways and that an increase in intracellular Ca^{2+} additionally opens TEA- and Ba^{2+}-insensitive K^+ channels.

Evaluation of Ca^{2+}-dependent K^+ Efflux

To identify the Ca^{2+}-dependent K^+ channels involved in the ionomycin-induced K^+ efflux, the effect of several Ca^{2+}-dependent K^+ channels blockers was evaluated. Figure 3 shows the effect of apamin, iberiotoxin, clotrimazole and charybdotoxin on K^+ efflux in isolated crypt cells. Under basal conditions, none of the tested K^+ channel inhibitors had any effect on K^+ efflux (Fig. 3*A*). In contrast, ionomycin-induced K^+ efflux was totally inhibited by 100 μM clotrimazole and by 100 nM charybdotoxin, inhibitors of IK_{Ca} channels. However, apamin, an inhibitor

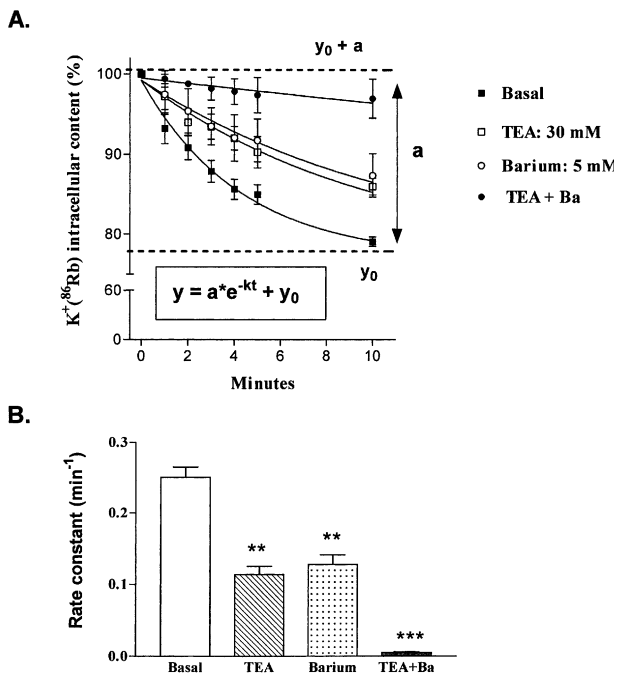


Fig. 1. K^+ (^{86}Rb) efflux in isolated colonic crypt cells. Effect of Ba^{2+} and TEA. (*A*) Time course of ^{86}Rb efflux. Colonic crypt cells were pre-incubated in mammalian medium, containing 20 mM KCl and ^{86}Rb as a tracer, at 25°C for 30 min, in the absence (basal condition) or the presence of 5 mM $BaCl_2$ and/or 30 mM tetraethylammonium (TEA). At the end of this period, cells were washed at 4°C in the same medium, without K^+ or ^{86}Rb, and ^{86}Rb efflux was evaluated for 10 min at 25 °C in the presence of 1 mM ouabain and 50 μM bumetanide, in addition to channel blockers. Cells were immediately diluted in ice-cold mammalian medium, separated from the medium by centrifugal filtration through an oil layer and intracellular radioactivity was determined as described in Methods. Content at time 0 was considered as 100% (initial content). Points show experimental data ± SEM of three different experiments and curves are the best fit to a single-exponential decay equation ($y = y_0 + a \cdot e^{-kt}$) using non-linear regression, where a is the span of the efflux, y_0 is the plateau, t is the time in minutes and k is the rate constant. (*B*) Effect of 30 mM TEA and 5 mM barium on the rate constant of the ^{86}Rb efflux. Values were analyzed by Analysis of Variance and difference between mean considered as significant at $P < 0.05$.

of small-conductance K^+ channels (SK_{Ca}), and iberiotoxin, inhibitor of large-conductance K^+ channels (BK_{Ca}) (Fig. 3*B*) were unable to inhibit it. Figure 4 presents the dose-response curves for clotrimazole (*A*) and for charybdotoxin (*B*) on inomycin-induced K^+ efflux, showing an IC_{50} of 25.3 ± 0.39 μM and 64.7 ± 4.95 nM for clotrimazole and charybdotoxin, respectively. These results suggest that ionomycin induces the opening of intermediate-conductance K^+ channels.

If the effect of ionomycin is produced by the induction of Ca^{2+} entry from the extracellular space, the elimination of Ca^{2+} from the incubation medium should abolish it. However, as shown in Fig. 5*A*, the absence of extracellular Ca^{2+} did not affect iono-

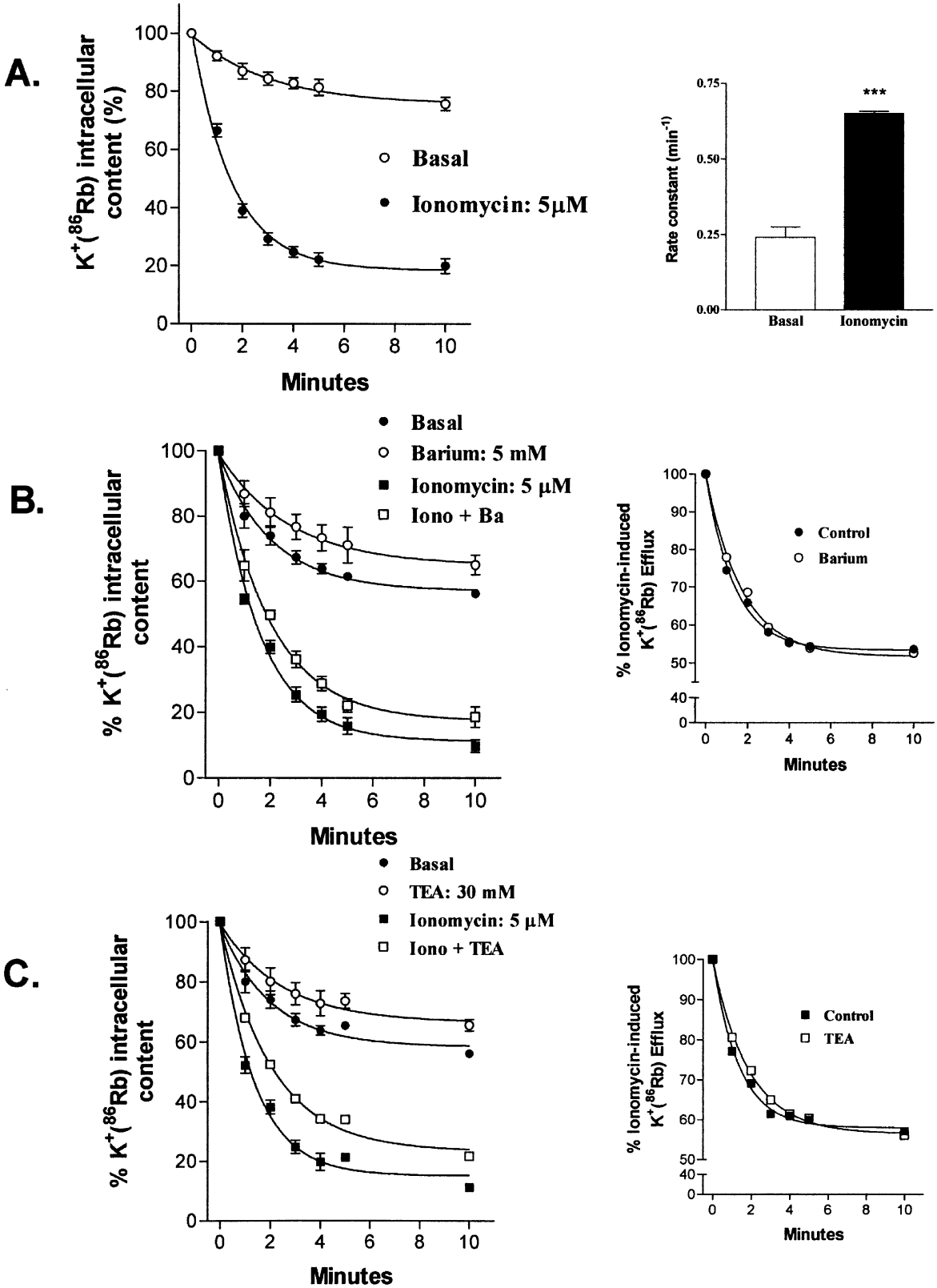


Fig. 2. Effect of Ba^{2+} and TEA on the ionomycin-induced K^+ (^{86}Rb) efflux in isolated colonic crypt cells. (*A*) Ionomycin-induced K^+ efflux. (*B*) Effect of 5 mM Ba^{2+} on basal and ionomycin-induced K^+ efflux. (*C*) Effect of 30 mM TEA on basal and ionomycin-induced K^+ efflux. Cells were pre-incubated with 5 mM $BaCl_2$ or 30 mM TEA and loaded with ^{86}Rb as indicated in Materials and Methods. K^+ (^{86}Rb) efflux was evaluated during 10 min at 25°C. Points show mean ± SEM of experimental data of three different preparations and curves are the best fit to a single-exponential equation, as described in Materials and Methods.

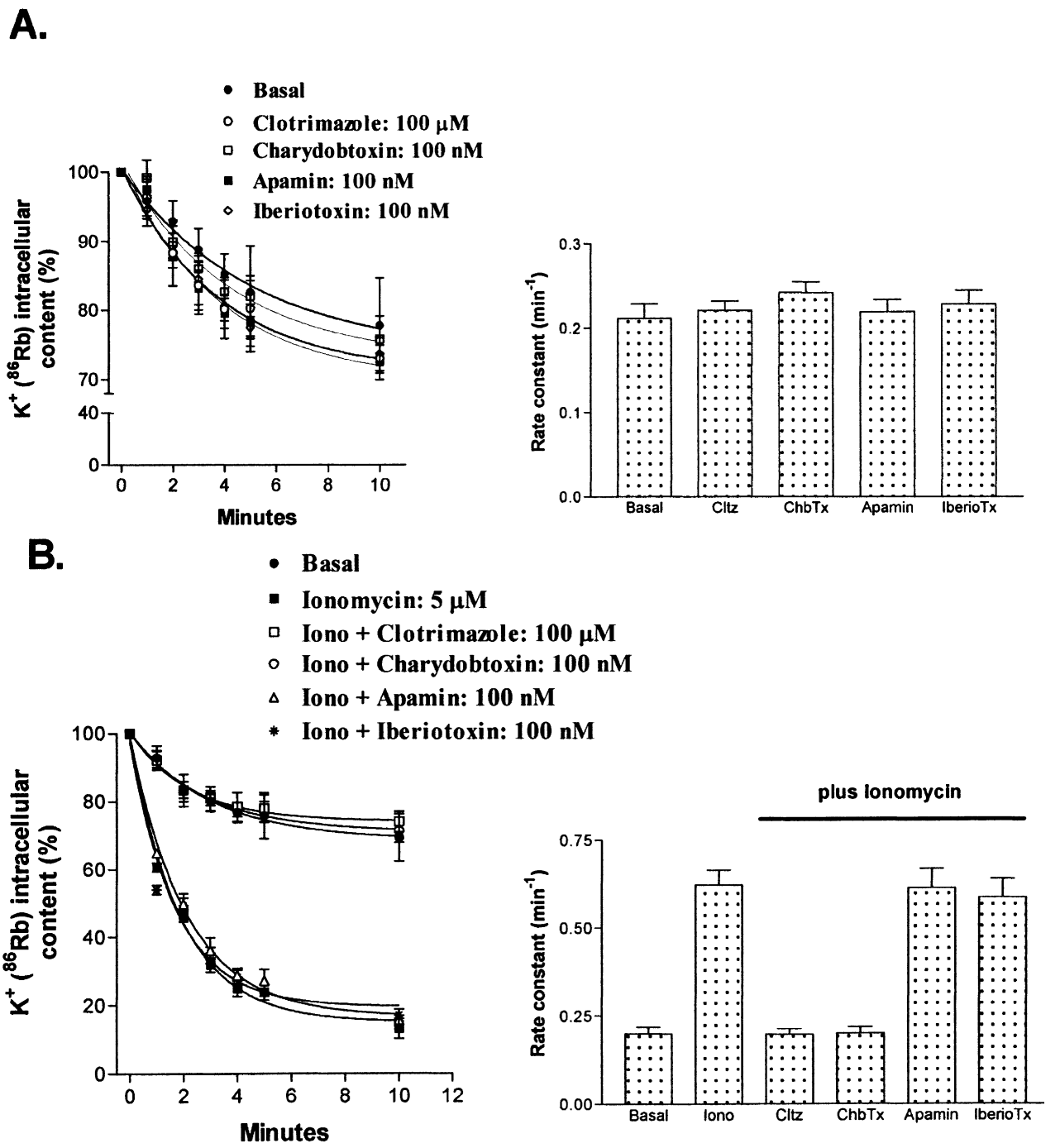


Fig. 3. Effect of clotrimazole, charybdotoxin, iberiotoxin and apamin on basal (*A*) or ionomycin-induced (*B*) K^+ efflux in isolated colonic crypt cells. Colonocytes were pre-incubated in the presence of 100 μM clotrimazole or 100 nM charybdotoxin or 100 nM apamin or 100 nM iberiotoxin during 30 min and loaded with ^{86}Rb as indicated in Materials and Methods. $K^+(^{86}Rb)$ efflux was evaluated during 10 min at 25°C, under basal conditions (*A*) and in the presence of 5 μM ionomycin (*B*) in the incubation medium. Results are mean ± SEM of three different experiments. Points represent experimental data and curves are the best fit to a single-exponential decay equation.

mycin-induced K^+ efflux. When cells were pre-incubated with BAPTA-AM, an intracellular Ca^{2+} chelant, the ionomycin-induced K^+ efflux was only partially inhibited (Fig. 5*B*). Depletion of intracellular Ca^{2+} stores, by preincubation of isolated cells in the absence of extracellular Ca^{2+} and BAPTA-AM, completely inhibited the ionomycin-induced K^+ efflux (Fig. 5*C*). These results indicate that intracellular Ca^{2+} stores play an important role in the activation of intermediate K^+ channels (IK_{Ca}) in colonic crypt cells.

To find experimental conditions that permit the evaluation of the effect of intracellular Ca^{2+} on $K^+(^{86}Rb)$ efflux, we evaluated different pre-incuba-

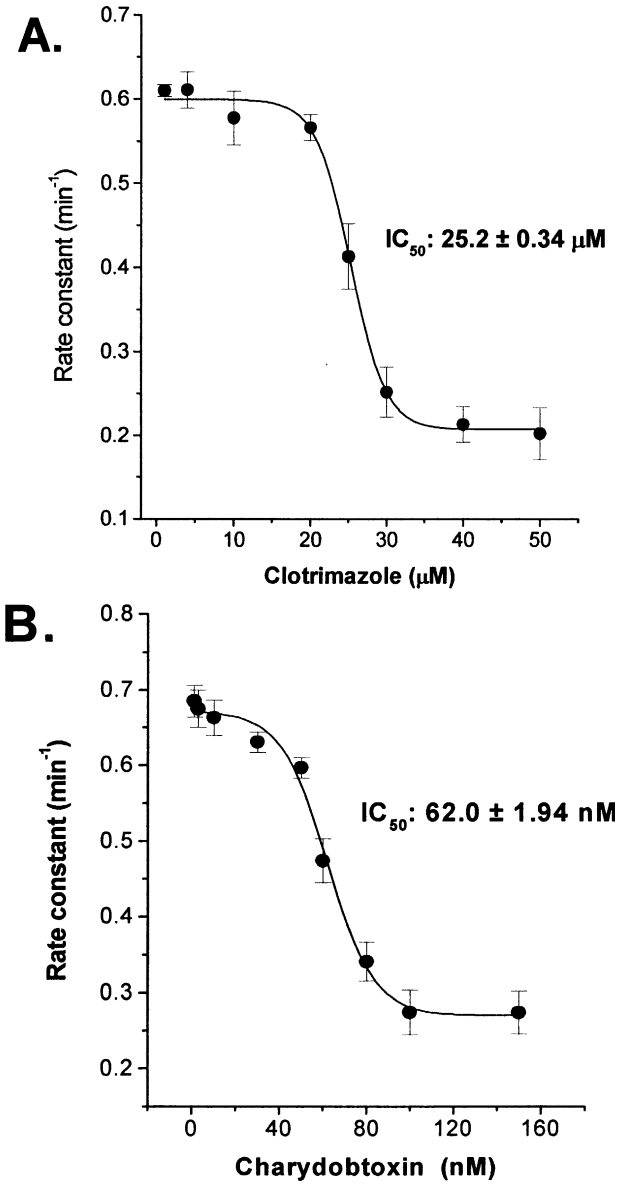


Fig. 4. Dose-response curve for clotrimazole (*A*) and charybdotoxin (*B*) on ionomycin-induced K^+(^{86}Rb) efflux in isolated colonic crypt cells. Results are means ± SEM of three different preparations.

tion conditions that allowed us to deplete the intracellular Ca^{2+} stores and to control the intracellular Ca^{2+} concentration by using known extracellular Ca^{2+} concentrations and ionomycin.

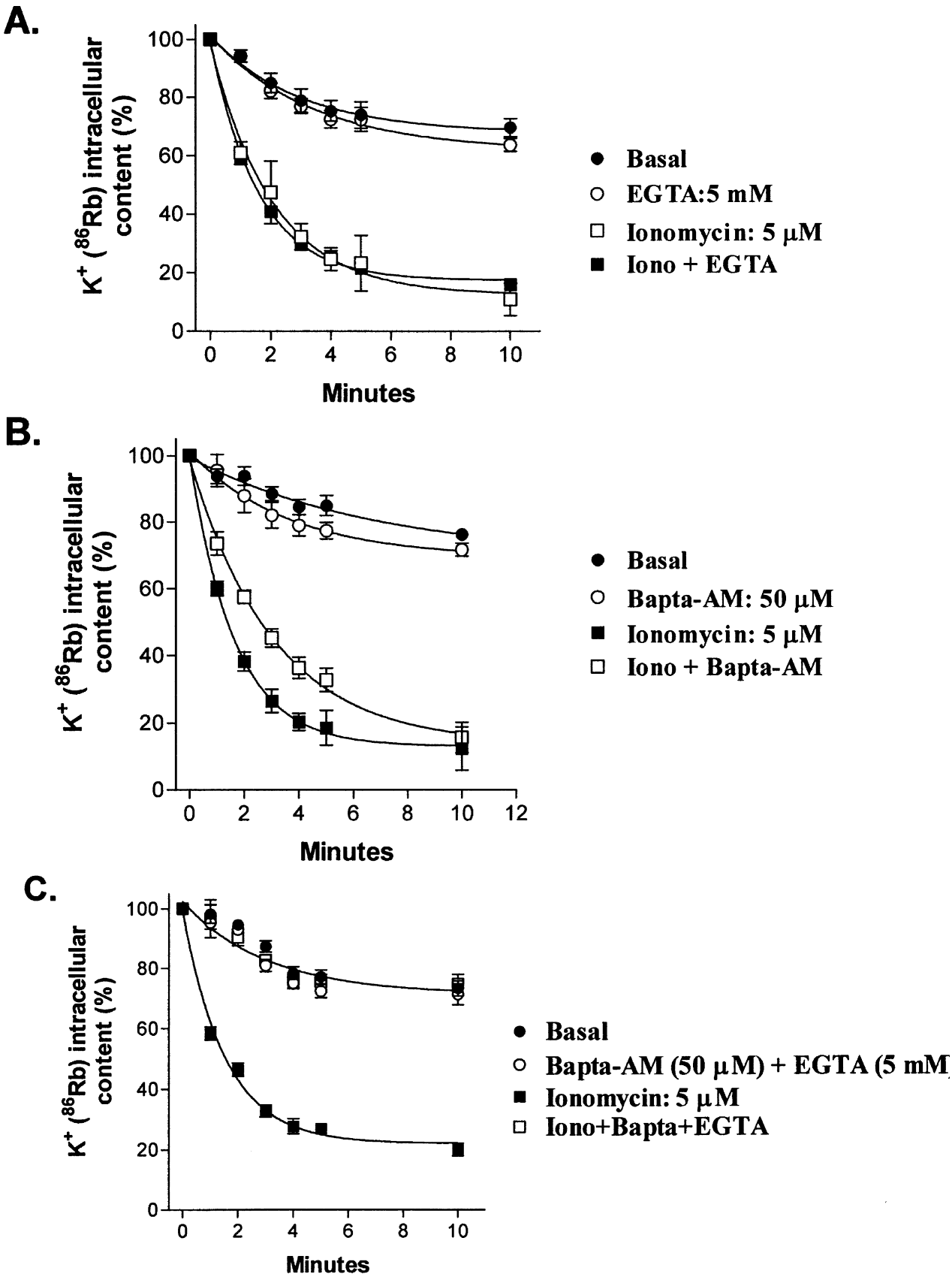
Figure 6 presents intracellular Ca^{2+} concentrations under different experimental conditions. Basal intracellular Ca^{2+} was 128 ± 1 nM. Thapsigargin, an inhibitor of endoplasmic reticulum Ca^{2+} ATPase, increased intracellular Ca^{2+} to 190 ± 1.6 nM and ionomycin augmented intracellular Ca^{2+} over 1 μM (Fig. 6*A*). The pre-incubation of isolated cells in the absence of extracellular Ca^{2+} (10 mM EGTA) reduced intracellular Ca^{2+} to 93 ± 0.3 nM, abolished the effect of thapsigargin and reduced the increase in intracellular Ca^{2+} concentration induced by ionomycin to 179 ± 1.2 nM (Fig. 6*B*). Preincubation of epithelial cells in the absence of extracellular Ca^{2+} (10 mM EGTA) and 1 μM thapsigargin for 60 min reduced intracellular Ca^{2+} to 60 ± 0.3 nM and decreased the response to ionomycin to 115 ± 0.5 nM (Fig. 6*C*). Only cell preincubation in the absence of extracellular Ca^{2+} (10 mM EGTA) for 120 min reduced the intracellular Ca^{2+} to 33 ± 0.3 nM and almost abolished the response to ionomycin (Fig. 6*D*). Using the last pre-incubation conditions, different extracellular Ca^{2+} concentrations (estimated in the presence of added $CaCl_2$ over EGTA) and 5 μM ionomycin in the incubation medium, it was possible to evaluate a dose-response curve for Ca^{2+} on the ionomycin-induced K^+ efflux (Fig. 6*E*). Under these experimental conditions, Ca^{2+} stimulated ionomycin-induced K^+ efflux with an EC_{50} of 1.07 ± 0.034 μM.

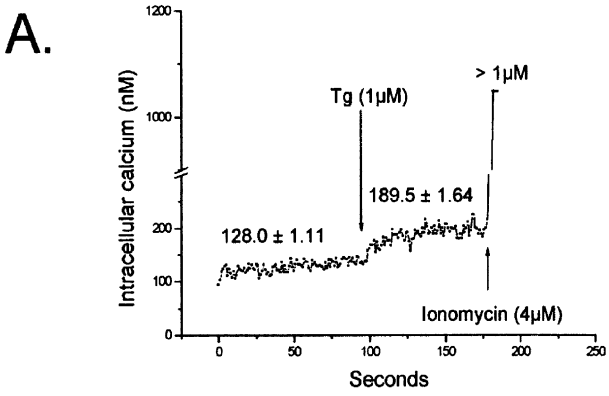
Finally, K^+ efflux was evaluated in colonic surface cells (Fig. 7). In contrast to crypt cells, basal efflux was not affected by either 30 mM TEA or 5 mM Ba^{2+}. However, ionomycin-induced K^+ efflux showed characteristics identical to that observed in colonic crypt cells.

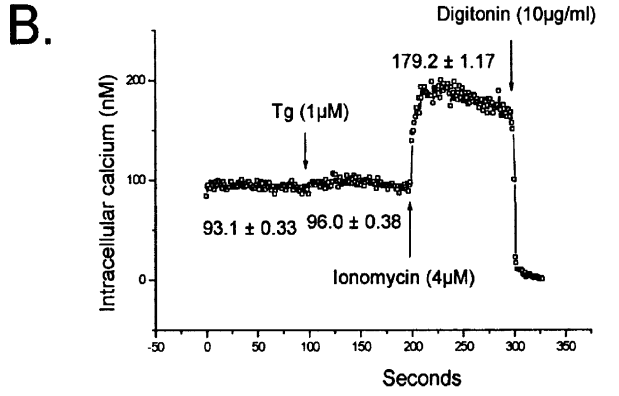
Discussion

The following model has been proposed to explain potassium and chloride secretion in the colon. Sodium, potassium, and chloride ions enter epithelial cells across the basolateral plasma membrane through the Na/K/2Cl cotransporter. Na^+ is extruded by the Na/K pump and K^+ is recycled across the basolateral membrane through K^+ channels or is secreted to the lumen through Ba^{2+}- and TEA-sensitive apical K^+ channels. Potassium exit induces cell hyperpolarization, increasing the driving force for Cl^- exit across apical Cl^- channels, inducing chloride secretion. An increase in intracellular Ca^{2+}, produced by secretagogues, would open basolateral Ca^{2+}-dependent K^+ channels, inducing basolateral K^+ exit, cell hyperpolarization and stimulation of

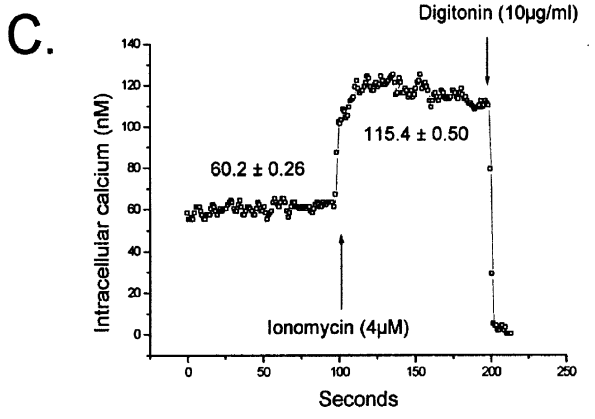
Fig. 5. Effect of the absence of extracellular Ca^{2+} and of cell Ca^{2+} depletion on K^+(^{86}Rb) efflux in isolated colonic crypt cells. (*A*) Cells were pre-incubated with 5 mM EGTA in the medium, which reduced extracellular free Ca^{2+} to zero, for 30 min and loaded with ^{86}Rb as indicated. Efflux was determined under basal conditions and in the presence of 5 μM ionomycin. (*B*) Cells were pre-incubated with 50 μM Bapta-AM for 30 min at 25°C and loaded with ^{86}Rb. Efflux was determined under basal conditions and in the presence of 5 μM ionomycin. (*C*) Cells were pre-incubated with 5 mM EGTA and 50 μM BAPTA-AM for 30 min at 25°C and loaded with ^{86}Rb as described in Materials and Methods. Results are mean ± SEM of three different experiments. Points are experimental data and curves represent the best fit to a single-exponential decay equation.


A.
K+ (86Rb) intracellular content (%)
100
80
60
40
20
0
0
2
4
6
8
10
Minutes
Basal
EGTA:5 mM
Ionomycin: 5 μM
Iono + EGTA
B.
K+ (86Rb) intracellular content (%)
100
80
60
40
20
0
0
2
4
6
8
10
12
Minutes
Basal
Bapta-AM: 50 μM
Ionomycin: 5 μM
Iono + Bapta-AM
C.
K+ (86Rb) intracellular content (%)
100
80
60
40
20
0
0
2
4
6
8
10
Minutes
Basal
Bapta-AM (50 μM) + EGTA (5 mM
Ionomycin: 5 μM
Iono+Bapta+EGTA

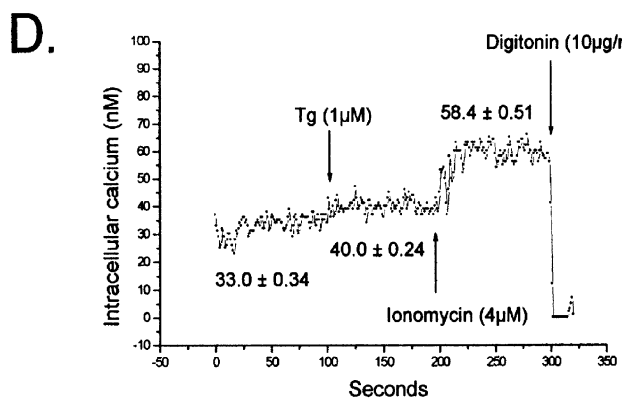




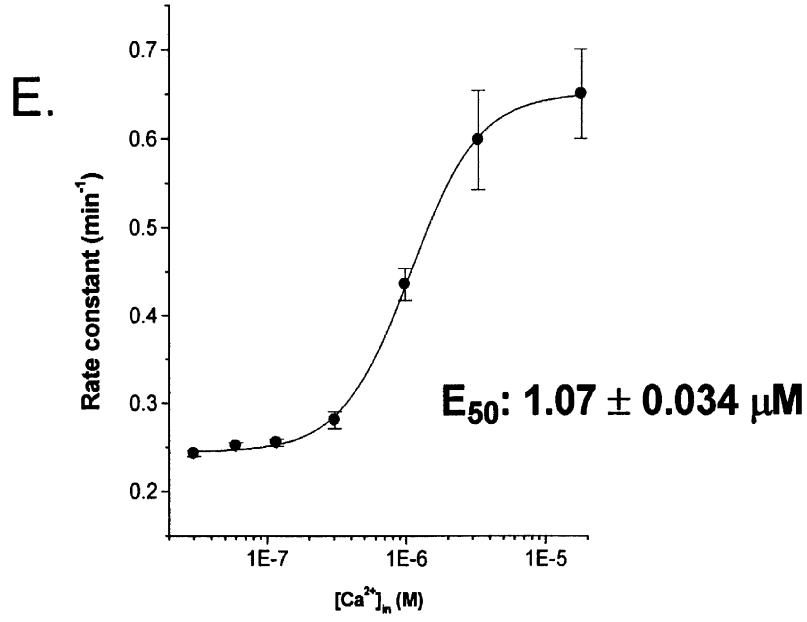
Cells preincubated 60 min with 10mM EGTA



Cells preincubated for 60 min with 1μM Tg + 10mM EGTA



Cells preincubated 120 min with 10mM EGTA



Cl^- and K^+ secretion. Reduction of Cl^- exit would decrease the Na/K/2Cl entry through the cotransporter. So, intracellular K^+ concentration would drop and apical K^+ efflux would decrease, reducing K^+ secretion. Sustaining this model, it has been shown, in the distal colon, that carbachol stimulates mucosal ^{86}Rb efflux, which is inhibited by mucosal TEA and serosal quinine [17], suggesting the partic-

ipation of Ca^{2+}-activated K^+ channels in the secretory process. Additionally, Ca^{2+}-dependent K^+ channel inhibitors induce cell depolarization and reduction of the driving force for Cl^- exit through the apical membrane [9]. Moreover, in rat proximal colon, it has been suggested that basolateral IK_{Ca} and apical BK_{Ca}, SK_{Ca} and IK_{Ca} channels could participate in both K^+ and Cl^- secretion induced by cholinergic agonists [18].

Although various Ca^{2+}-activated K^+ channels (K_{Ca}) have been identified in the colon, the specific role of these conductive pathways in transepithelial K^+ transport is unknown.

The participation of K^+ channels in K^+ absorption and secretion has been deduced from flux studies [9, 13, 17, 33]. In the mammalian colon, three different Ca^{2+}-activated K^+ channels have been described. The large-conductance channel (BK_{Ca}) has been identified in apical [4] and basolateral membranes [3, 22] of surface and crypt colonic cells. Small-(SKca) and intermediate (IK_{Ca})-conductance K^+ channels have been found in the basolateral membrane of crypt cells from mammalian colon [2, 3, 9, 22, 24]. Apical channels may facilitate K^+ secretion [4, 17, 28], while basolateral K^+ channels are thought to facilitate recycling of K^+ that enters the cell via Na/K/2Cl cotransporter or Na/K pump. K^+ efflux through basolateral Ca^{2+}-activated K^+ channels might also hyperpolarize the basolateral and apical membranes, thereby enhancing the driving force for the Ca^{2+}-induced apical Cl^- secretion [8, 9, 13, 25, 28].

In the present study, the role of Ca^{2+}-activated K^+ channels in the overall K^+ efflux in colonic surface and crypt cells was evaluated using Ca^{2+}-activated K^+ channel inhibitors. In crypt cells, under basal conditions (intracellular Ca^{2+}: ≈100 nM), neither apamin, iberiotoxin nor charybdotoxin affected K^+ (^{86}Rb) efflux, only Ba^{2+} and TEA had an inhibitory effect, indicating that Ca^{2+}-activated K^+ channels do not participate in basal efflux. In contrast, ionomycin (a calcium ionophore) largely stimulated K^+ (^{86}Rb) efflux, suggesting the opening of Ca^{2+}-dependent K^+ channels. These channels were insensitive to Ba^{2+}, TEA, iberiotoxin and apamin but inhibited by clotrimazole and charybdotoxin, suggesting that these channels are IK_{Ca} channels.

The lack of effect of TEA and Ba^{2+} (inhibitors of K^+ secretion) on ionomycin-induced K^+ (^{86}Rb) efflux indicates that Ca^{2+}-activated K^+ channels do not directly participate in the K^+ secretory process and their role in the transepithelial K^+ transport is probably located at the basolateral plasma membrane, where they would be involved in the recycling of K^+ and the opening of conductive pathways for this ion under Ca^+-mediated secretion. In isolated colonic crypt cells, Ca^{2+} stimulated ionomycin-induced K^+ efflux with an EC_{50} of 1.07 ± 0.034 μM (Fig. 6*E*).

Ionomycin-induced K^+ efflux was totally inhibited by both 100 μM clotrimazole and 100 nM charybdotoxin, inhibitors of IK_{Ca} channels, but not affected by apamin, an inhibitor of SK_{Ca} channels, nor iberiotoxin, inhibitor of BK_{Ca} channels. Although three types of Ca^{2+}-activated K^+ channels have been identified in the mammalian colon [18, 33], our results seem to indicate that only IK_{Ca} participate, in an important way, in the homeostasis of cellular potassium. In this sense, it has been shown that IK_{Ca} is the prevalent K^+ channel expressed in rat distal colon [18].

Finally, Ca^{2+}-activated K^+ channels, with identical characteristics, were identified in surface and crypt cells. In contrast, only crypt cells showed TEA- and Ba^{2+}-sensitive K^+ channels (Figs. 1 and 7), suggesting the cryptal localization of the K^+ secretory process.

Recently, the role of K_{Ca} channels in mediating K^+ secretion has been investigated in rat proximal colon [18]. In the absence of elevated intracellular Ca^{2+}, none of the K_{Ca}-inhibitors have any effect on ^{86}Rb fluxes, indicating that K_{Ca} channels do not participate in basal secretion. In contrast, carbachol-induced ^{86}Rb secretion is partially inhibited by

Fig. 6. Intracellular Ca^{2+} concentration in isolated colonic crypt cells. Cells were incubated with 10 μM fura-2/AM at 25°C for 30 minutes, then washed in efflux buffer by centrifugation and resuspended in the same solution. Fluorescence emitted at 510 nm was alternately determined at 350 nm and 380 nm excitation wavelengths. Fluorescence was corrected for background and cell autofluorescence before intracellular Ca^{2+} concentration calculations from the 350/380 emission ratio. (*A*) Intracellular Ca^{2+} concentration under basal conditions and after the addition of 1 μM thapsigargin and 5 μM ionomycin. (*B*) Intracellular Ca^{2+} concentration in cells pre-incubated for 60 minutes at 25°C with 10 mM EGTA. The effect of thapsigargin and ionomycin and 10 mg/ml digitonin was evaluated. (*C*) Cells were pre-incubated for 60 min with 1 μM thapsigargin and 10 mM EGTA and the intracellular Ca^{2+} concentration was determined. The effect of ionomycin and digitonin was tested. (*D*) Cells were pre-incubated with 10 mM EGTA for 120 min at 25°C. The effect of the sequential addition of thapsigargin, ionomycin and digitonin was evaluated. Graphs represent a typical experiment, which was repeated three times. (*E*) Dose- response curve for the effect of Ca^{2+} on the rate constant of the K^+(^{86}Rb) efflux in isolated colonic crypt cells. Isolated cells were pre-incubated for 120 min at 25°C with 10 mM EGTA and loaded with ^{86}Rb as described in Materials and Methods. Then cells were incubated at different extracellular Ca^{2+} concentrations in the presence of 5 μM ionomycin and K^+(^{86}Rb) efflux was determined. Results are means ± SEM of three different experiments. Points are experimental data and curves the best fit to a sigmoidal curve (Boltzman). Extracellular Ca^{2+} concentrations in EGTA-Ca^{2+}-containing solutions were estimated using a commercial program (Max-chelator). As a control, cells incubated at different extracellular Ca^{2+} concentrations were loaded with fura2-AM, for Ca^{2+} concentrations up to 1 μM, and 5-fluo-AM, for Ca^{2+} concentrations higher than 1 μM, and intracellular Ca^{2+} was measured. In all conditions, intracellular Ca^{2+} was very similar to extracellular Ca^{2+} concentrations.

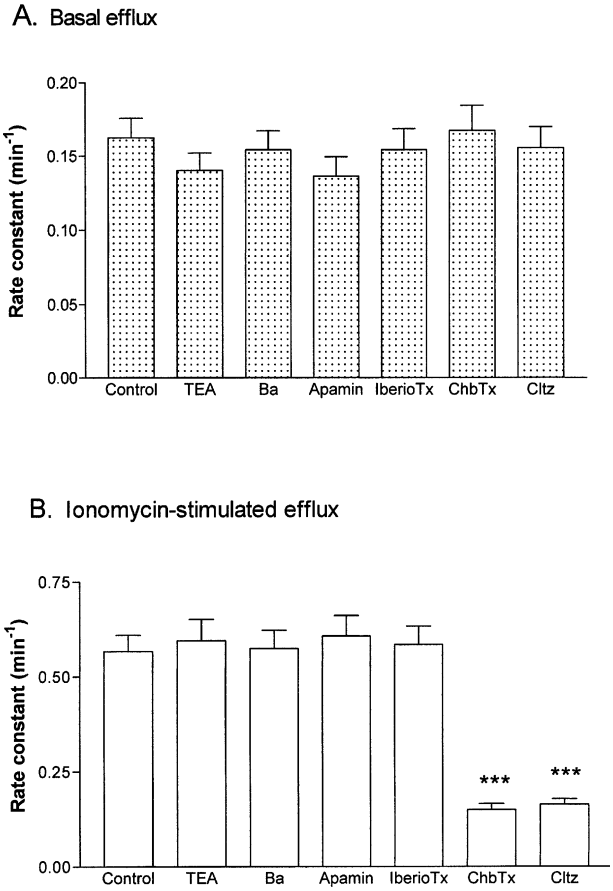


Fig. 7. Rate constant of K^+ (^{86}Rb) efflux in isolated colonic surface cells. Effect of different K^+ channel inhibitors. Colonic surface cells were pre-incubated in mammalian medium, containing 20 mM KCl and ^{86}Rb as a tracer, at 25°C for 30 min, in the absence (*Control*) or the presence of one of the following inhibitors: 30 mM TEA, 5 mM Ba^{2+}, 100 nM apamin, 100 nM iberiotoxin, 100 nM charydobtoxin or 100 μM clotrimazole. At the end of this period, cells were washed at 4°C in the same medium, without K^+ or ^{86}Rb, and ^{86}Rb efflux was evaluated for 10 min at 25°C in the presence of 1 mM ouabain and 50 μM bumetanide, in addition to channel blockers. Rate constants were determined under basal conditions (*A*) or in ionomycin-stimulated cells (*B*). Results are mean ± SEM of three different experiments. ** $P < 0.01$.

mucosal addition of IbTx or apamin or mucosal and serosal CLT, suggesting that apical BK_{Ca}, $SK2_{Ca}$ and IK_{Ca} channels probably contribute to K^+ secretion in the proximal colon. However, CLT applied to the mucosal side probably crosses the plasma membrane to inhibit basolateral IK_{Ca} [34]. As a consequence, CLT added to the mucosal side also abolished carbachol-induced K^+ secretion as an indirect consequence of blocking basolateral IK_{Ca} channels. Blocking basolateral IK_{Ca} channels depolarizes basolateral membrane, abolishing the driving force for apical Cl^- efflux, which leads to a decrease in basolateral $Na^+/K^+/2Cl^-$ cotransporter activity and, in this way, reduces K^+ uptake across the basolateral membrane, intracellular K^+ activity and apical K^+ efflux. Consistent with this model, serosal CLT inhibits both K^+ and Cl^- secretion in the proximal colon [18].

Furthermore, the localization of IK_{Ca} channels was explored using confocal immunofluorescence and western blot [18]. Confocal immuno-fluorescence data suggest that IK_{Ca} channels are predominantly expressed at the basolateral plasma membrane of colonic crypt. In contrast, western blot analysis demonstrates that IK_{Ca} channels are present in both apical and basolateral plasma membranes of the colonocyte. This discrepancy between immunofluorescence and western blot studies has been interpreted as a limited accessibility of the anti-IK_{Ca} antibody to the crypt lumen, but the presence of IK_{Ca} channels in apical membrane fractions could be due to cross contamination with basolateral plasma membranes during the membrane isolation procedure. In the same study, Joiner et al. [18] reported the presence of NHE1, a basolateral membrane marker, in the apical membrane fraction, indicating the cross contamination of the preparation. In addition, the basolateral location of IK_{Ca} has been confirmed by patch-clamp studies [2, 22, 24].

In summary, the predominant K^+-conductive pathway, in both surface and crypt cells, is the Ca^{2+}-dependent, intermediate-conductance K^+ channel (IK_{Ca}) but only crypt cells have TEA- and Ba^{2+}-sensitive K^+ channels, which would determine their participation in colonic K^+ secretion.

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