Anion secretion induced by capacitative Ca^{2+} entry through apical and basolateral membranes of cultured equine sweat gland epithelium

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1. Anion secretion induced by capacitative Ca^{2+} entry through apical and basolateral membranes of cultured equine sweat gland epithelium was studied using the short-circuit current (I_{SC}) technique.
2. Thapsigargin induced an increase in I_{SC} that could be inhibited when external Ca^{2+} was chelated by EGTA.
3. The inhibition of the thapsigargin-induced I_{SC} could be reversed by re-addition of Ca^{2+} to apical or basolateral solutions. The magnitude of the reactivated I_{SC} depended predominantly on basolateral Ca^{2+} concentration.
4. The magnitude of the reactivated I_{SC} upon basolateral Ca^{2+} addition increased with the thapsigargin concentration, indicating its dependence on the emptied state of the Ca^{2+} store induced by thapsigargin.
5. The thapsigargin-induced I_{SC}, as well as the Ca^{2+}-dependent reactivation of I_{SC} in EGTA-treated epithelia, was inhibitable by apical, but not basolateral, addition of flufenamate, and by basolateral addition of La^{3+}. Other Ca^{2+} channel blockers, verapamil and nifedipine, had no effect when applied to either membrane.
6. The results suggest that thapsigargin-induced anion secretion by the equine sweat gland epithelial cells is crucially dependent upon the Ca^{2+} influx occurring primarily through the basolateral membrane, and that apical and basolateral membranes may possess different pathways for Ca^{2+} entry.

Regulated changes in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) constitute a centrally important mechanism that allows hormones and neurotransmitters to exert control over cellular functions, including fluid and electrolyte secretion (Petersen, 1992; Berridge, 1993). In non-excitable cells, the exact mechanism of Ca^{2+} influx from the extracellular medium is still controversial. In many cases Ca^{2+} influx takes place through the plasma membrane and the degree of influx depends on the content of the intracellular Ca^{2+} stores. This type of Ca^{2+} entry has been termed 'capacitative Ca^{2+} entry' and has been widely demonstrated in various types of epithelia using fluorescence techniques (Tsunoda, Stuenkel & Williams, 1990; Illek, Fischer & Machen, 1992; Klär, Leipziger, Nitschke & Greger, 1993; Nitschke, Leipziger & Greger, 1993; Missiaen, Smedt, Pary, Oike & Casteels, 1994; Tojyo, Tanimura, Matsumoto & Sugiya, 1995). However, it is still not well established whether there is any difference in Ca^{2+} influx through apical and basolateral membrane domains, and the relationship of the differential influx to electrolyte transport across the epithelium is unclear.

An equine sweat gland epithelial cell line (E/92/3) has been reported to respond to a number of calcium-mobilizing agonists, such as ATP and thapsigargin (Ko *et al.* 1994*a*; Ko, Wilson & Elder, 1994*b*; Wilson, Ko, Pediani, Rakhit, Nichol & Bovell, 1995). A previous study on this cell line has shown that the microsomal Ca^{2+}-ATPase inhibitor thapsigargin can increase $[Ca^{2+}]_i$ (Ko *et al.* 1994*b*). This action was due to a slow but persistent leak of Ca^{2+} from the intracellular Ca^{2+} stores followed by a sustained Ca^{2+} influx from extracellular fluid via an as yet unknown mechanism. This phenomenon is consistent with the 'capacitative Ca^{2+} entry' model (Kwan & Putney, 1990; Putney, 1993). In another study, using the short-circuit current (I_{SC}) technique, thapsigargin proved to be a potent secretagogue stimulating both electrogenic chloride and bicarbonate secretion across the monolayers of equine sweat gland epithelial cells grown on a permeable support (Ko, Chan, Cheng Chew & Wong, 1996). These studies suggest a link between thapsigargin-induced Ca^{2+} mobilization and anion secretion in the equine sweat gland epithelial cells.

The present study was designed to examine the anion secretion induced by 'capacitative Ca^{2+} entry' in equine sweat gland epithelial cells using the short-circuit current technique. This technique has the advantage over most of the conventional fluorescence techniques in that: (1) it provides easy access to the apical and basolateral membranes of an epithelium; and (2) by manipulating the Ca^{2+} concentration of solution bathing either the apical or basolateral membrane, the contribution of extracellular Ca^{2+} from the apical/basolateral side to anion secretion can be delineated. The present study has provided the first piece of evidence that the action of thapsigargin in stimulating anion secretion in equine sweat gland epithelial cells is crucially dependent upon Ca^{2+} influx occurring primarily through the basolateral membrane domain. Furthermore, apical and basolateral membranes may possess different pathways for Ca^{2+} entry.

METHODS

Cell culture

Standard techniques were used to maintain a spontaneously transformed epithelial cell line (E/92/3) derived from the equine sweat gland as described previously (Wilson *et al.* 1993). The standard culture medium was William's medium E, supplemented with 5% fetal calf serum and L-glutamine (1 mM), penicillin (100 i.u. ml^{-1}), streptomycin (100 $\mu g\ ml^{-1}$), bovine insulin (100 $\mu g\ ml^{-1}$), hydrocortisone (10 ng ml^{-1}) and sodium selenite (10 ng ml^{-1}). Cells were removed from the culture flasks by 0·25% trypsin–EDTA (0·05 mM). Aliquots of 0·25 ml of cell suspension containing 1×10^5 cells were seeded onto 0·45 cm^2 Millipore filter assemblies as described by Cuthbert, George & MacVinish (1985). Briefly, a silicone washer with a 0·45 cm^2 hole was stuck to the Millipore filter (type HAWP, 0·45 μm) using Silastic® 3140 RTV adhesive (Dow Corning), creating a small well into which cells could be seeded. The epithelial cells were therefore grown on permeable support floating on the culture medium. Cultures were incubated for 3–4 days at

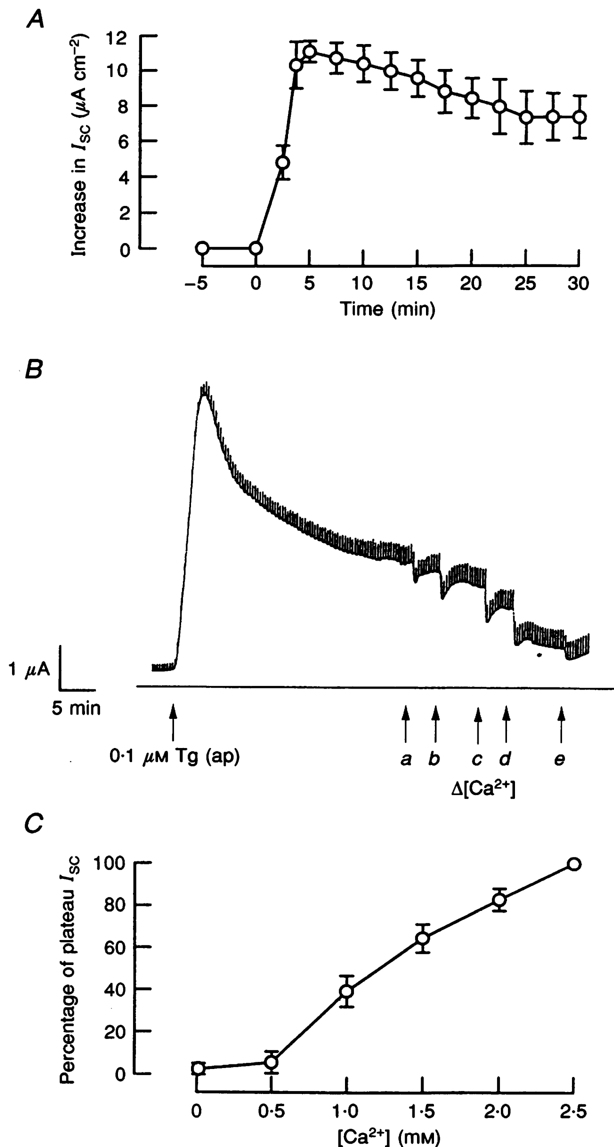

Figure 1. Dependence of thapsigargin-mediated I_{SC} on external Ca^{2+} concentration

A, thapsigargin (0·1 μM)-induced I_{SC} in cultured equine sweat gland epithelia bathed in normal KH solution. Each point shows the mean ± S.E.M. of 5 experiments. *B*, effect of chelating extracellular Ca^{2+} by EGTA on the I_{SC} response to thapsigargin applied apically (0·1 μM Tg (ap)). When I_{SC} had stabilized, EGTA was added (from 0·5 to 2·5 mM) to the apical and basolateral bathing solutions to reduce the Ca^{2+} concentration to 2 (*a*), 1·5 (*b*), 1 (*c*), 0·5 (*d*) and 0·01 mM (*e*). The horizontal lines represent zero I_{SC}. The record is representative of 5 experiments.
C, percentage plateau I_{SC} plotted against free Ca^{2+} concentrations. Values are means ± S.E.M.

37 °C in 5% CO_2. Thereafter, the monolayers reached confluency and were ready for I_{SC} measurement.

Short-circuit current measurement

The method used for the short-circuit current measurement was described elsewhere (Cuthbert *et al.* 1985). In short, the confluent monolayers were mounted vertically between the two halves of the modified Ussing chambers. Monolayers were short circuited (transepithelial potential difference clamped at zero) using a voltage-clamp amplifier (DVC 1000; World Precision Instruments Inc.). The resulting I_{SC} was displayed on-line on a pen recorder (Kipp and Zonen, Delft, The Netherlands). Transepithelial resistance was obtained from the ohmic relationship by clamping the tissue intermittently at a value of 0·1 mV every 10 s. In most experiments, tissues were bathed in normal Krebs–Henseleit (KH) solution.

Materials and solutions

William's medium E, fetal calf serum, penicillin–streptomycin and trypsin–EDTA were purchased from Gibco Laboratories. Bovine insulin, hydrocortisone, transferrin, sodium selenite, epidermal growth factor and flufenamate were purchased from Sigma. Thapsigargin and A23187 were purchased from Calbiochem Novabiochem (La Jolla, CA, USA).

Normal KH solution had the following composition (mM): NaCl, 117; KCl, 4·7; $CaCl_2$, 2·5; KH_2PO_4, 1·2; $MgSO_4$, 1·2; $NaHCO_3$, 25; glucose, 11·1. The solution was gassed with 5% CO_2–95% O_2 to give a pH of 7·4. To prevent precipitation when La^{3+} was used, $NaHCO_3$ and KH_2PO_4 were omitted from the solution and $MgSO_4$ was replaced with $MgCl_2$. The solution was buffered with 10 mM Hepes and bubbled with 100% O_2 to maintain pH at 7·4. The osmolalities of all the solutions were 290 mmol kg^{-1}. Free Ca^{2+} concentrations of the solution were calculated using the EQCAL software (Biosoft®, Cambridge, UK).

Statistical analysis

Results are expressed as means ± S.E.M. Comparison between groups of data were made by Student's unpaired *t* test. A *P* value < 0·05 was considered statistically significant.

RESULTS

Dependence of the thapsigargin-induced I_{SC} on extracellular Ca^{2+}

Thapsigargin (0·1 μM) added apically caused the I_{SC} to rise to a peak level of 11·07 ± 0·61 μA cm^{-2} (basal I_{SC} of 0·1 ± 0·03 μA cm^{-2}, $n = 50$) followed by a decline to a plateau level (7·35 ± 1·19 μA cm^{-2}) after 25 min (Fig. 1*A*, $n = 5$). At the steady state, EGTA was added to both sides at various concentrations to reduce the free Ca^{2+} concentration. Figure 1*B* and *C* shows that the increase in I_{SC} elicited by thapsigargin (0·1 μM) was dependent upon the external Ca^{2+} concentration. When increasing concentrations of EGTA (from 0·5 to 2·5 mM) were added to both sides of the bathing solution to reduce Ca^{2+} concentrations from 2 to 0·01 mM, the plateau I_{SC} fell with decreasing [Ca^{2+}] and at 0·01 mM Ca^{2+} the plateau I_{SC} was suppressed to the basal level (Fig. 1*B*). Figure 2 shows the complete abolition of the thapsigargin-induced I_{SC} by addition of 2·6 mM EGTA to the bathing solution (free Ca^{2+} was estimated to be about 1 μM), and the reappearance of the I_{SC} upon restoration of free Ca^{2+} (final free Ca^{2+} concentration was estimated to be 2·5 mM). The Ca^{2+}-reactivated I_{SC} had two components: a transient increase of I_{SC} to 21·1 ± 2·9 μA cm^{-2} was followed by a decline to a

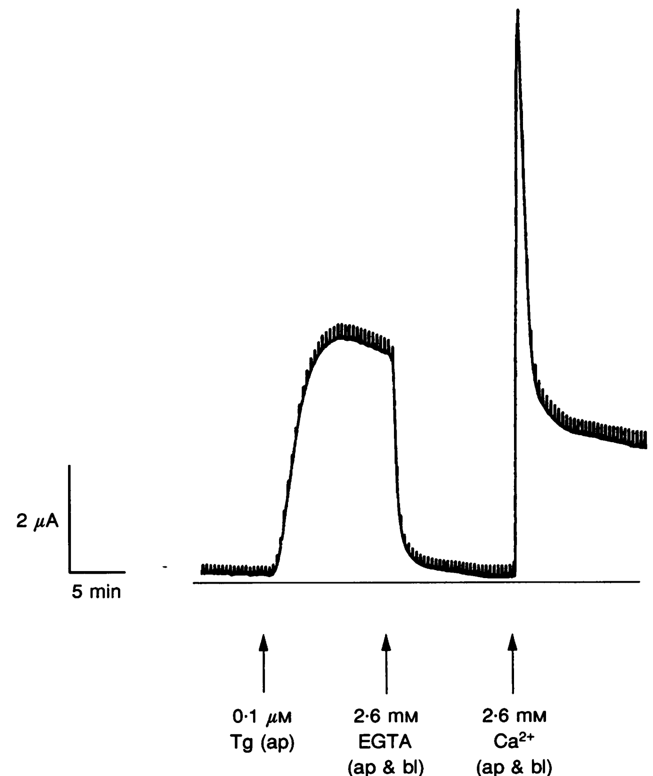

Figure 2. Effect of Ca^{2+} chelation and re-addition on the thapsigargin-induced I_{SC}

After a I_{SC} response was elicited, 2·6 mM EGTA was added to both sides (ap & bl) to chelate free Ca^{2+}. I_{SC} was reduced to the basal level. $CaCl_2$ (2·6 mM) was then added to restore the free Ca^{2+} concentration to 2·5 mM. The transient current pulses were the results of intermittently clamping the potential at 0·1 mV. The record is representative of 5 experiments.

plateau phase ($3{\cdot}0 \pm 0{\cdot}1\ \mu A\ cm^{-2}$). Addition of EGTA did not cause any significant changes in the transepithelial resistance of the monolayers.

After EGTA (2·6 mM, apical and basolateral) was added to the baths to prevent Ca^{2+} influx, thapsigargin (0·1 μM, apical) caused a small increase in I_{SC} ($0{\cdot}7 \pm 0{\cdot}1\ \mu A\ cm^{-2}$, $n = 25$; Fig. 3*A*). This small rise in I_{SC} could reflect a transient increase in $[Ca^{2+}]_i$ elicited by thapsigargin in the absence of extracellular free Ca^{2+} (Ko *et al.* 1994*b*). When Ca^{2+} was added to both sides of the bath (final free Ca^{2+} concentration was estimated to be 2·5 mM), a large increase in I_{SC} ($20{\cdot}7 \pm 2{\cdot}5\ \mu A\ cm^{-2}$; Fig. 3*A*) similar to that shown in Fig. 2 was observed, indicating that Ca^{2+} entry through plasma membranes was responsible for the thapsigargin-stimulated anion secretion. In the absence of thapsigargin, the addition of Ca^{2+} to an EGTA-pretreated epithelium did not evoke a rise in I_{SC} (Fig. 3*B*, $n = 5$).

Predominant dependence of the I_{SC} on basolateral Ca^{2+}

The difference in Ca^{2+} entry between the apical and basolateral membrane domains was also examined. Monolayers were bathed in one of the following solutions: (i) control solution containing a normal Ca^{2+} concentration in both apical and basolateral compartments; (ii) apical and basolateral solutions containing 2·6 mM EGTA; (iii) apical solution containing 2·6 mM EGTA; and (iv) basolateral solution containing 2·6 mM EGTA. In (iii) and (iv), the contralateral side contained normal Ca^{2+}. Monolayers were then challenged with 0·1 μM thapsigargin and the respective increase in I_{SC} is shown in Fig. 4*A*. Under control conditions (normal Ca^{2+} concentration on both sides), apical application of thapsigargin stimulated a large increase in I_{SC} of $12{\cdot}0 \pm 0{\cdot}6\ \mu A\ cm^{-2}$, measured at the peak of the response ($n = 6$). When free Ca^{2+} was chelated by EGTA in both the apical and basolateral bathing solutions, the I_{SC} elicited by thapsigargin was almost completely abolished (I_{SC} increase of $0{\cdot}4 \pm 0{\cdot}2\ \mu A\ cm^{-2}$, $n = 4$). However, chelating Ca^{2+} in the apical solution alone did not affect the thapsigargin-induced I_{SC} to a great extent (the increase in I_{SC} was $9{\cdot}5 \pm 1{\cdot}0\ \mu A\ cm^{-2}$ compared with $12{\cdot}0 \pm 0{\cdot}6\ \mu A\ cm^{-2}$ under control conditions, $n = 6$). In contrast, the increase in I_{SC} was substantially reduced to $1{\cdot}3 \pm 0{\cdot}3\ \mu A\ cm^{-2}$ ($n = 5$) when the monolayers were incubated basolaterally in EGTA solution, suggesting that the thapsigargin-induced I_{SC} is predominantly dependent on basolateral Ca^{2+} influx.

To illustrate further the differential dependence of the thapsigargin-induced I_{SC} on Ca^{2+} entry through the two membrane domains, in another set of experiments the Ca^{2+} concentrations of the apical and/or basolateral bathing solutions were varied from 0 to 2·5 mM by adjusting the amount of EGTA added to the bath(s). Figure 4*B* shows that when the Ca^{2+} concentration in the apical solution was kept constant, the magnitude of the thapsigargin-induced I_{SC} increased as the Ca^{2+} concentration in the basolateral bathing solution increased. The dependence of the I_{SC} on the basolateral Ca^{2+} concentration was similar to that observed when the Ca^{2+} concentration in both apical and basolateral solutions was adjusted. However, when the basolateral Ca^{2+} concentration was kept constant, the I_{SC} response to

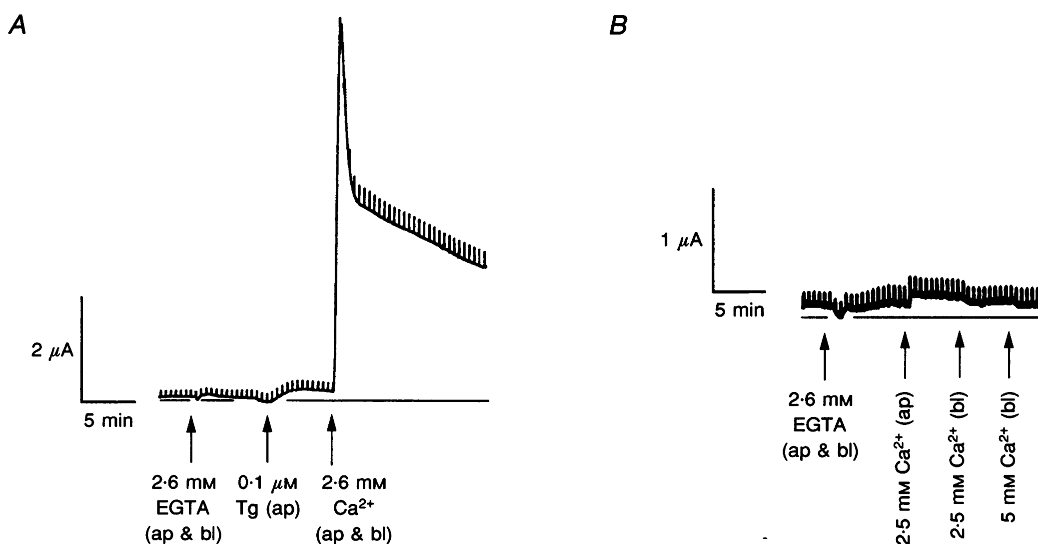

Figure 3. Inhibition of thapsigargin-induced I_{SC} by pretreatment with EGTA and activation by re-addition of Ca^{2+}

A, I_{SC} response to thapsigargin in Ca^{2+}-free bathing solution. Free Ca^{2+} was removed by adding 2·6 mM EGTA. After the response to thapsigargin (0·1 μM Tg (ap)) had stabilized, $CaCl_2$ was added to the bath to restore free Ca^{2+} concentration to 2·5 mM. The record is representative of 3 experiments. *B*, a control experiment showing that the EGTA-treated epithelium did not respond to Ca^{2+} re-addition to either side in the absence of thapsigargin. The record is representative of 5 experiments.

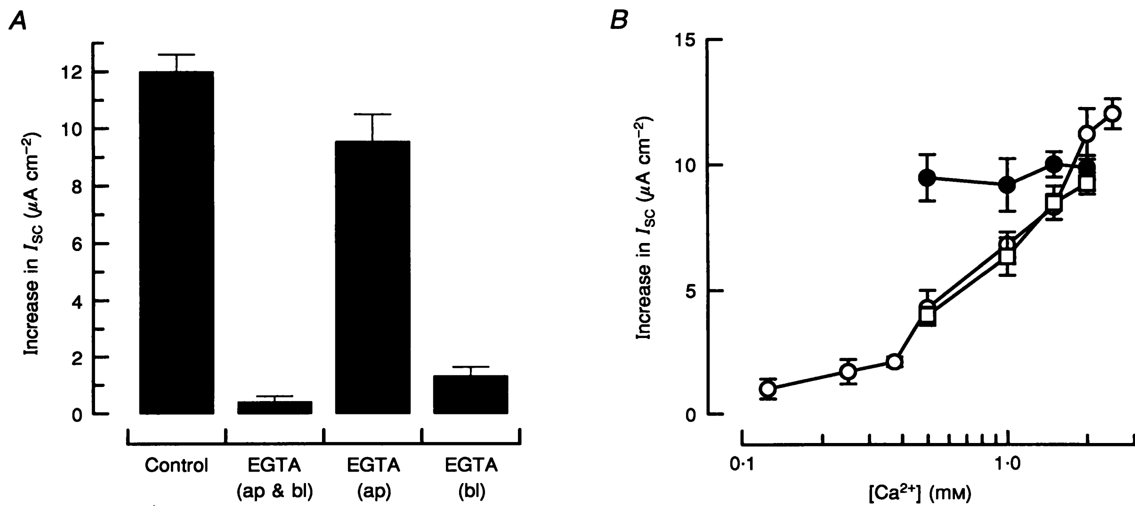

Figure 4. Dependence of the thapsigargin-induced I_{SC} on apical and basolateral Ca^{2+} concentrations

A, thapsigargin-induced I_{SC} responses obtained from epithelia incubated in: (i) control solution with normal apical and basolateral Ca^{2+} concentrations; (ii) apical and basolateral solutions with added EGTA (2·6 mM); (iii) apical solution with added EGTA and normal basolateral solution; and (iv) basolateral solution with added EGTA and normal apical solution. *B*, effect of varying free Ca^{2+} concentrations in apical and/or basolateral bathing solutions on thapsigargin-induced I_{SC}. ○, variable [Ca^{2+}] both sides. ●, apical [Ca^{2+}] variable; basolateral [Ca^{2+}] = 2·5 mM. □, basolateral [Ca^{2+}] variable; apical [Ca^{2+}] = 2·5 mM. Values are means ± S.E.M. for 6 separate epithelia.

thapsigargin remained relatively the same (the increase was around 9·6 μA cm^{-2}), regardless of the changes in apical Ca^{2+} concentrations. Taken together, these results indicate that the secretory response to thapsigargin is determined by the amount of Ca^{2+} present in the basolateral solution.

The dependence of the I_{SC} induced by a Ca^{2+} ionophore, A23187, on the influx of Ca^{2+} was also examined. Figure 5*A* shows that apical addition of A23187 (1 μM) to the monolayers bathed in control solution evoked a large transient increase in I_{SC} (29·9 μA cm^{-2} measured at the

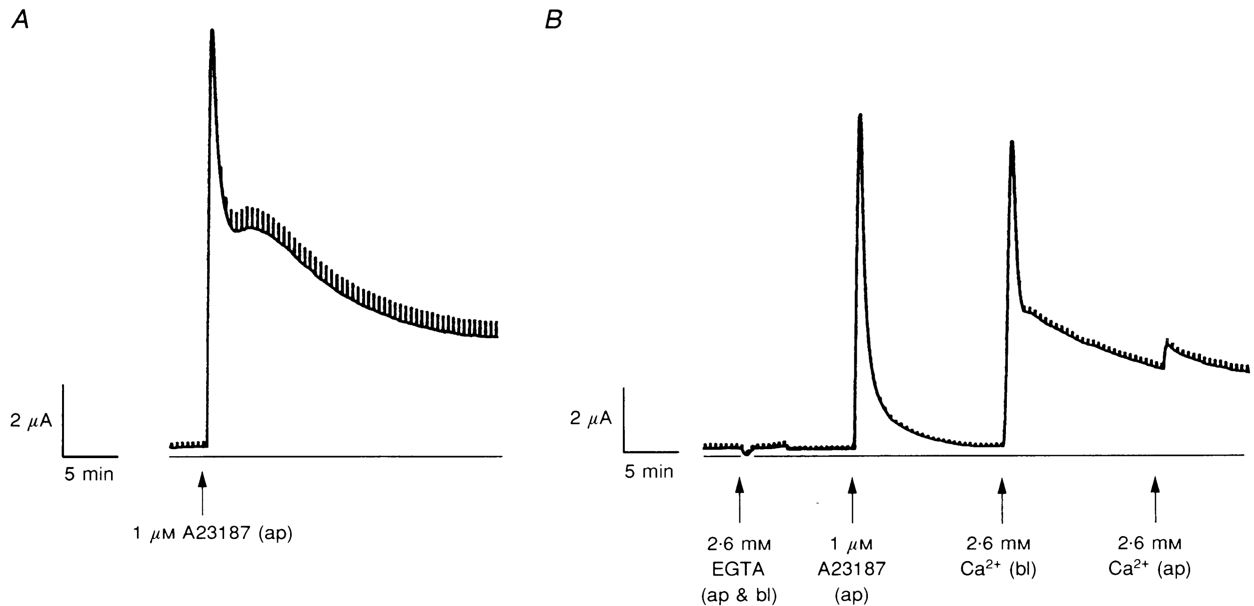

Figure 5. Effect of removal and re-addition of Ca^{2+} on A23187-induced I_{SC}

A, I_{SC} response to A23187 applied apically (1 μM A23187 (ap)) in normal Ca^{2+}-containing solution. *B*, A23187-induced I_{SC} in Ca^{2+}-free bathing solution (2·6 mM EGTA (ap & bl)) and upon re-addition of Ca^{2+} basolaterally followed by apical addition. The record is representative of 5 experiments.

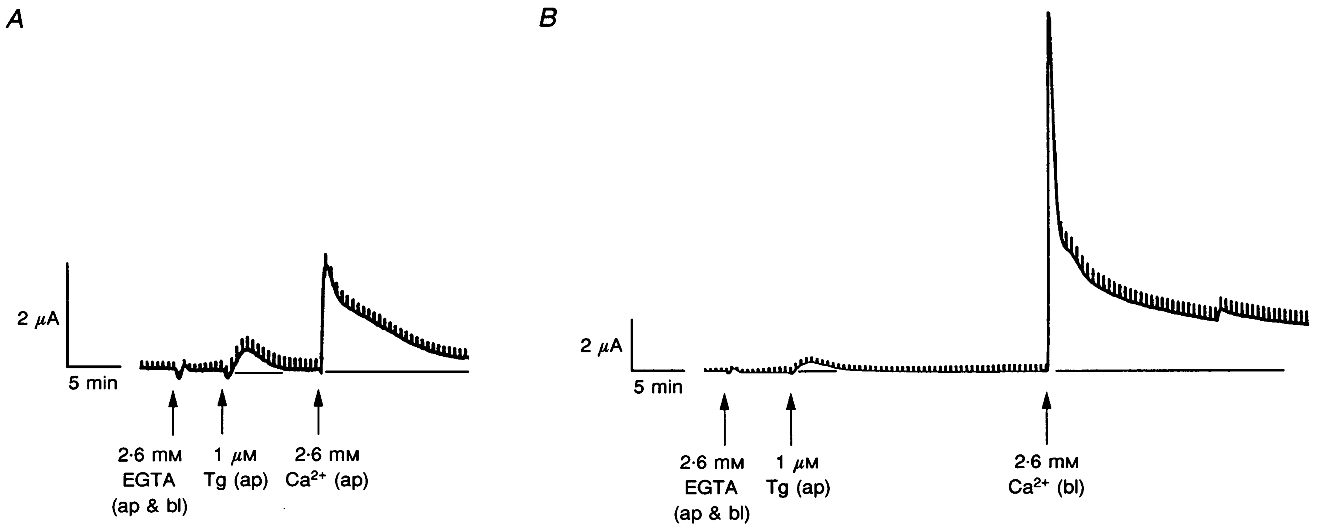

Figure 6. Dependence of the thapsigargin-induced I_{SC} on Ca^{2+} entry through apical or basolateral membranes

Monolayers were challenged with thapsigargin (1 μM, ap) in Ca^{2+}-free bathing solution (with 2·6 mM EGTA added). Ca^{2+} was restored to apical (*A*) or basolateral (*B*) bathing solutions. Each record is representative of 5 experiments.

peak) followed by a sustained phase ($9{\cdot}5 \pm 0{\cdot}8\ \mu A\ cm^{-2}$). When free Ca^{2+} in both apical and basolateral bathing solutions was chelated by EGTA, addition of A23187 stimulated only a transient increase in I_{SC} (Fig. 5*B*). The transient increase in I_{SC} could be due to the release of Ca^{2+} from the intracellular store in the absence of Ca^{2+} influx. Subsequent current activation with both a transient and a sustained phase was observed upon addition of extracellular free Ca^{2+}, with the I_{SC} response to basolateral addition being larger than that to apical addition.

Emptying of the thapsigargin-sensitive Ca^{2+} pool increases Ca^{2+} entry

In other cell types it has been reported that the depletion of the Ca^{2+} stores can be graded by using different concentrations of thapsigargin and the Ca^{2+} influx can be correlated with the content of the stores (Villalobos & García-Sancho, 1995). In the present study, we investigated whether anion secretion stimulated by the influx of Ca^{2+} through the apical and basolateral membranes of the cells was also correlated with the content of the Ca^{2+} store. Experiments were performed in such a manner that EGTA was added to chelate all extracellular free Ca^{2+} and the monolayers were then challenged with different concentrations of thapsigargin (from 1 nM to 1 μM) to deplete the intracellular Ca^{2+} store to various extents. Calcium was then added to the apical (Fig. 6*A*) or basolateral bathing solutions (Fig. 6*B*). As summarized in Fig. 7, for a given concentration of thapsigargin, the increase in the I_{SC} upon basolateral addition of Ca^{2+} was always larger than that upon apical Ca^{2+} addition. The increase in I_{SC} upon basolateral addition of Ca^{2+} was also dependent on the

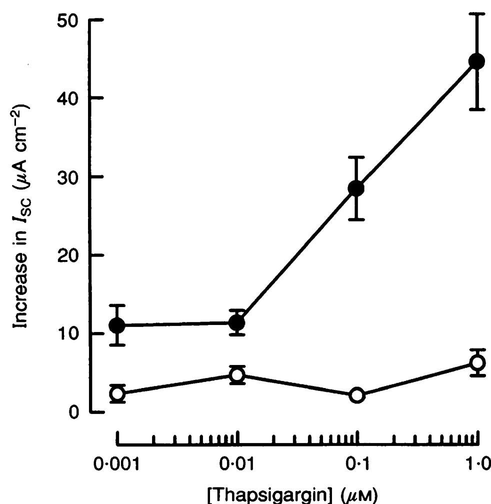

Figure 7. Effect of different concentrations of thapsigargin on I_{SC}

I_{SC} was elicited by re-addition of Ca^{2+} to the apical (○) or basolateral (●) side of EGTA-treated epithelia. Values are means ± S.E.M. for 5 separate epithelia.

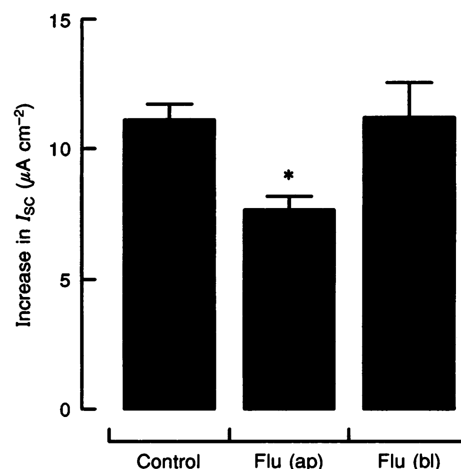

Figure 8. Inhibition of thapsigargin-mediated I_{SC} by pretreatment with apical or basolateral flufenamate (Flu, 100 μM)

Mean I_{SC} response to 0·1 μM thapsigargin applied apically. Values are means ± S.E.M. for 5 separate epithelia (* $P < 0.05$ compared with control).

thapsigargin concentration, suggesting that Ca^{2+} influx through the basolateral membrane depends on the content of the thapsigargin-sensitive Ca^{2+} store. The increase in I_{SC} upon apical addition of Ca^{2+} was seemingly rather insensitive to the concentration of thapsigargin (Fig. 7).

Differential effects of flufenamate and La^{3+}

It has been reported in other epithelial cell types that a non-selective cation channel is responsible for the capacitative-Ca^{2+} entry which can be blocked by flufenamate (Gögelein, Dahlem, Englert & Lang, 1990; Siemer & Gögelein, 1993). Therefore, flufenamate was used to test whether this type of channel is responsible for the Ca^{2+} influx through the plasma membrane in E/92/3 cells. Monolayers were pretreated with flufenamate (0·1 μM, apical or basolateral) and then challenged with 0·1 μM thapsigargin. As summarized in Fig. 8, the thapsigargin-induced I_{SC} was insensitive to basolateral pretreatment with flufenamate (I_{SC} increase was 11·2 ± 1·3 μA cm^{-2} compared with the control value of 11·1 ± 0·6 μA cm^{-2}, n.s., $n = 6$). In the presence of apically applied flufenamate, however, the thapsigargin-induced I_{SC} was inhibited by 32% (from 11·1 ± 0·6 to 7·6 ± 0·5 μA cm^{-2}, $P < 0.05$, $n = 5$), which was similar to the reduction in the thapsigargin-induced I_{SC} when Ca^{2+} was absent from the apical solution (Fig. 4A).

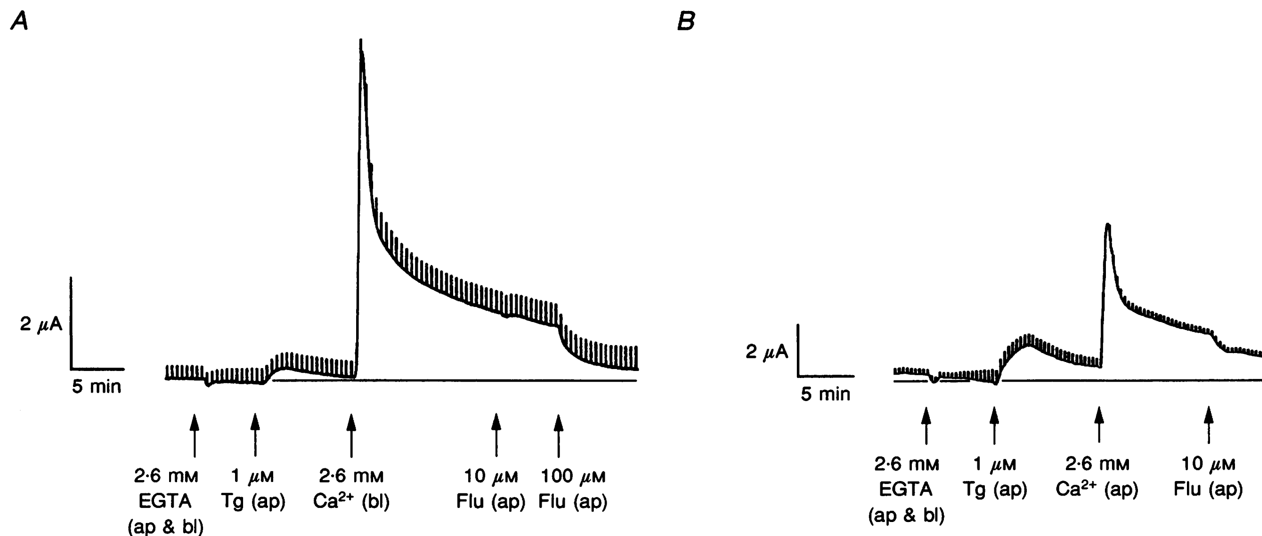

Figure 9. Demonstration of an effect of flufenamate on apical Ca^{2+} entry

A, I_{SC} recording showing ineffectiveness of 10 μM apical addition of flufenamate on basolateral Ca^{2+}-induced I_{SC}. Monolayers ($n = 9$) were challenged with thapsigargin (1 μM Tg (ap)) in Ca^{2+}-free bathing solution (2·6 mM EGTA (ap & bl)). After the response to thapsigargin had stabilized, Ca^{2+} (2·6 mM) was added to the basolateral bathing solution followed by apical application of 10 and 100 μM flufenamate (Flu). *B*, I_{SC} recording showing inhibitory effect of flufenamate on apical Ca^{2+}-activated I_{SC}. Monolayers ($n = 6$) were challenged with thapsigargin in Ca^{2+}-free bathing solution. After the response to thapsigargin had stabilized, Ca^{2+} was added to the apical bathing solution followed by an apical application of flufenamate (Flu, 10 μM).

Further experiments were carried out to demonstrate that flufenamate inhibited the thapsigargin-activated I_{SC} by blocking apical Ca^{2+} entry, and not by a direct effect on Cl^- channels. Monolayers were incubated with EGTA-containing solution on both sides of the chamber and then stimulated with thapsigargin (1 μM, apical) followed by re-addition of Ca^{2+} to the basolateral bathing solution. The increase in I_{SC}, which was solely dependent on basolateral Ca^{2+} influx, was inhibited by apical addition of flufenamate at 100 μM but not at 10 μM (Fig. 9*A*, $n = 9$), indicating that flufenamate had an effect on Cl^- channels at the higher concentration only. However, as shown in Fig. 9*B*, at 10 μM, a concentration shown to be ineffective in blocking Cl^- channels (see above), flufenamate was able to inhibit the I_{SC} induced by apical re-addition of Ca^{2+} (in the absence of basolateral Ca^{2+}, $n = 6$), indicating that flufenamate had an effect of on apical Ca^{2+} entry.

Voltage-dependent Ca^{2+} channel blockers, such as nifedipine and verapamil (0·1 mM), did not have any effect on the thapsigargin-induced I_{SC} when added to either the apical or basolateral side of the bath (data not shown). However, the cation La^{3+} was able to inhibit the increase in I_{SC} induced by re-addition of Ca^{2+} to the basolateral bathing solution (Fig. 10*A*, $n = 5$) in a concentration-dependent manner with an IC_{50} of 4·7 ± 1·5 μM (Fig. 10*B*), indicating that the basolateral membrane of the epithelium could have a different mechanism for Ca^{2+} entry from that observed in the apical membrane. The experiments were performed in HCO_3^--free solution to prevent precipitation of La^{3+}, but under HCO_3^--free conditions thapsigargin did not elicit any discernible changes in I_{SC} upon re-addition of apical Ca^{2+}. Therefore, it was not possible to test whether La^{3+} had any effect on the I_{SC} induced by re-addition of Ca^{2+} to the apical bathing solution.

DISCUSSION

The present study has clearly demonstrated the dependence of the thapsigargin-induced anion secretion on extracellular Ca^{2+}. The supporting evidence includes the following observations. (1) The thapsigargin-induced I_{SC} is inhibited by external addition of the Ca^{2+} chelator EGTA applied either before or after stimulation with thapsigargin. (2) EGTA-inhibited thapsigargin-induced I_{SC} can be reactivated upon addition of extracellular Ca^{2+}. (3) The thapsigargin-induced I_{SC} is also inhibited by pharmacological agents known to block Ca^{2+} channels.

In the presence of EGTA, thapsigargin induced a small rise in I_{SC} which was only 3 % of the rise observed in the absence of EGTA (Fig. 4*A*), indicating that a substantial proportion of the thapsigargin-induced anion secretion relies on extracellular Ca^{2+}. This is consistent with results from previous fluorescent studies performed in E/92/3 cells which showed that thapsigargin induced a biphasic increase

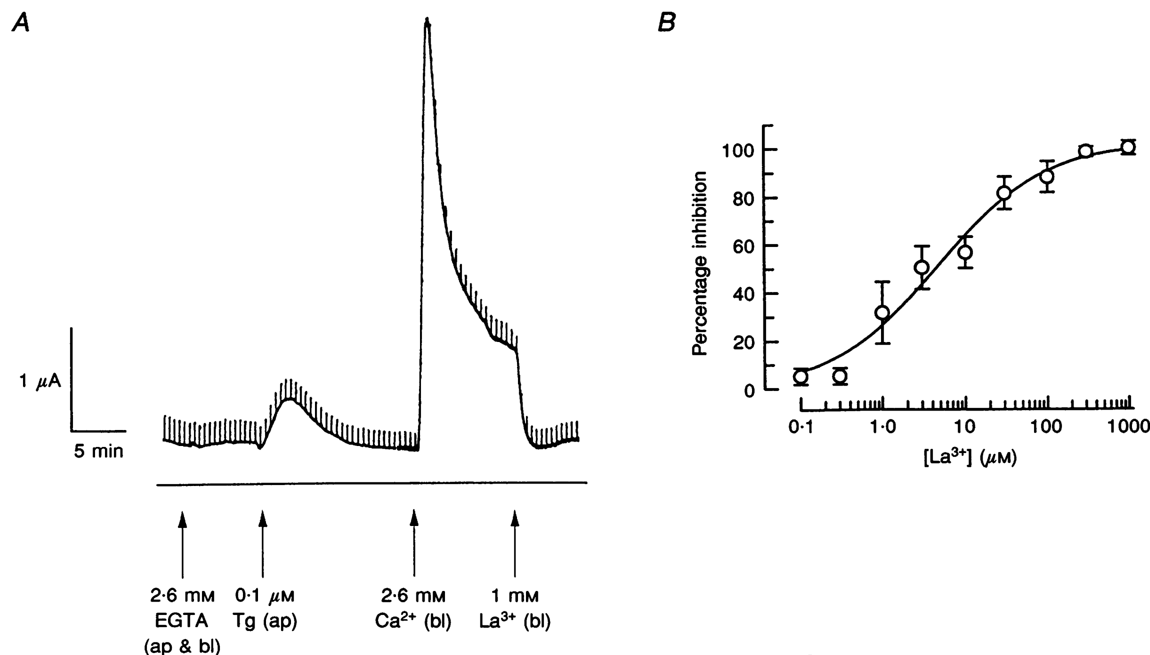

Figure 10. Inhibitory effect of La^{3+} on I_{SC}

A, inhibitory effect of La^{3+} on reactivated I_{SC} upon basolateral re-addition of Ca^{2+}. Monolayers were challenged with thapsigargin (0·1 μM Tg (ap)) in Ca^{2+}-free bathing solution (2·6 mM EGTA (ap & bl)). After the response to thapsigargin had stabilized, Ca^{2+} (2·6 mM) was added to the basolateral bathing solution and the subsequent I_{SC} was blocked by La^{3+} (1 mM). The record is representative of 5 experiments. *B*, concentration-dependent inhibition of La^{3+} on reactivated I_{SC} upon basolateral re-addition of Ca^{2+}. Values are means ± S.E.M. for 9 separate epithelia.

in $[Ca^{2+}]_i$, a small transient increase followed by a sustained increase, but that in Ca^{2+}-free medium thapsigargin elicited only a transient increase in $[Ca^{2+}]_i$ (Ko *et al.* 1994*b*). Although it could be argued that the thapsigargin-induced I_{SC} could be due to a direct effect of thapsigargin on anion secretion and not to a Ca^{2+}-dependent effect, this possibility is excluded by the present observation that thapsigargin was not able to elicit substantial I_{SC} in the presence of EGTA. Taken together, the results show that the dependence of the thapsigargin-induced I_{SC} on extracellular Ca^{2+} appears to reflect thapsigargin-induced Ca^{2+} mobilization in equine sweat gland epithelial cells.

It should be noted that a similar dependence of thapsigargin-induced anion secretion on extracellular Ca^{2+} has been observed previously in human colonic epithelial cells (Brayden, Hanley, Thastrup & Cuthbert, 1989). However, the details of the mechanisms by which Ca^{2+} enters the cells have not been elucidated. The present study has taken advantage of the Ussing chamber (Ussing & Zerahn, 1951), with separated apical and basolateral compartments, which allows separate manipulation of extracellular Ca^{2+} concentration in the apical or basolateral solutions to study in detail the mechanism of Ca^{2+} entry. A previous study has shown that equine sweat gland epithelial cells, when grown on permeable support, form polarized monolayers (Ko *et al.* 1996). The apical and basolateral surfaces can then be accessed separately via the two halves of the Ussing chamber. Most of the conventional fluorescent studies have been performed on cells grown on glass coverslips, which do not allow differentiation of epithelial cells and separate manipulation on either surface.

The present study has demonstrated for the first time that thapsigargin-induced anion secretion by an epithelium depends upon differential Ca^{2+} entry through apical and basolateral membrane domains. It appears that in equine sweat gland epithelial cells the thapsigargin-induced Ca^{2+} influx is predominantly through the basolateral membrane. First, varying apical concentrations of Ca^{2+} by adjusting the EGTA concentration while keeping the basolateral Ca^{2+} concentration constant did not seem to affect the thapsigargin-induced I_{SC} to a great extent. On the contrary, the magnitude of the thapsigargin-induced I_{SC} increased as basolateral Ca^{2+} concentration increased, indicating a significant role for basolateral Ca^{2+} influx in thapsigargin-induced anion secretion. Second, the dependence on concentration of the thapsigargin-induced I_{SC} was observed only in the presence of basolateral Ca^{2+}, regardless of the apical Ca^{2+} concentration. It has been reported in other cell types that Ca^{2+} influx correlates with a gradual emptying of Ca^{2+} stores which is achieved by applying different concentrations of thapsigargin (Villalobos & García-Sancho, 1995). The dependence of the I_{SC} on thapsigargin concentration suggests that the emptying of the Ca^{2+} store induced by thapsigargin evokes a predominantly basolateral Ca^{2+} influx which is important in stimulating anion secretion. In other words, thapsigargin-induced anion secretion may be mediated by capacitative Ca^{2+} entry through the basolateral membrane. Lastly, a Ca^{2+} ionophore, A23187, also induced an increase in I_{SC}, which also appears to be predominantly dependent on basolateral Ca^{2+} influx. The ionophore may act mainly by permeabilizing the store membrane and hence by activation of capacitative Ca^{2+} entry in equine sweat gland epithelial cells.

There has long been controversy over whether capacitative Ca^{2+} entry into non-excitable cells occurs through channels or through a carrier mechanism (review by Putney & Bird, 1993). The two major obstacles associated with identifying Ca^{2+} influx pathways in epithelial cells are the lack of specific agonists and antagonists for the channels, and the rather low conductance of this pathway. Recently, a patch-clamp study in HT_{29} colonic carcinoma cells has revealed a inward cation current which could be activated by Ca^{2+} store release and cytosolic Ca^{2+} chelation (Kerst, Fischer, Normann, Kramer, Leipziger & Greger, 1995). This inward cation current was inhibited by removal of Ca^{2+} and/or Na^+ from the bathing solution, and was sensitive to flufenamate, Gd^{3+} and La^{3+}. It has been proposed that this flufenamate-, Gd^{3+}- and La^{3+}-sensitive inward cation current is responsible for capacitative Ca^{2+} entry in HT_{29} cells. The present study in equine sweat gland cells has also demonstrated that the thapsigargin-induced I_{SC}, which is dependent on thapsigargin-induced Ca^{2+} influx, is inhibitable by flufenamate and La^{3+}, but not by other Ca^{2+} channel blockers, such as verapamil and nifedipine. The present study has further demonstrated that the thapsigargin-induced Ca^{2+} influx through apical and basolateral membranes exhibits differential sensitivity to flufenamate and La^{3+}, indicating for the first time that the apical and basolateral membranes of an epithelium may possess different Ca^{2+} influx pathways, i.e. different ion channels. The observed inhibitory effect of apically but not basolaterally applied flufenamate suggests that the thapsigargin-induced apical Ca^{2+} influx may be through a flufenamate-sensitive non-selective cation channel (Gögelein *et al.* 1990; Schumann, Greger & Leipziger, 1994; Kerst *et al.* 1995). One could argue that the effect of flufenamate could be due to a direct effect on Cl^- channels (Wangemann *et al.* 1986). However, a previous patch-clamp study has shown that flufenamate is likely to act by attenuating the agonist-induced increase in $[Ca^{2+}]_i$, rather than by affecting Cl^- channels directly (Kerst *et al.* 1995). The present results also indicate that flufenamate blocks apical Ca^{2+} entry directly. In the absence of apical Ca^{2+}, apical addition of flufenamate inhibited I_{SC} induced by basolateral Ca^{2+} influx at a concentration of 100 μM, indicating that flufenamate has an effect on Cl^- channels at this concentration. However, 10 μM flufenamate was sufficient to block I_{SC} induced by apical Ca^{2+} influx, suggesting that it affected Ca^{2+} entry rather than Cl^- channels at this low concentration. In contrast, La^{3+} has been found to block basolateral Ca^{2+} entry in the present study. It should be noted that the experiments were performed in HCO_3^--free solution to prevent precipitation of La^{3+}, but that, for reasons we

cannot explain, under HCO_3^--free conditions thapsigargin did not elicit any discernible changes in I_{SC} upon re-addition of apical Ca^{2+}. Therefore, it is not certain whether La^{3+} has an effect on apical Ca^{2+} influx. What is clear from the present study is that the basolateral Ca^{2+} influx is inhibitable by La^{3+} but not flufenamate. The previously observed inhibitory effects of both flufenamate and La^{3+} on the Ca^{2+}-dependent inward cation current in HT_{29} cells (Kerst *et al.* 1995) could be due to the fact that voltage-clamped patches may access channels present in both apical and basolateral membranes. La^{3+} is known to block many Ca^{2+} channels and has been shown to block Ca^{2+} entry in a number of epithelia (Brayden *et al.* 1989; Negulescu & Machen, 1995). Although we cannot provide information on which specific Ca^{2+} channels are involved, the present study has clearly indicated that different mechanisms, i.e. flufenamate-sensitive and La^{3+}-sensitive ion channels, are involved in apical and basolateral Ca^{2+} entry. The asymmetrical activation of the thapsigargin-induced I_{SC} by apical and basolateral Ca^{2+} influx observed in the present study (Fig. 6) could be explained by different Ca^{2+} entry kinetics governed by different ion channels present in the apical and basolateral membranes.

The present finding of a differential dependence of the thapsigargin-induced I_{SC} on apical and basolateral Ca^{2+} influx, and the possible involvement of different Ca^{2+} entry pathways in the two membranes, is consistent with the results obtained from a recent fluorescent study demonstrating membrane-restricted regulation of Ca^{2+} release and influx in polarized airway epithelia (Paradiso, Mason, Lazarowski & Boucher, 1995). It was found that internal Ca^{2+} release and activation of the Ca^{2+} influx pathway were confined to the membrane of receptor activation. This implies the existence of two topographically distinct Ca^{2+} stores, each associated with apical and basolateral membrane of the cells. Although the present study employing thapsigargin has demonstrated different membrane-associated Ca^{2+} influx pathways, it is not certain that in E/92/3 cells receptor-regulated Ca^{2+} release and influx is membrane restricted, since thapsigargin is a Ca^{2+}-mobilizing agent that bypasses the receptor. Further investigation using a receptor agonist, such as ATP, is required to clarify this point.

Although the present study did not measure cellular Ca^{2+} directly, our results, together with previous Ca^{2+} measurements (Ko *et al.* 1994*a*,*b*; Ko, Pediani, Bovell & Wilson, 1995), have established a link between thapsigargin-induced I_{SC} and thapsigargin-induced Ca^{2+} influx. With this link established, we have been able to provide the first evidence demonstrating anion secretion induced by capacitative Ca^{2+} entry through apical and basolateral membranes via different mechanisms, e.g. different ion channels. The present study has also indicated a predominant role of basolateral Ca^{2+} influx in regulating anion secretion in equine sweat gland epithelial cells. It remains to be elucidated whether the regulation of the apparently separate Ca^{2+} influx pathways is also membrane restricted, i.e. whether the apical (or basolateral) receptor activates only the apical (or basolateral) Ca^{2+} influx, as has been observed in airway epithelia (Paradiso *et al.* 1995). The answer to this question may provide a better understanding of how epithelia respond to various luminal and serosal environments and yet maintain cellular homeostatic functions.

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