

## Ionic mechanisms of $\text{Ca}^{2+}$ -dependent electrolyte transport across equine sweat gland epithelium

W. H. KO, H. C. Chan\*, S. B. Cheng Chew and P. Y. D. Wong

*Department of Physiology, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, NT, Hong Kong*

1. The ionic mechanism involved in  $\text{Ca}^{2+}$ -stimulated electrolyte transport in cultured equine sweat gland epithelial cells was studied using the short-circuit current ( $I_{\text{SC}}$ ) technique.
2. Microscopy revealed that the cultured cells grown on Millipore filters formed polarized monolayers with tight junctions. Monolayers exhibited a mean transepithelial resistance of  $333.9 \pm 40.4 \Omega \text{ cm}^2$ .
3.  $\text{Ca}^{2+}$ -mobilizing agents, A23187 ( $1 \mu\text{M}$ ) or thapsigargin ( $0.01$ – $1 \mu\text{M}$ ), stimulated  $I_{\text{SC}}$  while forskolin exerted little effect on the  $I_{\text{SC}}$ .
4. Replacement of external  $\text{Cl}^-$  by gluconate significantly reduced the  $I_{\text{SC}}$  by 63% when stimulated by  $0.1 \mu\text{M}$  thapsigargin. Residual  $I_{\text{SC}}$  could be abolished (>99%) by elimination of  $\text{HCO}_3^-$  from the bathing solution.
5. Basolateral addition of bumetanide ( $0.1 \text{ mM}$ ), ouabain ( $0.01 \text{ mM}$ ) and acetazolamide ( $45 \mu\text{M}$ ) and apical addition of methyl isobutyl amiloride (MIA,  $1$ – $100 \mu\text{M}$ ) all had inhibitory effects on the thapsigargin-stimulated  $I_{\text{SC}}$  to various extents.
6. Substantial current inhibition could be obtained using 4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid (DIDS) and diphenylamine-2-carboxylate (DPC) in a concentration-dependent manner.
7. The  $\text{K}^+$  channel blocker barium ( $5 \text{ mM}$ ) was effective on both sides of the epithelium with a much larger effect on the basolateral side.
8. The inhibitory effects of acetazolamide, amiloride, MIA, DIDS and DPC on the thapsigargin-stimulated  $I_{\text{SC}}$  were also observed when a  $\text{Cl}^-$ -free solution was used.
9. The results provide evidence for  $\text{Ca}^{2+}$ -stimulated  $\text{HCO}_3^-$  as well as  $\text{Cl}^-$  secretion by equine sweat gland epithelium.

Sweat production in equine species and primates, especially in humans, is an important component of thermoregulation (Quinton, 1983). The mechanism by which sweating is controlled in equines appears to be quite different from that in primates. While the principal regulator of sweat secretion in primates is acetylcholine, equine glands do not respond to this agonist but secrete predominantly in response to  $\beta$ -adrenergic agonists (Snow, 1977; Johnson & Creed, 1982; Bijman & Quinton, 1984*a, b*). However, there is evidence that  $\text{Ca}^{2+}$  may also play a role (Bijman & Quinton, 1984*a, b*; Wilson, Pediani, Bovell, Smith, Elder & Jenkinson, 1993*a*). In fact, agonist-stimulated anion permeability of a cell line derived from the equine sweat gland epithelium has been shown to be mediated by  $\text{Ca}^{2+}$  and not cAMP (Ko *et al.* 1994*a*; Ko,

Wilson & Elder, 1994*b*; Wilson, Ko, Pediani, Rakhit, Nichol & Bovell, 1995). This cell line was originated from explanted and cultured sweat glands isolated from horse skin. It appears that functional cAMP-dependent  $\text{Cl}^-$  channels have been lost during culture but that the cultured cells respond to adrenaline with increased  $\text{Cl}^-$  permeability and intracellular  $\text{Ca}^{2+}$  concentration (Wilson *et al.* 1993*b*). Possessing this feature, these cells may provide a unique system for studying  $\text{Ca}^{2+}$ -dependent mechanisms involved in agonist-stimulated sweat secretion, which may also have implications for the human since the  $\text{Ca}^{2+}$ -dependent regulatory pathway via cholinergic stimulation is by far the most important control of sweating in humans. The present study attempted to establish an equine culture grown on permeable supports to be assessed by short-circuit current

\* To whom correspondence should be addressed.

( $I_{SC}$ ) measurement and elucidate the ionic mechanism of  $Ca^{2+}$ -activated electrolyte transport in the equine sweat gland epithelium.

## METHODS

### Tissue culture technique

Standard techniques were used to maintain an epithelial cell line (E/92/3) derived from the equine sweat gland as described previously (Wilson *et al.* 1993*b*). The standard culture medium was William's medium E, completed with 5% fetal calf serum and supplemented with L-glutamine (1 mM), penicillin (100 i.u. ml<sup>-1</sup>), streptomycin (100 µg ml<sup>-1</sup>), bovine insulin (100 µg ml<sup>-1</sup>), hydrocortisone (10 ng ml<sup>-1</sup>) and sodium selenite (10 ng ml<sup>-1</sup>). Cells were removed from the culture flasks by 0.25% trypsin-EDTA (0.05 mM). Aliquots of 0.25 ml of cell suspension containing  $1 \times 10^5$  cells were seeded into each 0.45 cm<sup>2</sup> Millipore filter assembly as described by Cuthbert, George & MacVinish (1985). Briefly, a silicone washer with a 0.45 cm<sup>2</sup> 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 a permeable support floating on the culture medium. Cultures were incubated for 3–4 days at 37 °C in 5% CO<sub>2</sub>. Thereafter, the monolayers reached confluency and were ready for the short-circuit current ( $I_{SC}$ ) measurement.

### Short-circuit current measurement

The method used for the  $I_{SC}$  measurement was described elsewhere (Cuthbert *et al.* 1985). In short, the confluent monolayers were clamped vertically between the two halves of an Ussing chamber. Monolayers were short circuited (transepithelial potential difference clamped at zero) using a voltage-clamp amplifier (DVC 1000; World Precision Instruments Inc., New Haven, CT, USA). The resulting  $I_{SC}$  was displayed on-line on a pen recorder (Kipp and Zonen, Delft, The Netherlands). Transepithelial resistance was obtained from Ohm's law by clamping the tissue intermittently at a value of 0.1 mV. In most experiments, tissues were bathed in normal Krebs-Henseleit (K-H) solution.

### Light and electron microscopy

Morphological examinations were made under light and electron microscopy. Three-day-cultured epithelial cells were stained with Toluidine Blue for light microscopical observation. For transmission electron microscopy, cells were fixed with 2.5% glutaraldehyde (pH 7.4) in phosphate buffer solution for 1 h at 4 °C, and washed in a phosphate buffer solution before post-fixation in osmium tetroxide (1%). Samples were dehydrated in an ethanol series, infiltrated with up-graded propylene oxide-spurr mixture (Merck, Germany and Electron Microscopy Sciences, PA, USA, respectively) and finally embedded in freshly made pure spurr. The resin was polymerized overnight at 65 °C. Sections were cut and stained with uranyl acetate and lead citrate, and examined in a Hitachi H7100 electron microscope.

### 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, 4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid (DIDS), barium chloride and bumetanide were purchased from Sigma. Thapsigargin, forskolin and A23187 were purchased from Calbiochem Novabiochem (La Jolla, CA, USA). Methyl isobutyl amiloride (MIA) was purchased from Research Biochemicals

International. Acetazolamide was obtained from American Cyanamid Company (Pearl River, NY, USA). Diphenylamine-2-carboxylate (DPC) was purchased from Riedel-de Haën (Germany). Normal K-H solution had the following composition (mM): NaCl, 117; KCl, 4.7; CaCl<sub>2</sub>, 2.5; KH<sub>2</sub>PO<sub>4</sub>, 1.2; MgSO<sub>4</sub>, 1.2; NaHCO<sub>3</sub>, 25; glucose, 11.1. The solution was gassed with 5% CO<sub>2</sub>-95% O<sub>2</sub> to give a pH of 7.4. In Cl<sup>-</sup>-free solution, ambient Cl<sup>-</sup> was removed by substitution of gluconate (141.7 mM). The osmolalities of the solutions were 290 mosmol kg<sup>-1</sup>. In Cl<sup>-</sup>- and HCO<sub>3</sub><sup>-</sup>-free solution, both anions were replaced with gluconate (141.7 mM) and the solution was buffered with 10 mM Hepes and bubbled with 100% O<sub>2</sub> to maintain pH at 7.4. The osmolality was adjusted to 290 mosmol kg<sup>-1</sup> with D-mannitol (25 mM). To prevent precipitation when barium was used, KH<sub>2</sub>PO<sub>4</sub> was omitted from the solution and MgSO<sub>4</sub> was replaced with MgCl<sub>2</sub>.

### Statistical analysis

Results are expressed as means ± s.e.m. Comparisons between groups of data were made by Student's unpaired *t* test. *P* < 0.05 was considered statistically significant.

## RESULTS

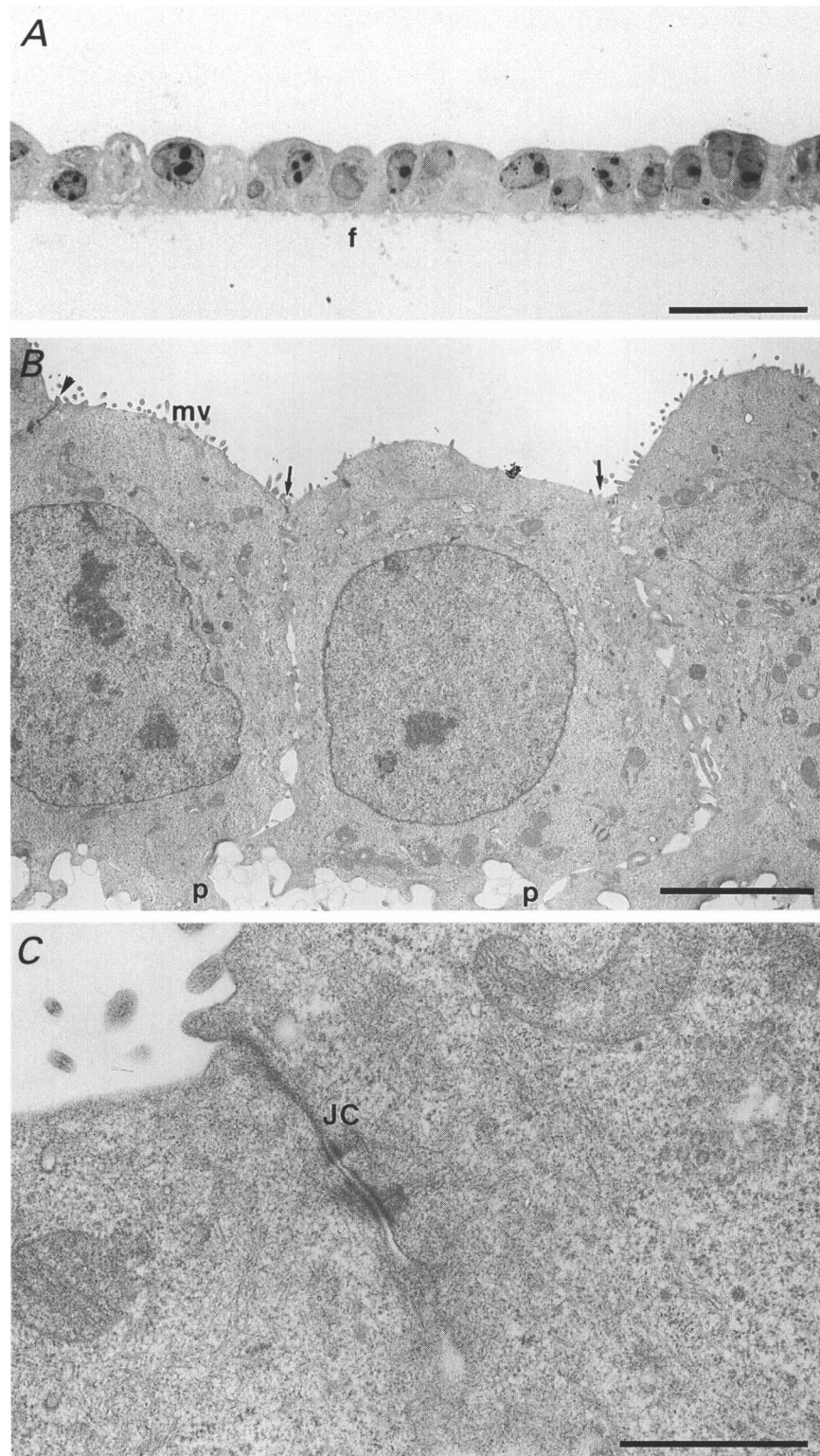
### Formation of polarized cultures on permeable supports

Epithelial sheet cultures were grown on Millipore filters as described in Methods. Satisfactory cultures required seeding with cells at a density of  $1 \times 10^5$  cells per well; densely seeded wells ( $3 \times 10^5$  cells per well) became multilayered in most of the area within 3 days of growth. Figure 1*A* shows a light microscopic section of a 3-day-cultured epithelial sheet stained with Toluidine Blue. A confluent sheet of monolayered cells was demonstrated, with cell nuclei and prominent nucleoli situated at the centre of the cells. The detailed appearance of these cells under similar culture condition is shown by the electron micrographs of Fig. 1*B* and *C*. The cells had microvilli on their apical surfaces as well as closely apposed intercellular membranes and well-developed junctional complexes. Cytoplasmic filaments and mitochondria were also encountered in the cytoplasm of the cell.

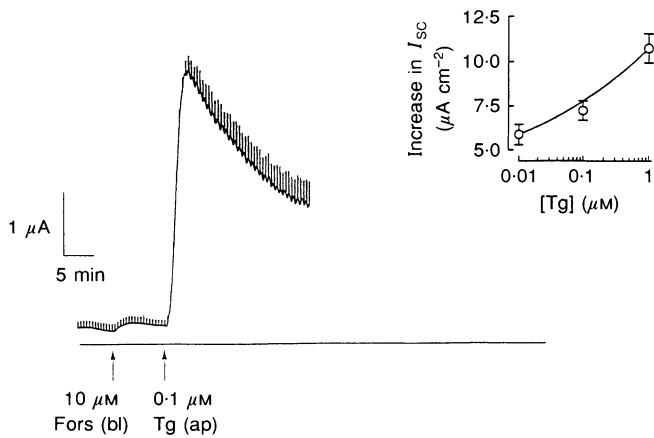
The electrophysiological parameters measured show that both the transepithelial potential and the basal  $I_{SC}$  were small but the cells did form a tight epithelium as indicated from the high transepithelial resistance. The average basal transepithelial potential was  $0.42 \pm 0.04$  mV (mean ± s.e.m., *n* = 42), with the apical side negative with respect to the basolateral. The basal  $I_{SC}$  was  $0.6 \pm 0.1$  µA cm<sup>-2</sup> (*n* = 42) and the transepithelial resistance was  $333.9 \pm 40.4$  Ω cm<sup>2</sup> (*n* = 42).

### Responses to Ca<sup>2+</sup>-mobilizing agents and insensitivity to cAMP-activating agents

<sup>125</sup>I efflux, measured in the same horse sweat gland cell line, has been shown to be insensitive to a cocktail of compounds designed to evoke a maximal cAMP-dependent response (Wilson *et al.* 1993*b*). In the present study, we wanted to confirm this finding using the  $I_{SC}$  technique. As shown in Fig. 2, addition of 10 µM forskolin did not alter



**Figure 1.** Light and electron micrographs of a 3-day cultured equine sweat gland epithelial cell line  
*A*, monolayered epithelial cells with basal region rested on a filter paper (*f*). Magnification,  $\times 460$ ; scale bar,  $50 \mu\text{m}$ . *B*, monolayered cells with cytoplasmic protrusions (*p*) growing down into the cellulose matrix. The apical region shows microvilli (*mv*) with apposed apical membranes (arrows) and tight junctions in between cells (arrowhead). Magnification,  $\times 4950$ ; scale bar,  $5 \mu\text{m}$ . *C*, a high magnification of part of *B* showing a well-developed junctional complex (*JC*). Formation of tight junction and desmosomes with filamentous material are clearly evidenced. Magnification,  $\times 31200$ ; scale bar,  $1 \mu\text{m}$ .



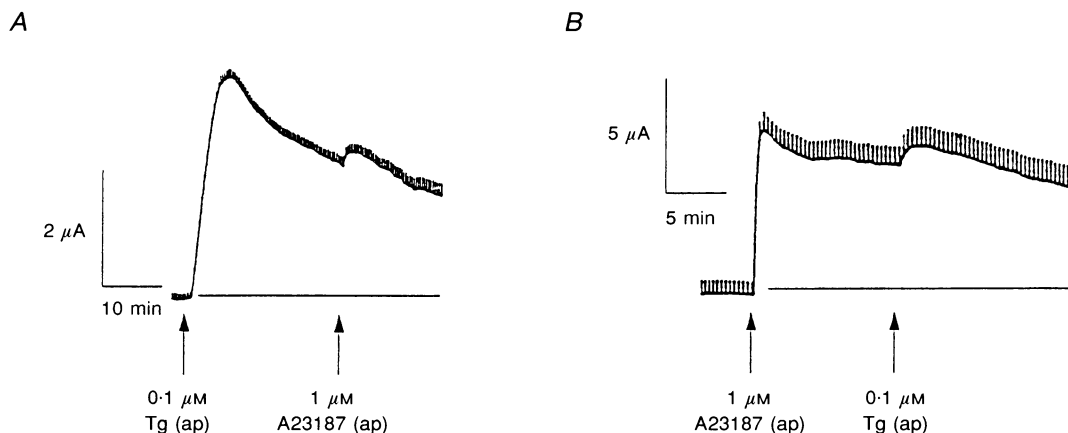
**Figure 2.** Effect of forskolin and thapsigargin on  $I_{SC}$  response in the cultured equine sweat gland epithelia

Transient current pulses were the results of intermittently clamping the potential at 0.1 mV. Arrows mark the time at which forskolin (Fors; basolateral, 10  $\mu$ M) or thapsigargin (Tg; apical, 1  $\mu$ M) was added. The horizontal lines represent zero  $I_{SC}$ . The inset shows the concentration-dependent increase in  $I_{SC}$  evoked by thapsigargin in the absence of forskolin. Values are means  $\pm$  s.e.m. for 10–13 separate epithelia.

the basal  $I_{SC}$  significantly ( $0.5 \pm 0.1 \mu\text{A cm}^{-2}$  as compared with a basal  $I_{SC}$  of  $0.4 \pm 0.1 \mu\text{A cm}^{-2}$ , n.s.,  $n = 6$ ). However, when the  $\text{Ca}^{2+}$ -mobilizing agent thapsigargin (0.1  $\mu\text{M}$ ), a microsomal  $\text{Ca}^{2+}$ -ATPase inhibitor (Thastrup, Cullen, Drobak, Hanley & Dawson, 1990), was added to the apical aspect, a rapid increase in  $I_{SC}$  was observed as illustrated in Fig. 2.  $I_{SC}$  responded to thapsigargin alone in a concentration-dependent manner as shown in the inset of Fig. 2. It was found that thapsigargin at a concentration of 3 nM did not cause any discernible change in  $I_{SC}$  while a significant increase in  $I_{SC}$  could be observed at 10 nM. No effect on the  $I_{SC}$  was observed when thapsigargin (up to 2  $\mu\text{M}$ ) was added to the basolateral side of the epithelia ( $n = 3$ , data not shown). Addition of a  $\text{Ca}^{2+}$  ionophore, A23187 (1  $\mu\text{M}$ ), to the apical side also produced a rapid increase in the  $I_{SC}$  (from  $0.03 \pm 0.03$  to  $11.2 \pm 2.2 \mu\text{A cm}^{-2}$ ,  $n = 3$ ). Neither thapsigargin nor A23187 could increase  $I_{SC}$  much further after treatment with A23187 or thapsigargin, respectively, as shown in Fig. 3, suggesting that the action of both agents might be mediated through a final common  $\text{Ca}^{2+}$ -dependent pathway as demonstrated in monolayers of human colonic epithelial cells (Brayden, Hanley, Thastrup & Cuthbert, 1989).

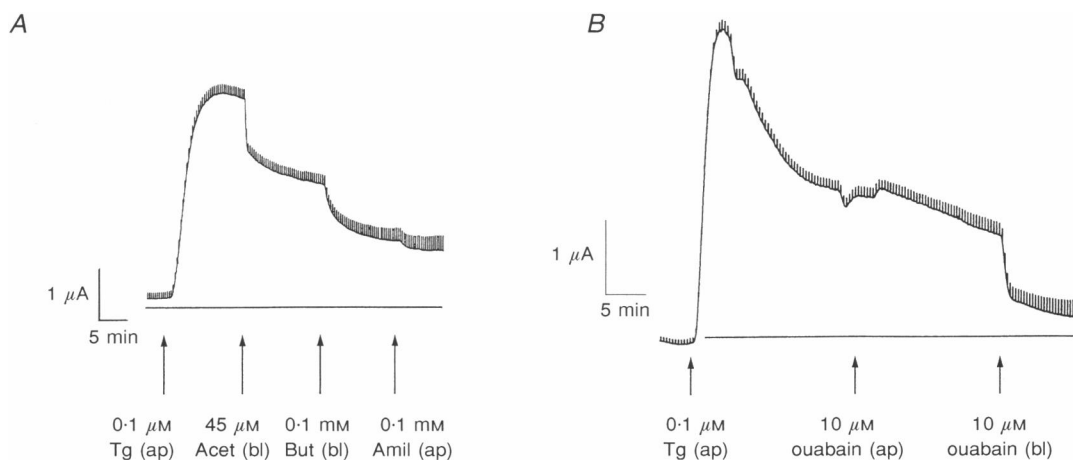
### Involvement of $\text{Cl}^-$ and $\text{HCO}_3^-$ in mediating thapsigargin-induced $I_{SC}$

It was expected that the  $I_{SC}$  response induced by the  $\text{Ca}^{2+}$ -mobilizing agents was largely due to the electrogenic anion secretion by these cells since they were derived from the secretory coil of equine sweat glands (Wilson *et al.* 1993b). Ion substitution experiments were performed to identify the ion-carrying species involved. When  $\text{Cl}^-$ -free (substitution with gluconate) K-H solution was used, the calcium concentration was re-adjusted to that in the normal K-H solution using a calcium-sensitive electrode to compensate for any  $\text{Ca}^{2+}$ -chelating effect exerted by gluconate.  $I_{SC}$  induced by thapsigargin (0.1  $\mu\text{M}$ ) was reduced in  $\text{Cl}^-$ -free K-H solution ( $2.68 \pm 0.18 \mu\text{A cm}^{-2}$ ,  $n = 44$ , as compared with  $7.23 \pm 0.55 \mu\text{A cm}^{-2}$ ,  $n = 13$ , in normal K-H solution,  $P < 0.001$ ), indicating that  $\text{Cl}^-$  was largely (63%) responsible for the increase in  $I_{SC}$ . Experiments were also performed in which both  $\text{Cl}^-$  and  $\text{HCO}_3^-$  in the bathing solution were replaced. The  $I_{SC}$  induced by 0.1  $\mu\text{M}$  thapsigargin was then completely abolished ( $n = 6$ ) suggesting that both  $\text{Cl}^-$  and  $\text{HCO}_3^-$  were involved in the  $I_{SC}$  response to thapsigargin.



**Figure 3.**  $I_{SC}$  responses to sequential addition of Tg and A23187 (A) or A23187 and Tg (B)

Arrows mark the time at which A23187 (apical, 1  $\mu\text{M}$ ) or thapsigargin (apical, 0.1  $\mu\text{M}$ ) was added. Each record is representative of 3 experiments.



**Figure 4**

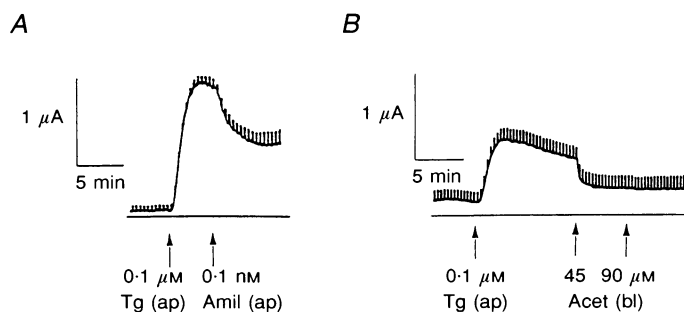
*A*, effect of sequential basolateral additions of acetazolamide ( $45 \mu M$ ) and bumetanide ( $0.1 \text{ mM}$ ) and apical addition of amiloride ( $0.1 \text{ mM}$ ) on the thapsigargin-evoked  $I_{sc}$ . *B*, effect of apical and basolateral additions of ouabain ( $0.01 \text{ mM}$ ) on the thapsigargin-evoked  $I_{sc}$ . Each record is representative of 4 experiments.

### Effects of inhibitors of ion transport on thapsigargin-induced $I_{sc}$

In order to elucidate the secretory mechanism in equine sweat gland cells, different inhibitors of transport processes were used. Figure 4*A* shows that the  $I_{sc}$  response to thapsigargin, obtained in normal K-H solution, was partially ( $31.3 \pm 3.3\%$ ,  $n = 13$ ) sensitive to basolateral application of a  $\text{Na}^+ - \text{K}^+ - \text{Cl}^-$  cotransporter inhibitor, bumetanide ( $0.1 \text{ mM}$ ). A fraction of the current,  $44.7 \pm 2.1\%$  ( $n = 13$ ), was also sensitive to a carbonic anhydrase inhibitor, acetazolamide ( $45 \mu M$ , basolateral). These two blockers acted additively to give about 75% inhibition of  $I_{sc}$  with the order of addition being immaterial. Also  $12.2 \pm 1.5\%$  ( $n = 11$ ) of the thapsigargin-induced  $I_{sc}$  was sensitive to apical addition of amiloride ( $0.1 \text{ mM}$ ). The presence of the basolaterally located  $\text{Na}^+ - \text{K}^+ - \text{ATPase}$  was also demonstrated by the inhibitory effect of ouabain on the thapsigargin-induced  $I_{sc}$  (Fig. 4*B*). Ouabain ( $0.01 \text{ mM}$ ) was found to be effective only when applied to the basolateral aspect of the epithelium, inhibiting  $77.6 \pm 8.4\%$  of the  $I_{sc}$  ( $n = 8$ ).

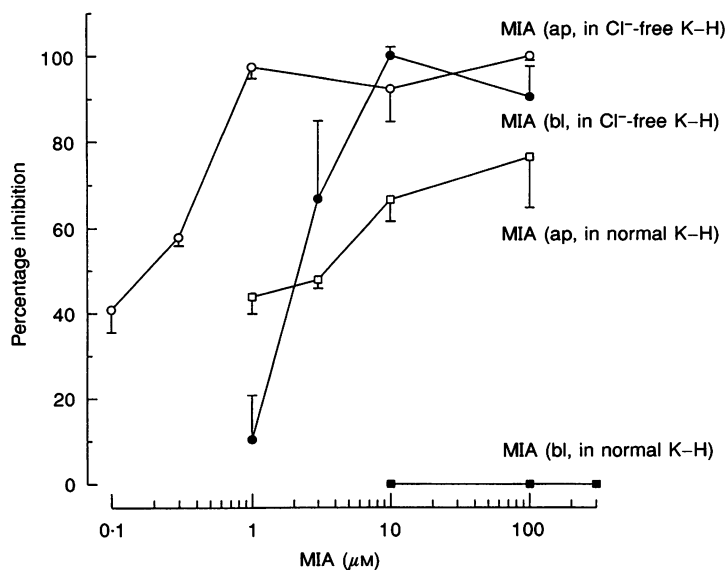
The effect of various inhibitors on  $\text{HCO}_3^-$ -dependent secretion was also examined, and the results are shown in Fig. 5. Under  $\text{Cl}^-$ -free condition, acetazolamide ( $45 \mu M$ , basolateral,  $n = 13$ ) was found to inhibit  $59.6 \pm 6.4\%$  of the thapsigargin-induced  $I_{sc}$ ;  $53.9 \pm 8.4\%$  of the  $I_{sc}$  could be inhibited by apical addition of  $0.1 \text{ mM}$  amiloride ( $n = 11$ ).

The effect of a specific  $\text{Na}^+ - \text{H}^+$  exchanger inhibitor, methyl isobutyl amiloride (MIA), on the thapsigargin-induced  $I_{sc}$  was also examined. In normal K-H solution, MIA was found to exert a significant inhibitory effect only when applied to apical but not to the basolateral side of the epithelium (Fig. 6). In  $\text{Cl}^-$ -free solution, MIA could exert its effect on both sides of the epithelium with a larger effect on the apical side (Fig. 6). However, MIA, as well as amiloride, was found to be ineffective in  $\text{HCO}_3^-$ -free solution ( $n = 6$ ), indicating a  $\text{HCO}_3^-$  dependence of the MIA and amiloride effect. Application of MIA was also found to abolish the effect of subsequent addition of amiloride ( $0.1 \text{ mM}$ , apical;  $n = 6$ ), suggesting that these two agents had a similar action (Fig. 7). The ineffectiveness of MIA or amiloride in



**Figure 5**

Effect of amiloride ( $0.1 \text{ mM}$ , apical; *A*) and acetazolamide ( $45 \mu M$ , basolateral; *B*) on thapsigargin-evoked  $I_{sc}$  in  $\text{Cl}^-$ -free bathing solution.



**Figure 6.** Concentration-dependent inhibition of thapsigargin-evoked  $I_{SC}$  by MIA in normal and chloride-free solution

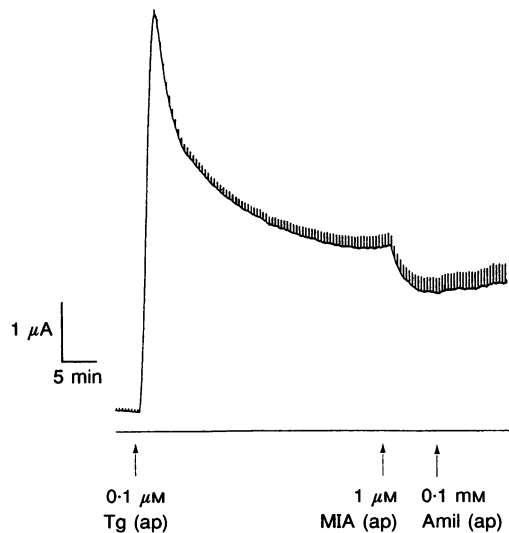
The effect of apical as well as basolateral addition of MIA was examined. Values are means  $\pm$  s.e.m. for 4 separate epithelia.

$\text{HCO}_3^-$ -free solution suggested that their action was on the  $\text{Na}^+-\text{H}^+$  exchanger rather than on apical  $\text{Na}^+$  channels. In  $\text{HCO}_3^-$ -free solution, there was no  $\text{CO}_2$  diffusing across the cells and hence no hydration of  $\text{CO}_2$  by carbonic anhydrase (inhibitible by acetazolamide) to form carbonic acid, which could be further dissociated to form  $\text{H}^+$  and  $\text{HCO}_3^-$ . Under this condition, the  $\text{Na}^+-\text{H}^+$  exchanger would not be in operation, and therefore no effect of MIA or amiloride could be observed.

We also examined the effect of two  $\text{Cl}^-$  channel blockers, DIDS and DPC, on the  $I_{SC}$  induced by either thapsigargin or A23187. In normal K-H solution,  $I_{SC}$  induced by both agents could be inhibited by apical addition, but not basolateral addition, of DIDS or DPC in a concentration-dependent manner. At concentrations of 0.9 mM (DPC) and 0.3 mM (DIDS), these two channel blockers were shown to inhibit 96% ( $n=3$ ) and 100% ( $n=3$ ) of the  $I_{SC}$ , respectively, suggesting that these  $\text{Cl}^-$  channel blockers were able to block both  $\text{Cl}^-$ - and  $\text{HCO}_3^-$ -dependent  $I_{SC}$ . The

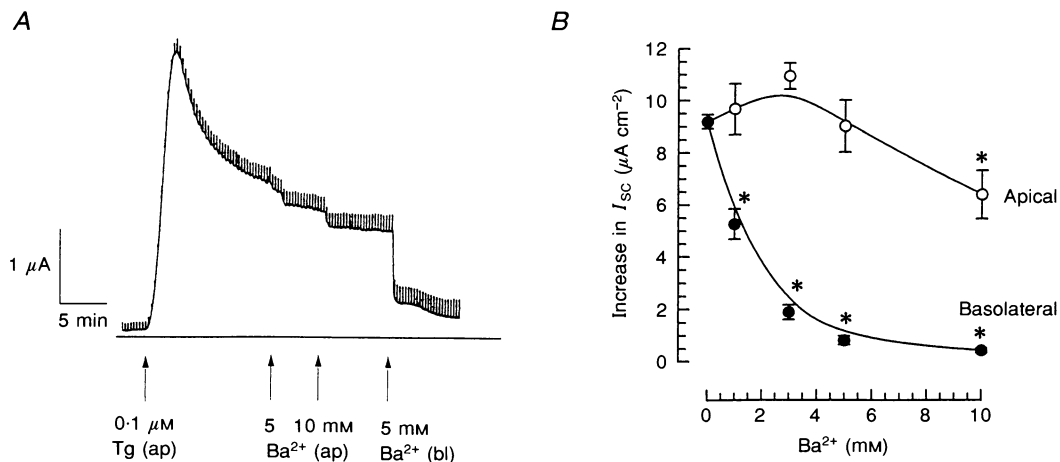
ability of the  $\text{Cl}^-$  channel blockers to block  $\text{HCO}_3^-$ -dependent secretion was further demonstrated in experiments using  $\text{Cl}^-$ -free solution. Inhibition of 79% ( $n=6$ ) of the  $\text{HCO}_3^-$ -dependent thapsigargin-induced  $I_{SC}$  was observed when 6  $\mu\text{M}$  DIDS or 0.5 mM DPC were used.

As shown in Fig. 8A, after stimulation with thapsigargin (0.1  $\mu\text{M}$ ), addition of the  $\text{K}^+$  channel blocker barium, to the apical side, only reduced the  $I_{SC}$  slightly even up to a concentration of 10 mM, whereas addition of 5 mM barium to the basolateral side caused a marked decrease in  $I_{SC}$  (96%;  $n=3$ ). Subsequent experiments were performed in which different concentrations of barium were added to either the apical or basolateral side of the monolayers followed by the agonist stimulation. The concentration-dependent inhibition of  $I_{SC}$  shown in Fig. 8B indicates a larger effect of basolateral addition of barium. Basolaterally administered barium achieved 95% ( $n=5$ ) inhibition of the thapsigargin-induced  $I_{SC}$  at a concentration of 10 mM ( $n=4$ ), whereas apical addition of barium exerted only



**Figure 7**

Effect of sequential apical addition of MIA (1  $\mu\text{M}$ ) and amiloride (Amil, 0.1 mM) on thapsigargin-evoked  $I_{SC}$  in normal K-H bathing solution.



**Figure 8.** Effect of apical (5 and 10 mM) and basolateral (5 mM) addition of Ba<sup>2+</sup> on thapsigargin-induced  $I_{sc}$

Each record is representative of 4 experiments. *B*, concentration-dependent inhibition of thapsigargin-induced  $I_{sc}$  by apically or basolaterally applied Ba<sup>2+</sup>. Values are means  $\pm$  s.e.m. for 6 separate epithelia; \*  $P < 0.05$  when compared with 0 mM Ba<sup>2+</sup>.

31% inhibition at the same concentration, suggesting that the basolateral barium-sensitive K<sup>+</sup> channels could be important in maintaining transepithelial anion secretion.

## DISCUSSION

An epithelial cell line derived from the coiled part of the equine sweat glandular tubule has been previously established (Wilson *et al.* 1993*b*). In contrast to the intact equine sweat glands whose control of secretion is almost exclusively via the  $\beta_2$ -adrenoceptor coupled to the cAMP-dependent secretory mechanism (Bijman & Quinton, 1984*a, b*), the anion permeability of the cultured cells appears to be regulated by Ca<sup>2+</sup> and not cAMP (Wilson *et al.* 1993*a*). Further studies on the equine sweat gland cell line have demonstrated that all elements of the well-documented Ca<sup>2+</sup>-dependent signal transduction pathway are present (Ko *et al.* 1994*a, b*), suggesting an important role of Ca<sup>2+</sup> in the regulation of secretory activity in these cells. Although this cell line may not be the true model of equine coil secretion, since cAMP-activated secretion is absent, its Ca<sup>2+</sup>-dependent secretory mechanism may have implication for sweat production in primates. The Ca<sup>2+</sup>-dependent regulatory pathway is important in the control of sweating in humans since cholinergic stimulation is by far the most physiologically relevant, because of the avidity and stability of fluid secretion it achieves (for review see Sato, Ohtsuyama, Suzuki & Sato, 1991). The Ca<sup>2+</sup>-dependent regulatory pathway is also important to the regulation of secretory activity in almost all mammalian exocrine cells (Petersen, 1992). In this regard, the cell line may provide a unique system for studying Ca<sup>2+</sup>-dependent secretory mechanisms as well as an inexhaustible source of easily handled experimental material.

To the best of our knowledge no previous studies of continuously short-circuited epithelia have been made with cultured monolayers of equine sweat glands, although it has been demonstrated in human sweat gland epithelial cells that sheet cultures do provide the simplest system yet available for quantitative analysis, having many advantages over perfused segments (Brayden, Cuthbert & Lee, 1988). In the present study, we have demonstrated that the equine sweat gland cells grown on permeable supports do form polarized monolayers with tight junction formation. The cultured monolayers exhibit a transepithelial potential of 0.42 mV, which is comparable to that found in human sweat gland sheet cultures. However, the horse epithelial sheets exhibit virtually no basal  $I_{sc}$  and a much higher transepithelial resistance (333  $\Omega$  cm<sup>2</sup>) when compared with that of human epithelial sheets (74  $\Omega$  cm<sup>2</sup>).

In the present study, we have confirmed in these cultured horse sweat gland cells that the  $I_{sc}$  does not seem to be altered by forskolin, an activator of adenylate cyclase, but does appear to respond to Ca<sup>2+</sup>-mobilizing agents. We have used thapsigargin in the present study for the following reasons: (1) it induces a rapid and pronounced increase in [Ca<sup>2+</sup>]<sub>i</sub> in a wide variety of cell types by specific and potent inhibition of the endoplasmic reticulum Ca<sup>2+</sup>-ATPase (Thastrup *et al.* 1990); and (2) thapsigargin has been employed in previous studies on the Ca<sup>2+</sup>-dependent signal transduction mechanism in the same equine sweat gland cell line (Ko *et al.* 1994*a, b*). Unlike the human cells whose  $I_{sc}$  could not be altered by thapsigargin, although they did respond to a number of other Ca<sup>2+</sup>-mobilizing agents such as bradykinin, carbachol and A23187 (Brayden, Pickles & Cuthbert, 1991; Pickles & Cuthbert, 1993), the equine cells in the present study responded to both thapsigargin and

A23187 with a rapid increase in  $I_{SC}$ . The inability of thapsigargin to give rise to an increase in  $I_{SC}$  in human cells has been attributed to a different spatiotemporal pattern of  $Ca^{2+}$  increase after thapsigargin as compared with that after other agonists. Regardless of the details of  $Ca^{2+}$  mobilization induced by either thapsigargin or A23187 in the equine cells, both agents do activate  $I_{SC}$ , which seems to be mediated by a common final  $Ca^{2+}$ -dependent pathway, e.g. a  $Ca^{2+}$ -activated conductance pathway, based on the observation that neither thapsigargin nor A23187 could increase  $I_{SC}$  much further after treatment with A23187 or thapsigargin, respectively. The effect of other physiological regulators of cellular  $Ca^{2+}$ -dependent pathways, such as ATP and bradykinin, on the  $I_{SC}$  has also been observed in this cell line (Ko *et al.* unpublished data), suggesting that the secretory activity of the equine sweat gland could be regulated under physiological condition via the  $Ca^{2+}$ -dependent pathway.

#### **$Cl^-$ and $HCO_3^-$ secretion by cultured equine sweat gland epithelium**

In contrast to the human cultures exhibiting reabsorptive ductal characteristic (Pedersen, Brandt & Hainau, 1985; Brayden *et al.* 1988), the present study shows that the cultured horse monolayers are predominantly anion secreting, based on the following observations: (1) at least 63% of the thapsigargin-induced  $I_{SC}$  could be abolished by replacement of  $Cl^-$  in the bathing solution; (2) an inhibitor of the  $Na^+-K^+-Cl^-$  cotransporter, bumetanide, was found to have a potent effect on the  $I_{SC}$ ; (3) apical addition of  $Cl^-$  channel blockers substantially inhibited the  $I_{SC}$ . Taken together, the cultured horse sweat gland epithelium exhibits predominantly secretory characteristics which are consistent with their origin, the coiled part of the equine sweat gland (Wilson *et al.* 1993b).

As far as we are aware no direct observation of  $HCO_3^-$  secretion has been obtained in any sweat gland of various species examined although such efforts have been made in the study of monkey sweat gland (Sato & Sato, 1987). The present study is the first to provide strong evidence for  $HCO_3^-$  secretion in equine sweat gland epithelium: (1) a significant portion of the thapsigargin-induced  $I_{SC}$  was dependent on extracellular  $HCO_3^-$ ; (2) an inhibitor of carbonic anhydrase, acetazolamide, had an inhibitory effect on the thapsigargin-induced  $I_{SC}$ ; and (3) a specific blocker of  $Na^+-H^+$  exchanger, MIA, as well as amiloride, also exerted an inhibitory effect on the  $I_{SC}$ . These results strongly suggest that the thapsigargin-induced  $I_{SC}$  was mediated by concurrent secretion of  $Cl^-$  and  $HCO_3^-$ . The involvement of the  $Na^+-H^+$  exchanger further indicates the existence of a cellular mechanism by which  $HCO_3^-$  could be transported outside the cell in parallel with the exit of  $H^+$  through the exchanger in order to maintain the intracellular pH. Although the physiological importance of  $HCO_3^-$  secretion in sweating remains unclear, it has been observed that the sweat of the horse is alkaline (Smith,

1890; Kerr & Snow, 1983), which may be due to significant  $HCO_3^-$  secretion as observed in the present study.

#### **Transport mechanisms in cultured equine sweat gland epithelium**

The present data could be best interpreted as though the cultured equine sweat gland epithelium possesses different transport mechanisms which could be operated concurrently or independently.

**$Cl^-$  secretion.**  $Cl^-$  secretion by the cultured equine sweat gland epithelium could be mediated by a transport system consisting of basolaterally located  $Na^+-K^+-ATPase$  (ouabain sensitive),  $Na^+-K^+-2Cl^-$  symport (bumetanide sensitive), the  $K^+$  channel (barium sensitive) and the apical  $Cl^-$  channel (DIDS and DPC sensitive). The  $Na^+-K^+-2Cl^-$  symport is driven by the low intracellular  $Na^+$  concentration maintained by the  $Na^+-K^+-ATPase$  and  $K^+$  channels on the basolateral side. The intake of  $K^+$  through the symport is balanced by its exit through the  $K^+$  channel. The net effect of these transporters is the accumulation of intracellular  $Cl^-$  above its electrochemical equilibrium, allowing secretion of  $Cl^-$  into the lumen through apical  $Cl^-$  channels upon agonist stimulation. The basic features of this transport system are similar to those described in monkey sweat gland epithelium (Sato & Sato, 1987) and a number of other epithelia (Welsh, 1983; Case, Hunter, Novak & Young, 1984; Wong, 1988).

**$HCO_3^-$  secretion.**  $HCO_3^-$  secretion by the cultured equine sweat gland epithelium has also been indicated by the present data. The sensitivity of the thapsigargin-induced  $I_{SC}$  to acetazolamide suggests that  $HCO_3^-$  could be produced inside the cell via carbonic anhydrase. The ability of apically applied  $Cl^-$  channel blockers, DIDS or DPC, to inhibit substantially the thapsigargin-induced  $I_{SC}$ , including both  $Cl^-$ - and  $HCO_3^-$ -dependent current, may imply that  $HCO_3^-$  secretion could be mediated by apical  $Cl^-$  channels, as suggested in epididymal epithelium (Wong, 1988). This notion is supported by the patch-clamp study on pancreatic duct cells demonstrating measurable permeability of  $HCO_3^-$  through  $Cl^-$  channels, though half that of  $Cl^-$  (Gray, Pollard, Harris, Coleman, Greenwell & Argent, 1990). However, the inhibitory effect of DPC and DIDS on both  $Cl^-$ - and  $HCO_3^-$ -dependent  $I_{SC}$  does not rule out the possibility that  $Cl^-$  and  $HCO_3^-$  are secreted through different anion channels since the inhibitory effect of these blockers could be non-specific.

The present data do not support a  $Na^+$  reabsorption mechanism via apical amiloride-sensitive  $Na^+$  channels in the cultured equine sweat gland cells since the action of amiloride appears to be similar to that of MIA, which affects the  $Na^+-H^+$  exchanger rather than apical  $Na^+$  channels. However, it has been suggested that intestinal  $Na^+$  reabsorption could be mediated in part via apical membrane  $Na^+-H^+$  exchanger (Gleeson, 1992). The observed inhibitory effect of apical MIA on both  $Cl^-$ - and



$\text{HCO}_3^-$ -dependent thapsigargin-induced  $I_{\text{SC}}$  suggests the presence of an apically located  $\text{Na}^+-\text{H}^+$  exchanger. However, the role of the apically located  $\text{Na}^+-\text{H}^+$  exchanger in equine sweat production remains unclear.

The present study has also observed an inhibitory effect exerted by basolaterally applied MIA on the thapsigargin-induced  $I_{\text{SC}}$  in  $\text{Cl}^-$ -free solution ( $\text{HCO}_3^-$  dependent) but not in normal K-H solution. The effect of basolaterally applied MIA is less than that exerted by apically applied MIA. This could be explained by the presence of both apical and basolateral  $\text{Na}^+-\text{H}^+$  exchangers with different sensitivity to MIA. The presence of both apical and basolateral  $\text{Na}^+-\text{H}^+$  exchangers has also been observed in the human intestinal Caco-2 cell monolayer (Osypiw, Gleeson, Loble, Pemberton & McMahon, 1994). It may also be argued that basolaterally applied MIA could leak across the monolayer and act on the apical  $\text{Na}^+-\text{H}^+$  exchanger. If this interpretation stands, it is difficult to explain why such an effect was not observed in normal K-H solution. A basolaterally located  $\text{Na}^+-\text{H}^+$  exchanger may indeed be required for  $\text{HCO}_3^-$  secretion (in  $\text{Cl}^-$ -free solution) as proposed in pancreatic ducts (Argent & Case, 1994). A basolaterally located  $\text{Na}^+-\text{H}^+$  exchanger has also been suggested in monkey sweat glands, based on the observation that basolateral addition of amiloride had a mild effect on sweat secretion (Sato & Sato, 1987).

Although our data suggest different transport mechanisms in equine sweat gland epithelium, it remains unresolved how these transport mechanisms are operated under physiological conditions. It is also unclear whether all cells of the epithelium possess different transport mechanisms or some cell population is specialized in a certain type of transport. The present study should prompt further investigation on the detailed mechanisms and interactions of agonist-coupled anion ( $\text{Cl}^-$  and  $\text{HCO}_3^-$ ) secretion.

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