INVESTIGATION OF STIMULUS–SECRETION COUPLING IN EQUINE SWEAT GLAND EPITHELIA USING CELL CULTURE TECHNIQUES

STUART M. WILSON, JOHN D. PEDIANI, WING HUNG KO, DOUGLAS L. BOVELL*, SUSAN KITSON, IAN MONTGOMERY, GODFREY L. SMITH, IAN MONTGOMERY, URSILLA M. O. BROWN, HUGH Y. ELDER AND DAVID McEWAN JENKINSON

The Institute of Physiology, University of Glasgow, Glasgow G12 8QQ, UK

Accepted 24 June 1993

Summary

When sweat glands isolated from samples of horse skin were explanted and cultured under favourable conditions, they could exhibit cellular outgrowth. This growth could be maintained for 2–4 weeks and these primary cultures were then disaggregated and the resultant cell suspensions used to initiate epithelial cell lines. Secretion from intact equine sweat glands is regulated by β2-adrenoceptors and appears to be mediated by cyclic AMP, but there is evidence that calcium may also play a role. Adrenaline could increase the cyclic AMP content of the cultured cells and this response was mediated by β2-adrenoceptors. Adrenaline was also able to evoke a small increase in intracellular free calcium ([Ca\(^{2+}\)]\(_i\)) but the pharmacology of this response remains obscure. Adrenaline thus activates at least two potentially important second-messenger signalling pathways which have the capacity to interact, because adrenaline-evoked cyclic AMP formation was inhibited if [Ca\(^{2+}\)]\(_i\) was raised with ionomycin. The chloride permeability of mammalian epithelial cells characteristically rises during secretion, and adrenaline could increase chloride permeability in the cultured epithelia but the cells did not contain cyclic-AMP-dependent chloride channels and so this response was mediated by [Ca\(^{2+}\)].

Introduction

Sweat glands are found in essentially all mammalian groups but it is only in certain Bovidae, the primates and the Equidae that they play a significant role in thermoregulation (Jenkinson, 1973). In primates, the principal regulator of sweat secretion is acetylcholine but equine glands are unresponsive to this substance. Both types of glands, however, secrete in response to β-adrenergic agonists (Lovatt-Evans and Smith, 1956; Robertshaw and Taylor, 1969; Anderson and Aitken, 1977; Sato, 1977; Snow, 1977; Sato and Sato, 1981; Johnson and Creed, 1982; Bijman and Quinton, 1984a; Sato and Sato, 1988; Bovell et al. 1989) and it has therefore been proposed that the equine

*Present address: Faculty of Health Studies, Glasgow Caledonian University, Glasgow G13 1PP, UK.

Key words: cell culture, equine sweat gland, stimulus–response coupling.
sweat gland is unusual in being predominantly under β-adrenergic control (Bijman and Quinton, 1984a).

At the cellular level, the ionic fluxes associated with the onset of sweating in primates and horses seem very similar (Wilson et al. 1988a,b; Takemura et al. 1991), but there have been relatively few physiological studies of equine sweat glands so the stimulus–response coupling mechanism in these organs is incompletely understood. In an attempt to clarify the mechanism by which adrenergic agonists evoke secretion in equine sweat glands, we have explored the ability of these agents to evoke second-messenger signals and to evoke increased membrane chloride permeability, an important component of the secretory mechanism in mammalian epithelia (Cliff and Frizzell, 1990). These experiments were undertaken using preparations of cultured equine sweat gland epithelial cells developed for this purpose.

Materials and methods

Primary culture

The methods used to initiate cultures of equine sweat gland cells are based on protocols presented elsewhere for human glands (Lee et al. 1984, 1986) and so only brief details are presented here.

Samples of skin from the necks of horses that had been destroyed for humane reasons were collected into isolation medium (composition given below), trimmed of fat and hair, cut into pieces (approximately 10cm²) and immersed in 70% (v/v) ethanol for 1 min; sterile techniques were used for subsequent procedures. Samples were placed in 15–20ml of isolation medium and homogenised by repeated shearing with scissors. Sweat glands were isolated from the resultant slurry (Lee et al. 1984), transferred to Williams medium E (supplemented as detailed below) and then incubated (37˚C, 5% CO₂ in air) in culture medium containing collagenase (Worthington, 294iu.ml⁻¹) until the glandular tubule began to uncoil (20–50min). Glands were then transferred to fresh medium and incubated overnight.

Sufficient culture medium (approximately 1ml) to form a film over the plastic bottom was added to each of a series of 25cm² culture flasks. Two to four collagenase-treated glands were explanted into each flask and incubated until they attached to the plastic (24–48h). A further 2–3ml of medium was then added to each flask and incubation continued for 14–28 days; the medium was changed every 3–4 days.

Secondary culture

Each primary culture was washed with phosphate-buffered saline (PBS) before treatment with trypsin (0.25%) in Hank’s balanced salt solution (HBSS) for 3 min to dislodge fibroblasts, which were then removed by washing with PBS. This procedure was repeated 2–3 times. Epithelioid cells were detached using trypsin (0.24%) plus EDTA (0.1mmol.l⁻¹) in HBSS after preincubation (10min, 37˚C) in 0.1mmol.l⁻¹ EDTA in PBS. The cells were washed twice by centrifugation (5 min at 800g) and resuspension in culture medium, and were then plated onto 24-well culture plates (5×10⁴ cells per well).
Initiation and maintenance of cell lines

Primary cultures were washed with HBSS and incubated (37°C) in Dispase II (Boehringer). This is a proteolytic enzyme of bacterial origin that characteristically dislodges epithelial cells as intact cell sheets or as large cell clumps. The detached cell sheets from four to five primary cultures were pooled and allowed to settle from the enzyme solution, which was then discarded, along with any cells that had remained in suspension.

The sheets were washed by repeated settling from HBSS and a suspension of small cell clumps was prepared by trituration (approximately 1 min) in approximately 0.2 ml of trypsin in EDTA. This enzyme was inactivated by addition of 9.8 ml of culture medium containing 5% foetal calf serum. Samples (approximately 1 ml) of the resultant suspension of cell clumps were plated in 25 cm² tissue culture flasks. 2–3 ml of culture medium was added to each flask when cellular attachment had occurred.

Light and electron microscopy

The expression of cytokeratin was used as a cytological marker of epithelial lineage. This antigen was detected using a monoclonal antibody (AE1/AE3, Boehringer Mannheim Biochemica, 1:100 dilution) in a standard immunoperoxidase technique (Vectastain Elite ABC Kit, Vector Labs). Normal mouse ascites fluid served as a negative control and the technique was verified by the detection of cytokeratin in rabbit epidermis.

Cultured cells were routinely examined using a Nikon Diaphot phase contrast microscope. Cells growing on glass microscope slides were mounted in a drop of physiological salt solution for differential interference contrast microscopy (Zeiss Axiophot).

Cells on glass coverslips or cells that had been removed from culture flasks using Dispase II were fixed (2% glutaraldehyde in 100 mmol l⁻¹ sodium cacodylate) and processed for electron microscopy as described elsewhere (Montgomery et al. 1982) and examined in a Zeiss 109 transmission electron microscopy.

Quantification of cell growth

Cells were plated on 24-well plates (5 × 10⁴ cells per well). 3 h later all wells were washed three times with HBSS to remove non-viable cells. Separate samples of cell suspension (5 × 10⁴ cells) were washed by centrifugation and resuspension in HBSS and the cells were then dissolved in approximately 1 ml of 1% (w/v) sodium dodecyl sulphate (SDS) in 300 mmol l⁻¹ sodium hydroxide (SDS–NaOH) to enable the protein content of the inoculum to be determined. At given time intervals after plating, the culture medium was withdrawn and the growing cells were thoroughly washed with HBSS to ensure complete removal of the serum present in the culture medium. Cellular protein was then extracted from each well using approximately 1 ml of SDS–NaOH. The protein contents of the inoculum and of each well were subsequently assayed (Lowry et al. 1951). Plating efficiency was defined as the proportion of cellular protein which remained attached to the plastic after washing at 3 h. Data are presented as means ± standard errors and values of N refer to the number of experiments.
Measurement of cellular cyclic AMP

Cells grown (3–4 days) on 24-well plates were washed twice with physiological salt solution containing theophylline (1 mM) and incubated at 37°C in this buffer for 10 min. Cellular cyclic AMP was extracted by adding 10 μl of perchloric acid (80% w/v) to each well and was subsequently quantified by radioimmunoassay (Amersham kit). Cellular protein content was also determined (see above) and data are expressed as pmol cyclic AMP per mg of cellular protein (pmol/mg). This method is described in detail elsewhere (Wong and Huang, 1990).

The effects of various drugs were studied by including them in the physiological salt solution throughout the incubation period. Unless otherwise stated, all measurements of cyclic AMP content were therefore made in the presence of theophylline (1 mM).

Cells at different passage number were considered to be statistically independent and data are usually presented as means ± standard errors, where the value of N refers to the number of different passages. In some instances, however, we present mean values that were derived from repeated measurements of cells at a single passage. These data are presented as means ± standard deviations and the values of N refer to the number of replicate measurements that were made.

Regulation of cellular chloride permeability

Cells grown in 35mm diameter Petri dishes (3–4 days) were loaded with 125I by incubation (1–1.5h, 37°C) in physiological salt solution containing Na125I (1–4 μCi/ml) and the rate of isotope efflux was subsequently quantified. Changes in 125I efflux rate were assumed to reflect changes in cellular chloride permeability (Venglarik et al. 1990). All experiments were undertaken using a paired experimental protocol in which one dish of cells served as a control. Data are presented as means ± standard errors and values of N refer to the number of experiments. All protocols were repeated using cells from at least three different passages. This method is described in detail elsewhere (Venglarik et al. 1990).

Measurement of [Ca2+]i

Coverslips bearing growing cells (2 days after plating) were washed with physiological salt solution (composition given below) and the cells then loaded with either Indo-1 or Fura-2. These calcium-sensitive fluorochromes were loaded by incubating the cells for approximately 30min in physiological salt solution containing the acetyoxymethyl ester (AM) form of the appropriate dye (5 μmol l−1) and dimethylsulphoxide (DMSO 0.5% v/v). The coverslips were then mounted in a small heated chamber fixed to the stage of a Nikon Diaphot inverted microscope where the cells were superfused (33–35°C, 5–6mL min−1) with physiological salt solution. An increase in [Ca2+]i will cause a rise in the fluorescence ratio recorded from cells loaded with such fluorochromes, thus allowing changes in [Ca2+]i to be recorded using standard, microspectrofluorimetric techniques (Grynkiewicz et al. 1985). Absolute calibration of these signals is, however, very difficult to achieve for cells loaded using the AM form of such dyes (Highsmith et al. 1986) and
Solutions

The isolation medium contained (in mmol l\(^{-1}\)): NaCl, 100; KCl, 4.8; KH\(_2\)PO\(_4\), 1.2; MgSO\(_4\), 1.2; CaCl\(_2\), 1.3; NaHCO\(_3\), 20; 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (Hepes), 25; and D-glucose, 10; pH adjusted to 7.4 with NaOH.

The physiological salt solution contained (mmol l\(^{-1}\)): NaCl, 130; KCl, 5; Hepes, 20; MgCl\(_2\), 1; CaCl\(_2\), 1; D-glucose, 10. The pH was adjusted to 7.4 with NaOH.

The culture medium was Williams Medium E containing: L-glutamine (1 mmol l\(^{-1}\)), penicillin (100 i.u. ml\(^{-1}\)), streptomycin (100 \(\mu\)g ml\(^{-1}\)), bovine insulin (100 \(\mu\)g ml\(^{-1}\)), hydrocortisone (10 ng/ml\(^{-1}\)), transferrin (10 \(\mu\)g ml\(^{-1}\)) and trace element mix (0.5%). Unless otherwise stated the medium also contained epidermal growth factor (EGF, 20 ng/ml\(^{-1}\)) and foetal calf serum (5%).

Results

Isolation of sweat glands and initiation of cultures

Equine sweat glands were smaller and more fragile than their human counterparts and this significantly complicated their identification, isolation and handling. With practice, however, 10–50 glands could be obtained from a skin specimen, although the glandular ducts invariably became detached during the isolation process.

Cellular outgrowth became evident from about half of the explanted glands after 24–48h in culture. Cells were initially seen migrating from points on the glandular tubule (Fig. 1A) or from the open ends of tubules and, after 3–4 days, active cultures consisted of roughly circular areas (0.2–0.5 mm in diameter) of polygonal and spindle-shaped cells (Fig. 1B). After 5–7 days these cells had adopted polygonal morphology, although fibroblasts also grew in most cultures. Growth could be maintained for 2–4 weeks, by which time cultures contained roughly circular areas (2–3 cm in diameter) of epithelioid cells (Fig. 1C).

Several attempts were made to disaggregate these cultures by simply incubating them in trypsin plus EDTA. Although the fibroblasts detached rapidly, the epithelioid cells were very resistant to this enzyme solution, and once they been detached (30–45 min) they were no longer viable. Primary cultures were, however, successfully disaggregated by sequential incubation in trypsin (which selectively removed fibroblasts), followed by EDTA and finally by trypsin plus EDTA, which detached the epithelioid cells within approximately 5 min. The cells obtained from two animals were pooled and successfully plated onto 24-well plates. They were allowed to grow for three days, by which time they had formed an essentially continuous layer of epithelioid cells (Fig. 1D). The mean protein content of each well was 42.8 ± 2.7 \(\mu\)g.

Cyclic AMP production by cells in secondary cultures

Adrenaline (10 \(\mu\)mol l\(^{-1}\)) increased the cyclic AMP content of the cells in secondary culture and this response was mimicked by the \(\beta\)-adrenoceptor agonist isoprenaline.
Forskolin, an alkaloid that directly activates adenylate cyclase, also increased cyclic AMP content but the response was larger than the responses to either adrenaline or isoprenaline. The calcium ionophore ionomycin did not affect cellular cyclic AMP content (Table 1).

### Initiation of cell lines

Dispase II disaggregated primary cultures slowly (0.5–2h). The epithelioid cells were removed from the plastic surface as intact sheets, whereas fibroblasts either remained attached to the plastic or were released as single cells. During this disaggregation process it was often apparent that the epithelioid cells had grown on top of a layer of fibroblasts. The large sheets of epithelioid cells could easily be separated from the fibroblasts because they settled out of suspension much faster. A suspension of small clumps of epithelioid cells (Fig. 2A) could then be prepared by very brief treatment with trypsin plus EDTA. These retained the capacity for vigorous growth (Fig. 2B) and gave rise to secondary cultures consisting primarily of epithelioid cells. These cultures were maintained by serial passage (Dispase II followed by trypsin plus EDTA) and, at each stage, fewer fibroblasts were present until, by passage 5, they consisted exclusively of epithelioid cells. At this stage it became apparent that suspensions of viable cells could be prepared by relatively brief (10–15min) exposure to trypsin plus EDTA. The properties of the epithelioid cells had thus changed such that they could be maintained as cell lines using standard techniques.

Two such lines have been established (E/91/15 and E/92/3) and have now been maintained for 40–70 passages (1:3 split at each passage). They thus represent a

### Table 1. Cyclic AMP production by cells in secondary culture

<table>
<thead>
<tr>
<th>Culture conditions</th>
<th>Cellular cyclic AMP content (pmol/mg(^{-1}))</th>
<th>Increase over basal (fold increase)</th>
<th>% of response to forskolin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>0.05±0.006</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>+Ionomycin (0.1 (\mu)mol l(^{-1}))</td>
<td>0.04±0.004</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>+Adrenaline (10 (\mu)mol l(^{-1}))</td>
<td>0.18±0.096</td>
<td>2.8</td>
<td>37</td>
</tr>
<tr>
<td>+Isoprenaline (10 (\mu)mol l(^{-1}))</td>
<td>0.20±0.011</td>
<td>3.2</td>
<td>42</td>
</tr>
<tr>
<td>+Forskolin (100 (\mu)mol l(^{-1}))</td>
<td>0.42±0.063</td>
<td>7.6</td>
<td>100</td>
</tr>
</tbody>
</table>

Each value is the mean ± standard deviation of three determinations. The experimental data have been further analysed to express the increases in cyclic AMP content both as a function of the unstimulated cyclic AMP content and as a percentage of the mean response to forskolin.

Forskolin, an alkaloid that directly activates adenylate cyclase, also increased cyclic AMP content but the response was larger than the responses to either adrenaline or isoprenaline. The calcium ionophore ionomycin did not affect cellular cyclic AMP content (Table 1).
Cultured equine sweat gland epithelia

Fig. 2. Phase contrast micrographs showing the growth of equine sweat gland cells in secondary culture. (A) A clump of cells prepared using Dispase II and photographed 4h after plating onto plastic; the cells have attached to the surface but no cellular growth is evident. (B) The same field 24h after plating: the clump has given rise to an area (approximately 0.5mm in diameter) of actively growing epithelioid cells. (C) Differential interference contrast micrograph of a coherent culture of equine sweat gland epithelial cells at passage 12. Cell borders are evident (cb), mitochondria (m) are visible as faint elongated profiles in many cells, and lysosomes (ly) are seen as light, spherical objects around the nuclei (n) which usually contain nucleoli. A dividing cell (dc) is present. Scale bars, 100 μm (A,B), 20 μm (C).

potentially inexhaustible supply of experimental material and subsequent experiments were directed towards exploring their properties.

Pattern of cellular growth

Both cell lines retained epithelioid morphology (Fig. 2C) and continued to express cytokeratin, a cytological marker of epithelial lineage (Fig. 3). The cells usually grew to an essentially coherent monolayer within 3–4 days of plating.

We explored the extent to which the attachment and growth of the cells are dependent

Fig. 3. Immunohistochemical demonstration of cytokeratin in a cryostat section of unfixed horse skin and in cultured equine sweat gland cells grown on glass microscope slides. (A) The sweat gland coil stains positively (dark) against the surrounding collagen/fibrocyte stroma. (B) This antigen was also present in the cultured cells, which appear variably dark according to the amount and thickness of stained cytokeratin. Scale bars, 100 μm (A), 50 μm (B).
upon the presence of foetal calf serum in the culture medium (Fig. 4). In these experiments the non-viable cells were washed away 3h after seeding so that plating efficiency could be determined. Plating efficiency was stimulated by foetal calf serum, although the effect was not concentration-dependent (Fig. 4C). The growing cells were then washed again at 8h so that the mass of cellular protein present could be determined as an indicator of growth. Although small colonies of growing cells were observed in each well, there was an apparent fall in the amount of protein present (Fig. 4A). Washing at 8h therefore removed significant numbers of cells from the cultures and so these data do not accurately reflect growth. After this time, however, there was a clear increase in cellular mass, and this growth was stimulated by foetal calf serum (Fig. 4A). The protein content at 3h was therefore subtracted from that present at the end of the experimental period (60h) in order to provide an index of total growth. These data have been plotted against the concentration of foetal calf serum present in the medium. Growth was erratic in 1.25% foetal calf serum but was stimulated by higher concentrations (Fig. 4B).

Fig. 4. Growth of cultured equine sweat gland cells. (A) The pattern of growth observed in a paired experimental protocol (N=6) either in serum-free medium (●) or in 5% foetal calf serum (■). The growth observed over the entire 60h period (B) and the plating efficiency (C) have been plotted against the concentration of foetal calf serum (FCS) in the culture medium. Values of N are given beside each point. *P<0.05, **P<0.02 (paired t-test).

Fig. 5. Electron micrographs of cultured equine epithelia. (A) Cells grown to near coherence, fixed whilst still attached to the coverslip and sectioned perpendicular to the plane of the coverslip. Lateral interdigitations, id; junctional complex, jc; secreted material, sm; coated vesicle, cv; mitochondria, m; microvillus, mv; nucleus, n; rough endoplasmic reticulum, rer. (B) Low-power view of a sheet of cells detached from the substratum with Dispase II and sectioned perpendicular to the sheet. Basal processes, bp; other lettering as in A. (C) Higher-power micrograph of the margins of adjacent Dispase-II-treated cells showing a zonula occludens (zo) and a zonula adherens (za). Glycogen granules, gly; lysosome, ly. Other lettering as in A. Scale bars, 0.5 μm (A,C), 5 μm (B).
Cultured equine sweat gland epithelia
Analogous experiments were undertaken to explore the extent to which growth was dependent upon epidermal growth factor (EGF). They were conducted in 5% serum and the cells were not washed at 8h. The absence of EGF did not significantly affect plating efficiency (EGF-free, 31.6±4.5%; control, 37.6±3.7%; \( N=7, P>0.05 \), paired t-test) or cellular growth (EGF-free, 80.4±9.9 \( \mu \)g protein; control, 97.4±12.5 \( \mu \)g protein; \( N=7, P>0.05 \), paired t-test) over the 60h experimental period.

**Ultrastructure**

Electron microscopy revealed that the cells grew as a monolayer on glass coverslips or on plastic (Fig. 5A). The cells were approximately 20 \( \mu \)m in diameter but were flattened, being only approximately 2 \( \mu \)m deep at their nuclear regions. Overlap of the peripheral regions of neighbouring cells was common and these cell contacts were maintained by lateral interdigitations, junctional complexes (Fig. 5A) and by numerous desmosomes. Sparse, stubby microvilli were present on the upward-facing side of the cells and some secreted material was present at the surface in contact with the coverslip.

Cells which had been removed from plastic with Dispase II had a cuboidal appearance (15–20 \( \mu \)m deep). The stubby microvilli were still present on the upward-facing surface, and basal processes were now evident on the surface that had been attached to the plastic. The lateral interdigitations were still present and the junctional complexes retained their integrity (Fig. 5B); both zonulae occidentes and zonulae adherentes could be identified (Fig. 5C). Desmosomes and gap junctions were also commonly seen.

**Cyclic AMP production by the cell lines**

There was no significant difference in the basal (i.e. unstimulated) cyclic AMP content of the two cell lines. Both lines also showed essentially the same response to forskolin and to adrenaline, although the latter response was smaller than the former (Table 2). Assuming that the response to forskolin represents the maximal rate of cyclic AMP production, the amount of cyclic AMP present in each cell line was quantified both under basal (i.e. unstimulated) conditions and also in the presence of either forskolin or adrenaline.

<table>
<thead>
<tr>
<th>Cellular cyclic AMP content (pmolmg(^{-1}))</th>
<th>E/91/15 (( N=4 ))</th>
<th>E/92/3 (( N=7 ))</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>0.026±0.006</td>
<td>0.024±0.005</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>+Forskolin (100 ( \mu )mol l(^{-1}))</td>
<td>2.155±0.700</td>
<td>3.788±0.744</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>+Adrenaline (1 ( \mu )mol l(^{-1}))</td>
<td>0.366±0.073</td>
<td>0.230±0.036</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>% under adrenergic control</td>
<td>18.0±2.4</td>
<td>6.9±1.6</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

The significance of any apparent differences between the two cell lines was tested using Student’s unpaired t-test.
production, it appears that more of the cellular cyclic-AMP-generating capacity is under hormonal control in the E/91/15 cells (Table 2). In subsequent analyses, hormonally induced cyclic AMP accumulation was therefore expressed as the fraction of the response to a standard dose of adrenaline.

This response to adrenaline (1 μmol l⁻¹) was essentially unaffected by the α-adrenoceptor antagonist phentolamine (1 μmol l⁻¹, 14.2±6.9% inhibition; N=3, P>0.05, paired t-test) but was essentially abolished by propranolol, a β-adrenoceptor antagonist (1 μmol l⁻¹, 98±2% inhibition; N=3, P<0.02, paired t-test). Cyclic AMP accumulation is thus mediated by β-adrenoceptors.

The response to adrenaline was dose-dependent and the concentration required to evoke a half-maximal response (EC₅₀) was estimated to be 0.044±0.004 μmol l⁻¹ (Fig. 6A). Noradrenaline could also evoke cyclic AMP production but, although the maximal response was the same as that evoked by adrenaline, noradrenaline was approximately 75-fold less potent (Fig. 6B, EC₅₀=3.2±1.4 μmol l⁻¹).

β-Adrenoceptors can be subdivided into two broad types, β₁ and β₂ (Lands et al. 1967). In further experiments cells were stimulated with 0.2 μmol l⁻¹ adrenaline, which normally increased cellular cyclic AMP content from 0.11±0.022 pmol mg⁻¹ to 0.538±0.107 pmol mg⁻¹ (N=6). Atenolol, a β₁ antagonist, caused a dose-dependent inhibition of this response (Fig. 7). The inhibitory actions of the β₂ antagonist butoxamine were explored in an analogous series of experiments in which adrenaline (0.2 μmol l⁻¹) normally increased cellular cyclic AMP content from 0.112±0.022 pmol mg⁻¹ to 0.527±0.109 pmol mg⁻¹ (N=6). Although the results (Fig. 7)
suggested that butoxamine was a slightly more potent inhibitor than atenolol, the concentrations required for half-maximal inhibition did not differ significantly (butoxamine, 0.31±0.16 μmol l⁻¹; atenolol 0.80±0.5 μmol l⁻¹). Analysis of variance, however, indicated that these two data sets did not belong to the same statistical population (P<0.05), and further examination of the data revealed that butoxamine was a more effective antagonist than atenolol but that this effect was only apparent within a limited concentration range (1–10 μmol l⁻¹, Fig. 7). The observation that adrenaline is a more potent agonist than noradrenaline, together with the finding that butoxamine can inhibit the response to adrenaline more effectively than atenolol, strongly suggests that adrenaline-evoked cyclic AMP accumulation is mediated by receptors belonging to the β₂ subclass (Lands et al. 1967).

Fig. 7. Inhibitory effects of atenolol (●) and butoxamine (■). The responses to 0.2 μmol l⁻¹ adrenaline evoked in the presence of these compounds were expressed as fractions of the control response and these data have been plotted against the concentration of the antagonist used. Sigmoid curves were fitted to the data using a least-squares regression procedure in which each data point was weighted according to the reciprocal of its standard deviation. Differences between the degree of inhibition produced by these two compounds were tested using a paired t-test. *P<0.05. N values are given beside each point.

The secretory responses of isolated equine glands can show some dependence upon calcium (Bijman and Quinton, 1984b) and so the effects of raising the intracellular free calcium concentration ([Ca²⁺]ᵢ) upon adrenaline-evoked cyclic AMP production were studied. In these experiments the cyclic AMP content of unstimulated cells was 0.091±0.019 pmol mg⁻¹ (N=9) and adrenaline (1 μmol l⁻¹) increased this by 0.830±0.229 pmol mg⁻¹. The calcium ionophore ionomycin did not affect resting cyclic
AMP content (0.088±0.018 pmol mg\(^{-1}\)) but did inhibit the response to adrenaline (0.277±0.068 pmol mg\(^{-1}\)). These data therefore suggest that raising [Ca\(^{2+}\)]\(_i\) can inhibit receptor-mediated cyclic AMP production.

**Regulation of cellular chloride permeability**

An increase in cellular chloride permeability is an important component of the secretory response of mammalian epithelia (Cliff and Frizzell, 1990) and so the ability of adrenaline to influence chloride permeability was monitored using the \(^{125}\)I efflux technique (Venglarik *et al.* 1990).

Under control conditions isotope efflux was essentially monoexponential. Adrenaline, in the presence of the phosphodiesterase inhibitor theophylline, evoked only a small and rather variable increase in the rate of efflux (Fig. 8A). The mean rate constant over the three samples collected immediately after addition of these drugs was greater than the corresponding value measured from the control cells (P<0.05, paired \(t\)-test); adrenaline can therefore increase cellular chloride permeability.

Adrenaline evokes cyclic AMP production (Fig. 6), but the data presented in Fig. 8B show that the permeability did not change when cells were exposed to a cocktail of compounds that activate cyclic-AMP-dependent responses in other cell types (Gray *et al.* 1989; Cliff and Frizzell, 1990). Raising the cyclic AMP concentration does not, therefore, evoke increased chloride permeability. Ionomycin, however, could consistently evoke a

![Fig. 8. Regulation of cellular chloride permeability. Rate constants for \(^{125}\)I efflux are plotted against superfusion time. (A) Data (N=5) from control cells (■) and from cells which were exposed to 10 \(\mu\)mol l\(^{-1}\) adrenaline and 1 \(\mu\)mol l\(^{-1}\) theophylline (●) for the period indicated: (B) Data (N=7) from control cells (■) and from cells which had been exposed to a cocktail of compounds (forskolin, 10 \(\mu\)mol l\(^{-1}\); isomethylbutylxanthine, 100 \(\mu\)mol l\(^{-1}\); dibutyryl-cyclic AMP, 100 \(\mu\)mol l\(^{-1}\)) designed to evoke a maximal cyclic-AMP-dependent response (●).](image-url)
large permeability increase, which strongly suggests that the chloride permeability of
these cells can be regulated by \([\text{Ca}^{2+}]_i\) (Fig. 9).

*Regulation of \([\text{Ca}^{2+}]_i\)*

Adrenaline (10 \(\mu\text{mol}\text{l}^{-1}\)) increased \([\text{Ca}^{2+}]_i\) in each of three Indo-1-loaded
preparations, although the responses were small and variable (Fig. 10). Forskolin
(10 \(\mu\text{mol}\text{l}^{-1}\)) caused no visible change in \([\text{Ca}^{2+}]_i\) over a 3 min period \((N=3)\) in parallel
experiments conducted using cells at the same passage, and further experiments on these
cells showed that this concentration of forskolin causes a large (approximately 35-fold)
rise in cellular cyclic AMP content (control, 0.047±0.001 pmol mg\(^{-1}\); forskolin,
1.75±0.36 pmol mg\(^{-1}\); mean ± s.d., \(N=4\)). The increase in \([\text{Ca}^{2+}]_i\) therefore does not
appear to be secondary to the rise in cyclic AMP.

The properties of this response to adrenaline were investigated further in cells which
had been loaded with Fura-2. In these experiments, 1 \(\mu\text{mol}\text{l}^{-1}\) adrenaline increased
\([\text{Ca}^{2+}]_i\) in 6 out of 8 preparations, and 10 \(\mu\text{mol}\text{l}^{-1}\) adrenaline increased \([\text{Ca}^{2+}]_i\) in 4 out of
5 preparations. Similar increases were evoked by isoprenaline, either at 1 \(\mu\text{mol}\text{l}^{-1}\) (2 out
of 2 preparations) or at 10 \(\mu\text{mol}\text{l}^{-1}\) (8 out of 12 preparations), whereas phenylephrine (10
\(\mu\text{mol}\text{l}^{-1}\)) increased \([\text{Ca}^{2+}]_i\) in 3 out of 9 preparations.

**Discussion**

*The culture of equine sweat gland epithelia*

If human skin is homogenised by repeated shearing, sweat glands ‘pop out’ from the
surrounding connective tissue and can be readily picked out of the skin slurry (Lee *et al.*
1984). If these glands are explanted and cultured under favourable conditions, outgrowth
of epithelial cells can occur (Pedersen, 1984; Collie *et al.* 1985; Lee *et al.* 1986).
Equine sweat glands could also be isolated and cultured using this approach, but with greater difficulty as horse skin did not readily homogenise and the equine glands were smaller and much more fragile. This significantly complicated both their isolation and subsequent handling. The ascending ducts were usually detached from the glands during isolation and so the growing cells were almost certainly all derived from the coiled part of the glandular tubule. In the horse, this contains only a secretory epithelium, as there is no equine analogue of the specialised reabsorptive epithelium found within the primate gland (Montgomery et al. 1982, 1985). Moreover, only a single secretory cell type is present, although the tubule also contains myoepithelial cells and fibrocytes (Montgomery et al. 1982). The possibility that at least some of the epithelioid cells may be derived from the myoepithelium cannot be excluded, but this does seem unlikely as cultures from human secretory coils do not contain cells with the electrophysiological characteristics of myoepithelial cells (Reddy et al. 1992; Reddy and Quinton, 1992).

Suspensions of viable equine cells could only be prepared using trypsin plus EDTA if the primary cultures were preincubated in trypsin and then in EDTA but, as with human pancreatic duct cells (Harris and Coleman, 1987), the use of Dispase II greatly facilitated the establishment of secondary cultures.

The life span of epithelial cell cultures established from human sweat glands or pancreatic ducts is limited by the restricted proliferative capacity of human epithelial cells (Lee et al. 1986; Harris and Coleman, 1987; Lee and Dessi, 1989; Buchanan et al.

---

**Fig. 10.** The effects of adrenaline (10 μmol l⁻¹) upon [Ca²⁺], in three preparations of cells loaded with Indo-1. Fluorescence ratios were recorded from areas containing 10–50 confluent cells, and the signals were filtered at 3Hz (−3 dB frequency). Cells were exposed to adrenaline as indicated.
1990). Equine cultures, however, have now been maintained for over 50 passages whilst retaining epithelioid morphology and continuing to express cytokeratin, a marker of epithelial lineage. Horse sweat gland epithelial cells thus appear to be capable of undergoing spontaneous transformation into essentially ‘immortal’ cell lines. The growth of these cells was not inhibited when EGF was withdrawn and so, at least over the time period of the present experiments, they do not appear to require this growth factor. They did, however, retain some dependence upon the growth factors present in serum. The equine cell lines therefore seem to display the low dependence upon exogenous growth factors that is typical of transformed cells (Freshney, 1987).

These cell lines therefore provide a potentially inexhaustible source of experimental material. Physiological studies of some other such ‘immortal’ epithelial cell lines have provided valuable insights into the mechanism of transepithelial salt and water transport (e.g. Dharmasathaphorn et al. 1984; Simmons, 1993). The epithelial cell lines established in the present study may therefore provide a valuable experimental model of the equine sweat gland epithelium, permitting physiological investigations of the factors that regulate secretion in these organs. This will, however, depend crucially upon the extent to which the cells retain the features of the parent epithelium, so subsequent experiments were directed towards exploring their physiological properties.

The generation of second-messenger signals

Earlier studies (Snow, 1977; Bijman and Quinton, 1984a) have shown that secretion from equine sweat glands is mediated by adrenoceptors belonging to the β₂ subclass (Lands et al. 1967). The pharmacological properties of cyclic AMP production in the cultured cells and of secretion in intact glands (Snow, 1977; Bijman and Quinton, 1984a) were essentially identical, supporting the view that cyclic AMP plays an important role in stimulus–secretion coupling in the equine sweat gland (Bijman and Quinton, 1984b).

Secretion from the sweat glands in small skin plugs of horse skin can become acutely dependent upon calcium after prolonged incubation in calcium-free solutions. Changes in \([\text{Ca}^{2+}]\), may therefore play a role in stimulus–secretion coupling (Bijman and Quinton, 1984b). Adrenaline increased \([\text{Ca}^{2+}]\), in the cultured cells. This rise was not secondary to the increased cellular cyclic AMP content. Changes in \([\text{Ca}^{2+}]\), are one of the key mechanisms by which responses to hormones and neurotransmitters are mediated (Petersen, 1992) and so, although the adrenaline-evoked rise in \([\text{Ca}^{2+}]\), was small and variable, it could constitute an important component of the signal transduction pathway.

In primate glands, receptor-regulated cyclic AMP production is augmented if \([\text{Ca}^{2+}]\), is elevated (Sato and Sato, 1983). This may also be true of equine sweat glands so, even if the secretory mechanism can only be activated by cyclic AMP, changes in \([\text{Ca}^{2+}]\), could still be important if they were able to modulate cyclic AMP production. This does not, however, appear to be the case because ionomycin, a calcium ionophore, did not affect the cyclic AMP content of the unstimulated cells and inhibited adrenaline-evoked cyclic AMP production. Large increases in \([\text{Ca}^{2+}]\), thus appear to antagonise receptor-regulated cyclic AMP formation, although this observation does not altogether eliminate the possibility that the much smaller adrenaline-evoked increase in \([\text{Ca}^{2+}]\), could augment cyclic AMP production.
Individual sweat glands in small biopsies of horse skin respond to adrenaline and to isoprenaline with a smooth increase in secretory rate that is entirely attributable to the activation of β-adrenoceptors (Bijman and Quinton, 1984a). These glands also secrete in response to membrane-permeant analogues of cyclic AMP, but with a longer latency and a slower onset than hormonally evoked responses (Bijman and Quinton, 1984b). Isoprenaline was observed to raise \([\text{Ca}^{2+}]_i\) in the cultured epithelial cells and, although the pharmacology of this response is not clear, activation of β-adrenoceptors can increase \([\text{Ca}^{2+}]_i\) in other epithelial cells (Takemura, 1985; Koizumi et al., 1991). It is possible, therefore, that increased \([\text{Ca}^{2+}]_i\) may mediate the initial phase of the response to isoprenaline in the horse sweat gland but that cyclic AMP production subsequently maintains secretion.

The regulation of cellular chloride permeability

A secretagogue-evoked increase in cellular chloride permeability is an important part of the secretory response of mammalian epithelial cells, which characteristically contain separate chloride conductances that are activated by \([\text{Ca}^{2+}]_i\) and by cyclic AMP (Cliff and Frizzell, 1990; Venglarik et al., 1990). The chloride permeability of the cultured equine cells could not be regulated by cyclic AMP, although \([\text{Ca}^{2+}]_i\)-dependent chloride channels did appear to be present.

There is good evidence that cyclic AMP does mediate secretion from equine sweat glands and it therefore appears that functional cyclic-AMP-dependent chloride channels have been lost during culture. A recent study of a cell line derived from the human colonic epithelium showed that chloride permeability could only be regulated by cyclic AMP in cells which had become differentiated into a polarised epithelial sheet. Increases in \([\text{Ca}^{2+}]_i\), however, could raise the chloride permeability of both differentiated and undifferentiated cells (Morris et al., 1992). The protein species that constitutes the cyclic-AMP-dependent chloride channel was, however, always present, and so the insertion of functional cyclic-AMP-dependent chloride channels into the plasma membrane seems to be an event that is closely associated with the development of cellular polarity (Morris et al., 1992).

There was, however, evidence of polarity among the observed ultrastructural characteristics of the cultured cells. Most significantly, junctional complexes were present between adjacent cells, and these junctions were proximate to the upward-facing membrane, which thus appears to correspond to the luminal membrane of the intact gland. Their presence strongly suggests that the epithelial cells have become integrated into epithelial sheets. In the cultured cells, however, only a few, sparse and stubby microvilli were present on this membrane, whereas the intact gland normally exhibits numerous, long microvilli (Montgomery et al., 1982).

In conclusion, although the permeability properties of the equine sweat gland epithelia have not been investigated directly, cyclic-AMP-dependent chloride channels do appear to be present (Bijman and Quinton, 1984b). Such channels were not found in the cultured cells, possibly because they do not express a fully differentiated phenotype under the present culture conditions (Morris et al., 1992). The cells did, however, retain the receptors and second-messenger pathways that appear to regulate secretion from intact
glands (Bijman and Quinton, 1984a,b). We have thus been able to make the first direct demonstration of receptor-regulated cyclic AMP production in equine sweat gland epithelia, and our observation that adrenaline can increase [Ca\(^{2+}\)], may well explain why secretion from the intact glands can, at least under certain circumstances, become dependent upon external calcium (Bijman and Quinton, 1984b).

The authors are grateful to the Royal Hong Kong Jockey Club for financial support. We also thank R. I. Freshney for his helpful suggestions and discussions.

References


Cultured equine sweat gland epithelia


