EXTRACELLULAR ATP CAN ACTIVATE AUTONOMIC SIGNAL TRANSDUCTION PATHWAYS IN CULTURED EQUINE SWEAT GLAND EPITHELIAL CELLS

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Summary
Changes in intracellular free calcium concentration ([Ca^{2+}]_i) were monitored in a cell line that was derived from the equine sweat gland epithelium. ATP and closely related compounds could increase [Ca^{2+}]_i, with a rank order of potency of UTP>ATP>ADP=AMP=adenosine=α,β-methylene-ATP. The responses to ATP and to UTP were initiated by the release of calcium from an internal store and subsequently sustained by calcium influx. The rise in [Ca^{2+}]_i thus seems to be mediated by P_2U receptors that are coupled to phosphoinositidase C. Some desensitisation of this response developed during repeated stimulation with ATP and this was blocked by staurosporine, an inhibitor of protein kinase C, and augmented by a phorbol ester which acts as an exogenous activator of this enzyme. A protein-kinase-C-dependent inhibitory pathway thus seems to become active during repeated stimulation with ATP. ATP and related compounds could also raise cellular cyclic AMP content. The order of potency was ATP>ADP=AMP=adenosine>UTP, suggesting that this response is mediated via a separate subclass of P_2 receptor. The present results demonstrate that ATP can activate autonomic signal-transduction pathways in cultured equine sweat gland cells and suggest that there may be a purinergic component to the control of secretory activity in the equine sweat gland.

Introduction
The secretion of sweat is an important thermoregulatory response in Equidae but the means by which sweating in these animals is controlled is not fully understood. Current evidence suggests that horse sweat glands are controlled via β_2-adrenoceptors and that the changes in membrane permeability underlying secretion are therefore mediated

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primarily by cyclic-AMP-dependent mechanisms (Snow, 1977; Johnson and Creed, 1982; Bijman and Quinton, 1984a,b). Preliminary experiments have shown, however, that external ATP can both increase intracellular free calcium concentration ([Ca$^{2+}$]$_i$) and modulate membrane permeability in cultured equine sweat gland cells (Wilson et al. 1993a). This raises the possibility of a purinergic component to the sudomotor control mechanism. In the present experiments, we have therefore characterised the effects of external ATP upon equine sweat gland epithelial cells to establish the extent to which this agonist can exert regulatory control over autonomic signal-transduction pathways.

Materials and methods

Standard techniques were used to maintain an epithelial cell line (E/92/3) derived from the equine sweat gland (Wilson et al. 1993b). The standard culture medium was serum-free William's medium E, supplemented as detailed below. Cells were removed from culture flasks using trypsin (0.24 %)/EDTA (0.05 mmol l$^{-1}$) and resuspended (approximately $10^5$ cells ml$^{-1}$) in medium containing 5 % (v/v) foetal calf serum. Samples of this suspension were plated either on glass coverslips (approximately 0.2 ml each) or in 24-well plates (0.5 ml well$^{-1}$). Once cellular attachment had occurred (after approximately 12 h), the plating medium was replaced with standard medium.

Measurements of intracellular free calcium concentration

Coverslips bearing growing cells (18–36 h after plating) were incubated (30–40 min, 37 °C) in culture medium containing the acetoxymethyl (AM) ester form of the Ca$^{2+}$-sensitive, fluorescent dye Fura-2 (3 μmol l$^{-1}$) in order to load the cells with this dye. The coverslips were then mounted in a small (approximately 100 μl) heated chamber attached to the stage of an inverted microscope, where the cells were superfused (approximately 5 ml min$^{-1}$, 37 °C) with physiological salt solution. An increase in [Ca$^{2+}$]$_i$ causes a rise in the Fura-2 fluorescence ratio (350 nm and 380 nm excitation wavelengths) recorded from cells loaded with this dye and this allows changes in [Ca$^{2+}$]$_i$ to be monitored using standard microspectrofluorimetric techniques (Grynkiewicz et al. 1985). Absolute calibration of such signals is, however, very difficult to achieve for cells loaded with the AM form of this dye (Highsmith et al. 1986), so experimental records are presented as fluorescence ratios obtained from groups of 2–5 cells. Increases in [Ca$^{2+}$]$_i$, were quantified by measuring the fluorescence ratio at the peak of the response and subtracting from it the ratio measured before application of the agonist.

In several experiments, cells were repeatedly stimulated with ATP by delivering 30 s pulses of 100 μmol l$^{-1}$ ATP at 2 min intervals. The cells were initially stimulated during superfusion with control solution and superfusion then continued under the experimental conditions specified in the Results section. The data from these experiments are presented as fractional responses. These were calculated by expressing all ATP-evoked increases in the Fura-2 fluorescence ratio as fractions of the response to the first application of ATP. The extent to which sensitivity to ATP was lost during these experiments was quantified using the equation:

$$\text{Percentage desensitisation} = (1 - R_{3,4,5}/R_0) \times 100,$$
where $R_0$ is the increase in Fura-2 fluorescence ratio evoked by the initial pulse of ATP and $R_{3,4,5}$ is the mean response evoked by the final three pulses of ATP. The statistical significance of any desensitisation was assessed by using a paired $t$-test to compare $R_0$ and $R_{3,4,5}$.

**Measurement of cellular cyclic AMP content**

Cells grown (3–4 days) on 24-well plates were washed twice with physiological salt solution containing papaverine (20 μmol l$^{-1}$) and incubated (10 min, 37 °C) in this buffer (0.5 ml well$^{-1}$) either under control conditions or under the experimental conditions specified in the Results section. Papaverine is a phosphodiesterase inhibitor that was included in the incubation medium to inhibit the hydrolysis of cyclic AMP. Concentrated perchloric acid (10 μl) was then added to each well to arrest cellular metabolism and to extract cyclic AMP; these extracts were neutralised with KOH, brought to 1 ml with assay buffer (50 mmol l$^{-1}$ sodium acetate/acetic acid buffer; pH 5.8) and stored at −18 °C until their cyclic AMP contents were determined. Cellular protein was also extracted from each well with 1 ml of 300 mmol l$^{-1}$ NaOH in 1% (w/v) sodium dodecyl sulphate and quantified (Lowry et al. 1951; Petersen, 1977) so that the experimental data could be expressed as pmoles of cyclic AMP per microgram of cellular protein (pmol μg$^{-1}$).

Cyclic AMP was determined by radioimmunoassay. This is a technique that is based upon competition between the variable amount of cyclic AMP present in each cell extract and a fixed amount of a radioactive cyclic AMP analogue (adenosine 3′,5′-cyclic phosphoric acid 2′-O-succinyl-3-[125I]iodotyrosine methyl ester; 125I-cyclic AMP, Amersham). A finite number of cyclic AMP binding sites was provided by a rabbit antibody against cyclic AMP, which had affinity only for acetylated cyclic AMP. Samples of the acid extracts and of cyclic AMP standards were therefore brought to 500 μl with assay buffer and excess (25 μl) acetylating reagent (1 volume of acetic anhydride:2 volumes of triethylamine) was added to each tube. Samples (100 μl) of the resulting solutions were transferred to assay tubes and 100 μl aliquots of primary antibody (Calbiochem rabbit anti-cyclic AMP diluted 1:10000 in 50 mmol l$^{-1}$ sodium acetate/acetic acid buffer; pH 4.75, containing 1 mg ml$^{-1}$ human gamma globulin) and of 125I-cyclic AMP (approximately 12.5 nCi) were added to each. The binding reaction was then allowed to reach equilibrium (18 h, 4 °C). A sample (500 μl) of a donkey anti-rabbit IgG antibody immobilised onto beads (Amersham Amerlex-M immunoassay separation reagent) was then added to each tube to bind the primary antibody together with any associated 125I-cyclic AMP. The binding reaction was then calculated by reference to a standard curve. In some experiments, cells were stimulated with ATP at concentrations of up to 1 mmol l$^{-1}$. This exogenous ATP was thus present in the acid extracts, but appropriate experiments established that this did not interfere with the assay of cyclic AMP.

**Solutions**

The culture medium was William’s medium E supplemented with 1-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 sodium selenite (10 ng ml\(^{-1}\)). The physiological salt solution contained (in mmol l\(^{-1}\)): NaCl, 130; KCl, 5; Hepes, 20; MgCl\(_2\), 1; CaCl\(_2\), 1; and d-glucose, 10. The pH was adjusted to 7.4 with NaOH. Nominally Ca\(^{2+}\)-free solutions were prepared by omitting CaCl\(_2\) from the control saline.

**Results**

*Effects of ATP and related compounds upon [Ca\(^{2+}\)]\(_i\).*

ATP (100 \(\mu\)mol l\(^{-1}\)) consistently increased [Ca\(^{2+}\)]\(_i\), but there was some variability in the form of this response (Fig. 1). The initial rise in [Ca\(^{2+}\)]\(_i\) was rapid and, in some instances, this developed into a clearly defined peak (Fig. 1A,C). The interval between switching to the ATP-containing solution and obtaining a half-maximal response was 16.4±1.3 s (N=26, range 7.1–37.4 s) and so, as the dead time in the superfusion system was 1–2 s, the onset of the response was associated with a latency of at least 4–5 s and most cells responded only after longer intervals. Withdrawal of ATP caused [Ca\(^{2+}\)]\(_i\) to fall to basal levels, although there was some delay before this became apparent (Fig. 1). The decline was usually rapid but, in some instances, [Ca\(^{2+}\)]\(_i\) fell very slowly (Fig. 1C). Small, spontaneous increases in [Ca\(^{2+}\)]\(_i\) were occasionally seen (Fig. 1B).

The ATP-evoked increase in [Ca\(^{2+}\)]\(_i\) was concentration-dependent (Fig. 2) and a half-maximal response (EC\(_{50}\)) was estimated to occur at 12±1 \(\mu\)mol l\(^{-1}\). The pyrimidine nucleotide UTP was also an effective Ca\(^{2+}\)-mobilising agonist and this compound appeared to be slightly more potent (EC\(_{50}\)=4.9±0.2 \(\mu\)mol l\(^{-1}\)) than ATP. The cells also responded to ADP, but higher concentrations of this compound were required to increase [Ca\(^{2+}\)]\(_i\) (Fig. 2), indicating that it was less potent than either UTP or ATP (EC\(_{50}\)=346±60 \(\mu\)mol l\(^{-1}\)). AMP (1 and 3 mmol l\(^{-1}\), N=4), adenosine (1 and 3 mmol l\(^{-1}\), N=6) and \(\alpha,\beta\)-methylene-ATP (30 \(\mu\)mol l\(^{-1}\), N=6) caused no discernible change in [Ca\(^{2+}\)]\(_i\), but all cells in which these compounds were tested subsequently responded to 100 \(\mu\)mol l\(^{-1}\) ATP.

Internal [Ca\(^{2+}\)] remained above basal levels throughout 2 min applications of either ATP or UTP, although it tended to decline in the presence of agonist (Fig. 3). These compounds subsequently caused a rapid rise in [Ca\(^{2+}\)]\(_i\) during superfusion with the nominally Ca\(^{2+}\)-free solution but, in this situation, the response could not be sustained (Fig. 3). Subsequent elevation of external [Ca\(^{2+}\)], in the continued presence of agonist, evoked a sustained and reversible rise in [Ca\(^{2+}\)]\(_i\) (Fig. 3). Such an elevation of external [Ca\(^{2+}\)] did not affect [Ca\(^{2+}\)]\(_i\) in unstimulated cells (N=3), so the responses to ATP and to UTP consisted of an initial, transient phase independent of external Ca\(^{2+}\) and a sustained, Ca\(^{2+}\)-dependent element.

*Desensitisation of the response to ATP*

Repeated applications of ATP evoked a series of increases in [Ca\(^{2+}\)]\(_i\). The peak change in the Fura-2 fluorescence ratio caused by the initial pulse was 1.50±0.10 (N=26) but the responses to subsequent stimuli were smaller, demonstrating that some loss of sensitivity developed during repeated stimulation (Fig. 4A, Table 1). In other cell types this has
been attributed to receptor-mediated activation of protein kinase C (Llano and Marty, 1987; Maruyama, 1989) and so, to establish whether this was also true of the equine cells, we explored the effects of staurosporine and of the phorbol ester 12-O-tetradecanoyl phorbol 13-acetate (TPA). These are compounds that, respectively, inhibit and activate protein kinase C.

The data presented in Fig. 4A suggested strongly that the desensitisation was blocked by staurosporine (0.1 μmol l−1) and potentiated by TPA (1.6 nmol l−1). The desensitisation that developed under the various experimental conditions was therefore quantified (see Materials and methods) and these data are presented in Table 1; the effects of staurosporine and TPA were statistically significant. Also shown in Table 1 are data from experiments in which higher concentrations of TPA were used; there is some evidence that the effect of this compound is concentration-dependent but it did not totally abolished the response to ATP (Table 1).
Fig. 2. Dose–response curves for ATP and related compounds. Small groups of cells were stimulated with a series of 30 s pulses of increasing concentrations of the appropriate nucleotide delivered at 2 min intervals. In the experiments in which the effects of ADP and UTP were explored, the cells were also stimulated with 100 μmol l⁻¹ ATP so that all of these data could be expressed as fractions of the response to this standard stimulus. These fractional responses are plotted (mean ± S.E.M.) against the concentration of the nucleotide used (● ATP; ■ ADP; ▲ UTP). Values of N are given beside each point and the solid lines are sigmoid curves fitted to the experimental data using a least-squares regression procedure.

Table 1. Desensitisation of the response to ATP

<table>
<thead>
<tr>
<th>Experimental conditions</th>
<th>N</th>
<th>(%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>26</td>
<td>12±3</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>0.1 μmol l⁻¹ staurosporine</td>
<td>12</td>
<td>−6±7</td>
<td>NS</td>
</tr>
<tr>
<td>1.6 nmol l⁻¹ TPA</td>
<td>8</td>
<td>58±8*</td>
<td>&lt;0.0002</td>
</tr>
<tr>
<td>16 nmol l⁻¹ TPA</td>
<td>9</td>
<td>60±9*</td>
<td>&lt;0.0002</td>
</tr>
<tr>
<td>160 nmol l⁻¹ TPA</td>
<td>6</td>
<td>80±2*</td>
<td>&lt;0.0002</td>
</tr>
</tbody>
</table>

In each experiment cells were repeatedly stimulated with ATP. The first pulse was delivered under control conditions and superfusion was then continued under the experimental conditions that are detailed. The loss of sensitivity that developed during each experiment was quantified and its statistical significance determined (see Materials and methods). These data (means ± S.E.M.) and the appropriate values of P are tabulated.

The extent of the desensitisation that developed in the presence of TPA was compared with the desensitisation that developed under control using an unpaired t-test. The results of this analysis are indicated by asterisks; *P<0.0001; values of N refer to the number of experiments; NS, not significant.
The ability of staurosporine to antagonise the action of TPA was also explored. Cells were repeatedly stimulated with ATP and exposed to TPA (1.6 nmol l\(^{-1}\)) from immediately after the first pulse of agonist. This caused a loss of sensitivity essentially identical to that described above, but sensitivity to ATP appeared to be restored when the cells were subsequently exposed to 0.1 \(\mu\)mol l\(^{-1}\) staurosporine (Fig. 4B). The mean fractional response measured immediately prior to addition of staurosporine (0.49±0.08) was less \((P<0.002, \text{paired } t\text{-test})\) than the mean response to the final two pulses of ATP (0.79±0.05); staurosporine can thus antagonise the action of ATP.

**Effects of ATP upon cellular cyclic AMP content**

High concentrations of extracellular ATP were able to increase the cyclic AMP content of the equine cells. Although similar concentrations of ADP, AMP or adenosine were also able to evoke this response, these compounds were less potent than ATP and the pyrimidine nucleotide UTP was ineffective (Fig. 5).
We also explored the possibility that the cells may contain type 1 adenosine (A₁) receptors that characteristically allow adenosine to exert inhibitory control over adenylate cyclase (Burnstock, 1990b). In these experiments (N=6), the cyclic AMP content of the unstimulated cells was 0.250±0.064 pmol mg⁻¹ and this was increased by 0.489±0.118 pmol mg⁻¹ (N=6) on exposure to 0.1 μmol l⁻¹ forskolin, a diterpene that evokes cyclic AMP production by a direct effect on the catalytic subunit of adenylate cyclase. The cells responded to forskolin during exposure to the relatively selective A₁ receptor agonist N⁶-cyclopentyladenosine (1 nmol l⁻¹), but the increase in cyclic AMP content (0.304±0.092 pmol mg⁻¹) was smaller than the control (P<0.05, paired t-test).

Discussion

Physiological basis of the increase in [Ca²⁺]ₐ

The present study showed that ATP and certain related compounds can increase [Ca²⁺]ₐ in equine sweat gland epithelial cells with an apparent rank order of potency of
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UTP>ATP>ADP>AMP=adenosine=α,β-methylene-ATP. These results indicate that the response is mediated by a type 2 purine (P₂) receptor (Burnstock, 1990b) with properties essentially identical to those of a receptor subclass first identified in Ehlich ascites tumour cells (Dubyak and Young, 1985). These receptors are generally said to belong to the P₂U subclass and are usually coupled to phosphoinositidase C. They provide a means by which ATP can regulate the hydrolysis of a membrane phospholipid, phosphatidylinositol-4,5-bisphosphate (PtdIns4,5P₂) (Dubyak et al. 1988). The hydrophilic product of this reaction, inositol 1,4,5-trisphosphate (Ins1,4,5P₃), is the intracellular second messenger that releases Ca²⁺ from internal stores, and the resultant rise in [Ca²⁺], which is characteristically sustained by Ca²⁺ influx, provides one of the central mechanisms by which hormones and neurotransmitters exert regulatory control (Berridge, 1993).

The present responses to ATP and to UTP could be attributed to the release of Ca²⁺ from a limited internal store and to receptor-regulated calcium influx. This result suggests very strongly that the P₂U receptors in these cells are functionally coupled via the production of Ins1,4,5P₃. Preliminary experiments have suggested that the resultant rise

Fig. 5. Effects of ATP and related compounds upon cellular cyclic AMP content. In each of four independent experiments, cellular cyclic AMP content was measured both under unstimulated conditions and in the presence of various concentrations of ATP or of other related compounds. The magnitude of the agonist-evoked accumulation of cyclic AMP was calculated by subtracting the cyclic AMP content of the unstimulated cells from all experimental values and these data are plotted (mean ± s.e.m.) against the concentration of agonist used. An asterisk indicates that the plotted concentration differed significantly (P<0.05, paired t-test) from that measured in the unstimulated cells (0.312±0.097 pmol μg⁻¹, mean ± s.e.m., N=4). (A) The effects of ATP (●), ADP (■) or UTP (▲); (B) the effects of AMP (●) and adenosine (■).

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The graph shows the effects of ATP, ADP, UTP, AMP, and adenosine on cellular cyclic AMP content. The x-axis represents the concentration of agonist (μmol l⁻¹), while the y-axis represents the increase in cellular cyclic AMP content (Δpmol μg⁻¹). The data are plotted as mean ± S.E.M. for each concentration of agonist. An asterisk indicates a significant difference from the unstimulated condition.

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in [Ca\textsuperscript{2+}]\textsubscript{i} can cause changes in membrane permeability that are characteristic of a secretory response (Wilson et al. 1993a). These findings are in excellent accord with data from recent studies of epithelial cells from the amphibian kidney (Middleton et al. 1993) and the human trachea (Mason et al. 1991) and mammary gland (Flezar and Heisler, 1993). It appears that P\textsubscript{2U} receptors provide a widespread mechanism that allows ATP to regulate transport processes in vertebrate epithelia.

ATP acts via P\textsubscript{2} receptors to increase [Ca\textsuperscript{2+}]\textsubscript{i} in rodent salivary and lacrimal acinar cells. These responses do not, however, involve either PtdIns\textsubscript{4,5}P\textsubscript{2} hydrolysis or the mobilisation of internal Ca\textsuperscript{2+} because the P\textsubscript{2} receptors in these cells are coupled to Ca\textsuperscript{2+}-permeable ion channels (McMillian et al. 1988; Sasaki and Gallacher, 1990). The possibility that this mechanism contributes to the sustained Ca\textsuperscript{2+}-dependent component of the present response cannot be excluded.

Some cell types contain adenosine receptors that are coupled to phosphoinositidase C (e.g. Ali et al. 1990). This does not, however, appear to be the case in the equine cells as adenosine evoked no discernible change in [Ca\textsuperscript{2+}].

**Mechanism of desensitisation to ATP**

The hydrophobic product of PtdIns\textsubscript{4,5}P\textsubscript{2} hydrolysis is a diacylglycerol molecule that remains in the plasma membrane and allosterically modulates the activity of protein kinase C: a Ca\textsuperscript{2+}- and phospholipid-dependent enzyme. Protein kinase C can influence many aspects of cellular physiology by regulating the phosphorylation of specific proteins. Although the activation of the enzyme appears to be part of the normal response to agonists that evoke PtdIns\textsubscript{4,5}P\textsubscript{2} hydrolysis, the role of this enzyme in stimulus–secretion coupling is not altogether clear (Nishizuka, 1984). Experiments on cholinergically stimulated pancreatic and lacrimal acinar cells have suggested that the activation of protein kinase C may allow inhibitory control over receptor-regulated Ins\textsubscript{1,4,5}P\textsubscript{3} production and thus provide a mechanism for desensitisation (Llano and Marty, 1987; Maruyama, 1989). The present data were consistent with this view, as desensitisation to ATP did not take place if protein kinase C was inhibited and the desensitisation was augmented by an exogenous activator of the enzyme. A protein-kinase-C-dependent inhibitory pathway thus appears to become active during repeated stimulation with ATP and this supports the view that the P\textsubscript{2U} receptors in these cells are coupled to phosphoinositidase C.

**Physiological basis of cyclic AMP production**

ATP and related compounds increased cellular cyclic AMP content with a rank order of potency of ATP>ADP=AMP=adenosine\textsubscript{5\prime}-UTP. The response thus appears to be mediated by a subclass of P\textsubscript{2} receptor (Burnstock, 1990b) with pharmacological properties different from those of the P\textsubscript{2U} receptors that underlie the rise in [Ca\textsuperscript{2+}]. To our knowledge, there are no previous reports that P\textsubscript{2} receptors can activate adenylate cyclase in epithelial cells (see, for example, Burnstock, 1990b). It is certainly clear that the P\textsubscript{2} receptors in epithelial cells from the amphibian kidney (Middleton et al. 1993) and the
human mammary gland (Flezar and Heisler, 1993) are not coupled to adenylate cyclase in this way.

These responses to ATP can be attributed to the activation of type 2 adenosine (A$_2$) receptors. These receptors characteristically activate adenylate cyclase but have only a very low affinity for ATP. In many experimental situations, however, extracellular nucleotidases can dephosphorylate ATP and thus provide sufficient adenosine to evoke cyclic AMP formation (Burnstock, 1990b). The possibility that a component of the present response to ATP was due to such an effect cannot be excluded, but this does not seem likely as ATP was more potent than adenosine. There is a recent report suggesting that some of the effects of ATP upon human platelets may be due to P$_2$-receptor-regulated cyclic AMP production (Soslau et al. 1993). It is therefore possible that such receptors may be involved in the present responses to ATP.

Experimental manoeuvres that increase [Ca$^{2+}$]$_i$ can augment receptor-regulated cyclic AMP formation in isolated primate sweat glands (Sato and Sato, 1983). It is therefore possible that the present response to ATP reflects a small, A$_2$-receptor-mediated response that was potentiated by the simultaneous, P$_2U$-receptor-mediated rise in [Ca$^{2+}$]$_i$. The response to adenosine would not be amplified in this way as this compound does not increase [Ca$^{2+}$]$_i$. It has not, however, been unequivocally established that the potentiating interactions between [Ca$^{2+}$]$_i$ and cellular cyclic AMP content that occur in primate sweat glands are due to intracellular actions (Sato and Sato, 1983); it is possible that they may be secondary to stimulation of the nerve terminals that are present in isolated glands. Our earlier studies of equine sweat gland cells in culture showed that ionomycin inhibits cyclic AMP formation (Wilson et al. 1993b), and we have obtained an essentially identical result from a virally transformed, epithelial cell line derived from the human sweat gland (Wilson et al. 1994). Large increases in [Ca$^{2+}$]$_i$ thus appear to inhibit cyclic AMP formation, although this result does not preclude the possibility that smaller, receptor-mediated elevations of [Ca$^{2+}$]$_i$ may exert potentiating effects (Wilson et al. 1993b).

The present data also suggested that equine cells may contain A$_1$ receptors that were able to inhibit cyclic AMP synthesis. Such receptors are present in epithelial cell lines derived from the rabbit collecting tubule (Spielman et al. 1992) and from the bovine and the human ocular ciliary epithelium (Wax et al. 1993). Such receptors do not, however, appear to be present in epithelial cells of the human trachea (Lazarowski et al. 1992). It is therefore possible that adenosine exerts both excitatory and inhibitory control over adenylate cyclase in the equine sweat gland epithelium. The physiological significance of this is not clear, but the present responses to adenosine would have been due to the activation of these mutually antagonistic signalling pathways. The net effect of this compound upon cellular cyclic AMP content would, therefore, be hard to predict, especially as the A$_1$- and A$_2$-receptor-linked pathways appear to desensitise at different rates (Ramkumar et al. 1991).

**Biological significance of the present results**

The principal new finding of the present work is that purinergic receptors are present on equine sweat gland epithelial cells in culture. This raises the possibility that such
receptors may also be present in the intact equine sweat gland, where they may be involved in the regulation of glandular activity. The present experiments were, however, undertaken using a permanent cell line that was derived from the secretory epithelium of the equine sweat gland by a spontaneous transformation process (Wilson et al. 1993a). The relevance of this work to the biology of the Equidae therefore depends crucially upon the extent to which these cells have retained the physiological features of the parent epithelium. We cannot unequivocally exclude the possibility that de-differentiation may have occurred in vitro and that the cells may now express physiological features that are not seen in vivo. Our initial studies of these cells did, however, suggest that they faithfully retained the adrenergic receptors and second-messenger pathways that regulate secretion from intact glands (Wilson et al. 1993a).

Although sweat glands are found in essentially all mammalian groups, it is only in primates, certain Bovidae and the Equidae, that they have become specialised as thermoregulatory organs (Jenkinson, 1973). Sweating in horses can be regulated both by adrenaline released from the adrenal medulla and by the action of postganglionic sympathetic nerves (Robertshaw and Taylor, 1969). Equine sweat glands contain $\beta_2$-adrenoceptors that characteristically have a high affinity for adrenaline. These receptors allow adrenaline to evoke the formation of cyclic AMP and this intracellular second messenger can evoke secretion in vitro; the mechanism of hormonally evoked sweating is thus reasonably clear (Robertshaw and Taylor, 1969; Snow, 1977; Bijman and Quinton, 1984a,b; Wilson et al. 1993b). The classic neurotransmitter released from the sympathetic nerve fibres is noradrenaline, and the postjunctional effects of this substance are mediated, primarily, via $\alpha$- and $\beta_1$-adrenoceptors. The equine sweat gland epithelium appears to be essentially devoid of these receptors since large injections of noradrenaline do not evoke secretion in vivo and high concentrations of this compound are required to evoke either secretion or cyclic AMP formation in vitro. It is thus not obvious how the sympathetic nerves may regulate sweating (Robertshaw and Taylor, 1969; Snow, 1977; Bijman and Quinton, 1984a,b; Wilson et al. 1993b).

It is now well known that ATP, as well as noradrenaline, is contained within sympathetic neurosecretory vesicles and that these compounds are released from the nerve terminals in parallel (Burnstock, 1990a,b). The present data thus suggest that ATP released from the periglandular sympathetic nerves may have the capacity to activate autonomic signal-transduction pathways within the glandular epithelium. This work, together with our earlier experiments (Wilson et al. 1993a), raises the possibility that purinergic neurotransmission may play a central role in regulating secretory activity in the equine sweat gland and that this process may therefore be an important part of the thermoregulatory response. It is now important to establish whether functional $P_{2U}$ receptors are present in intact equine sweat glands.

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