PERSISTENT DESENSITISATION OF THE β_2 ADRENOCEPTORS EXPRESSED BY CULTURED EQUINE SWEAT GLAND EPITHELIAL CELLS

SOMA RAKHIT, RUTH MURDOCH AND STUART M. WILSON*

Division of Neuroscience and Biomedical Systems, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow G12 8QQ, Scotland

*Present address and address for correspondence: Department of Child Health, Ninewells Hospital and Medical School, University of Dundee, Dundee DD1 9SY, Scotland (e-mail: s.m.wilson@dundee.ac.uk)

Summary

Adrenaline, forskolin and ATP all evoked accumulation of cyclic AMP in equine sweat gland epithelial cells, although the response to adrenaline was more transient than that to forskolin and ATP. Cells preincubated in adrenaline ($10\,\mu mol\,l^{-1}$, $32\,min$) showed essentially complete, homologous desensitisation, and this phenomenon reversed slowly (half-time $6.3\pm0.9\,h$). After $10\,min$ of recovery from preincubation in adrenaline, isobutylmethylxanthine (IBMX, $5\,mmol\,l^{-1}$) had no effect upon the desensitisation and the cells showed no loss of sensitivity to ATP and forskolin. After $10\,h$, however, the persistent desensitisation was partially reversed by IBMX and the cells showed reduced responses to ATP and forskolin. Increased phosphodiesterase activity may thus contribute to the persistent desensitisation. Experiments using forskolin-preincubated ($100\,\mu mol\,l^{-1}$, $32\,min$) cells suggested that increased cytosolic cyclic AMP levels did not underlie the initial loss of sensitivity to adrenaline but that this second messenger may initiate the series of events leading to the generalised loss of sensitivity seen after $10\,h$.

Key words: sweat glands, cell culture, stimulus–secretion coupling, cyclic AMP, phosphodiesterases, IBMX, equine anhydrosis.

Introduction

The evaporation of sweat from the body surface is an important route of thermoregulatory heat loss in horses, particularly during exercise (Bonner, 1994; Carlson, 1994; Hodgson *et al.* 1994), and it was Langley and Bennett (1923) who first showed that intradermal injections of adrenaline elicited profuse but local sweating in these animals. It is now clear that this response is mediated by glandular β_2 adrenoceptors which allow adrenaline to evoke cyclic 3′,5′ monophosphate (cyclic AMP) formation, and that it is a rise in the circulating level of adrenaline that normally triggers the sweating response seen during exercise (Robertshaw and Taylor, 1969; Snow, 1977; Johnson and Creed, 1982; Bijman and Quinton, 1984*a,b*). However, Langley and Bennett (1923) also noted that glands that had secreted in response to injected adrenaline failed to produce sweat when the animals were exercised later that day (see also Evans and Smith, 1956; Evans *et al.* 1957). The stimulated glands had thus entered a refractory state that lasted for several hours. Although it has since been shown that β_2 adrenoceptors lose their sensitivity to adrenaline during prolonged stimulation, this desensitisation usually reverses within 20–40 min of agonist removal (see e.g. Su *et al.* 1980; Levitzki, 1988; Hausdorff *et al.* 1990; Yu *et al.* 1993). There is, therefore, no obvious explanation for the persistent desensitisation seen in the equine sweat gland. The aim of the present study was to explore possible physiological factors responsible for this effect.

Materials and methods

Cell culture

Experiments were undertaken using a spontaneously transformed cell line derived from the secretory epithelium of the equine sweat gland (Wilson *et al.* 1993). Standard techniques were used to maintain these cells in William's medium E supplemented with L-glutamine ($1\,mmol\,l^{-1}$), penicillin ($100\,i.u.\,ml^{-1}$), streptomycin ($100\,\mu g\,ml^{-1}$), bovine insulin ($5\,\mu g\,ml^{-1}$), epidermal growth factor ($0.1\,\mu g\,ml^{-1}$), hydrocortisone ($10\,ng\,ml^{-1}$), transferrin ($5\,\mu g\,ml^{-1}$) and sodium selenite ($5\,ng\,ml^{-1}$). For experiments, cells were removed from culture flasks using trypsin/EDTA, plated onto 24-well plates (approximately 5×10^4 cells well^{-1}) and allowed to grow into monolayers that essentially covered the base of each well (3–4 days).

Measurement of cyclic AMP accumulation

The culture medium was withdrawn from each well and the cells were washed with physiological salt solution containing (in $mmol\,l^{-1}$): NaCl, 100; KCl, 4.8; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $CaCl_2$, 1.2; $NaHCO_3$, 20; 4-(2-hydroxyethyl)-1-piperazine

ethanesulphononic acid, 25, and D-glucose, 10 (pH adjusted to 7.4 with NaOH when bubbled with 5 % CO_2/95 % O_2). The cells were then incubated (10 min, 37 °C) in this solution until experiments were initiated by adding drugs to the appropriate wells. After carefully measured time intervals, the salt solution was rapidly replaced by 0.5 ml of 0.1 mol l^{-1} perchloric acid to arrest cellular metabolism and to extract cyclic AMP from the cells. The cyclic AMP content of each acid extract and the mass of protein in each well were then measured (Ko *et al.* 1994). All such measurements were made in duplicate or triplicate, and the results are expressed as pmoles of cyclic AMP per microgram of cellular protein (pmol µg^{-1}). Experimentally induced changes in cyclic AMP content were quantified by measuring the cyclic AMP content of stimulated cells and subtracting the basal cyclic AMP content, which was measured in unstimulated cells grown on the same plates. In some instances, the rate at which cyclic AMP had been exported from the cells was determined (pmol µg^{-1} h^{-1}) by measuring the cyclic AMP content of the salt solution that had bathed the cells for 32 min. The effects of isobutylmethylxanthine (IBMX) were studied by dissolving this phosphodiesterase (PDE) inhibitor directly in the physiological salt solution. In all instances, the IBMX-treated cells were exposed to the substance for 10 min before experiments were initiated by adding drugs to the appropriate wells.

Data analysis and experimental design

Preliminary experiments showed that there could be large variations in the magnitude of the responses seen in cells after different passage numbers, and so experiments were undertaken using strictly paired experimental designs in which control and experimental cells had been identically passaged and had been grown on the same plates under identical conditions. Data are presented as means ± standard errors, and values of N refer to the number of times a protocol was repeated using cells after a different number of passages. The significance of differences between mean values was evaluated using Student's paired *t*-test. For some analyses, commercially available software (Grafit 3.0, Erithacus Software Ltd, Staines, UK) was used to fit sigmoid curves to experimental data sets using a least-squares regression procedure. Adrenaline-preincubated cells were exposed to the hormone (10 µmol l^{-1}) for 32 min at 37 °C, washed with physiological saline and then incubated for carefully measured time intervals. Basal cyclic AMP levels and the increases in cellular cyclic AMP content evoked by adrenaline (10 µmol l^{-1}, 4 min), ATP (100 µmol l^{-1}, 16 min) or forskolin (100 µmol l^{-1}, 8 min) were then measured and compared with the responses evoked in control cells that had been grown on the same plates. To ensure that physically disturbing the cells did not affect their responsiveness, great care was taken to subject the control and experimental cells to the same pattern of washes/solution changes. The extent to which the preincubated cells had lost sensitivity to adrenaline (% desensitisation) was quantified using the expression:

$$\% \text{ desensitisation} = [1 - (E/C)] \times 100,$$

where E and C are the increases in cyclic AMP content evoked in the experimental and control cells, respectively. Initial experiments showed that cells did not tolerate prolonged incubation in physiological saline and so, in experiments involving recovery periods of longer than 1 h, monolayers were returned to complete culture medium once the desensitising stimulus was withdrawn.

To explore the effects of IBMX upon the extent to which desensitised cells recovered their sensitivity to adrenaline, adrenaline-preincubated cells were prepared as detailed above. These cells were then either allowed to recover under standard conditions or exposed to 5 mmol l^{-1} IBMX for the final 10 min of the recovery period. Basal cyclic AMP levels and the response to adrenaline (10 µmol l^{-1}, 4 min incubation) were then measured for both groups of desensitised cells. As part of each experiment, adrenaline-evoked increases in cellular cyclic AMP content were also measured under control conditions and in IBMX-treated cells. Each experiment thus provided paired estimates of the desensitisation seen under control conditions and in IBMX-treated cells. Forskolin-preincubated cells were exposed to this substance (100 µmol l^{-1}) for 32 min.

Results

Cyclic AMP accumulation by control cells

Adrenaline consistently evoked cyclic AMP accumulation, and this response reached a clearly-defined peak after 4 min (Fig. 1). Once this peak was reached, however, cyclic AMP levels fell towards the basal value and, after 32 min, the response had decayed by 86.5±2.1 % (Fig. 1). There was no significant difference between the rates at which cyclic AMP was exported from unstimulated (0.003±0.001 pmol µg^{-1} h^{-1}) and adrenaline-stimulated (0.006±0.003 pmol µg^{-1} h^{-1}) cells, and so it was clear that the falling phase of the response to
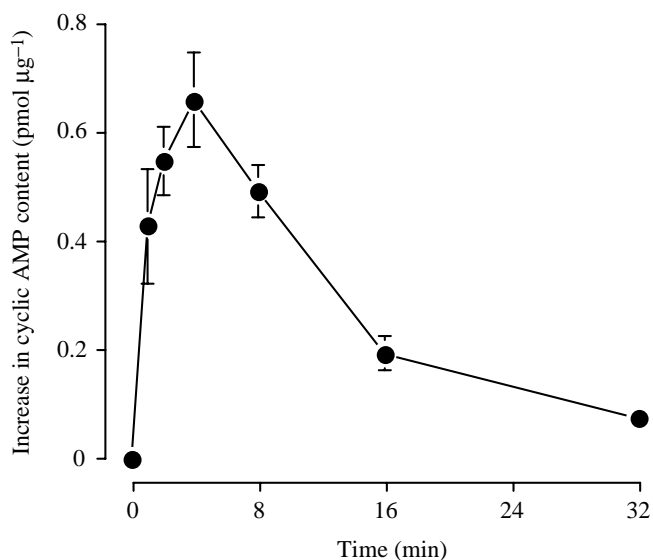

Fig. 1. Time course showing the changes in cyclic AMP content that occur during exposure to 10 µmol l^{-1} adrenaline. Values are means ± S.E.M., N=18; basal cyclic AMP content was 0.028±0.007 pmol µg^{-1}.
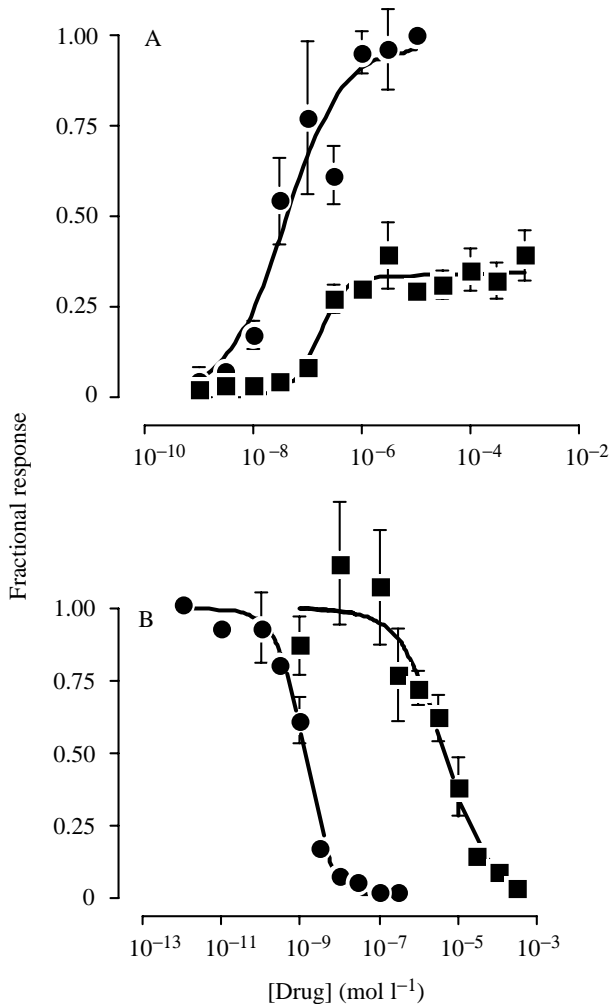
Fig. 2. (A) The increases in cyclic AMP content evoked by 4 min incubations in either adrenaline (●, $N=4$) or salbutamol (■, $N=6$) were normalised to the response evoked by $10 \mu mol\,l^{-1}$ adrenaline ($0.195\pm0.038\,pmol\,\mu g^{-1}$) and plotted against the concentration of agonist used. Basal cyclic AMP content was $0.012\pm0.002\,pmol\,\mu g^{-1}$. (B) Responses to $1 \mu mol\,l^{-1}$ adrenaline (4 min incubation) were measured both under control conditions ($0.418\pm0.100\,pmol\,\mu g^{-1}$) and in the presence of ICI-118,551 (●, $N=7$) or atenolol (■, $N=4$). The resultant increases evoked in each experiment were then expressed as fractions of the appropriate control response, and these data are plotted against the concentration of antagonist used. Basal cyclic AMP content was $0.018\pm0.003\,pmol\,\mu g^{-1}$. Values are means ± S.E.M.

adrenaline was not due to an increased efflux of cyclic AMP. The magnitude of the response to adrenaline was concentration-dependent (Fig. 2, ●), and the concentration required to evoke a half-maximal response (EC_{50}) was estimated to be $0.036\pm0.018 \mu mol\,l^{-1}$. Although salbutamol, a selective β_2 adrenoceptor agonist, also elicited cyclic AMP accumulation ($EC_{50}=0.164\pm0.036 \mu mol\,l^{-1}$), the maximal response was only $33.9\pm1.5\%$ of that evoked by a maximally effective concentration ($10 \mu mol\,l^{-1}$) of adrenaline (Fig. 2A, ■). ICI-118,551 (Fig. 2B, ■) and atenolol (Fig. 2B, ■) caused concentration-dependent inhibition of the response to
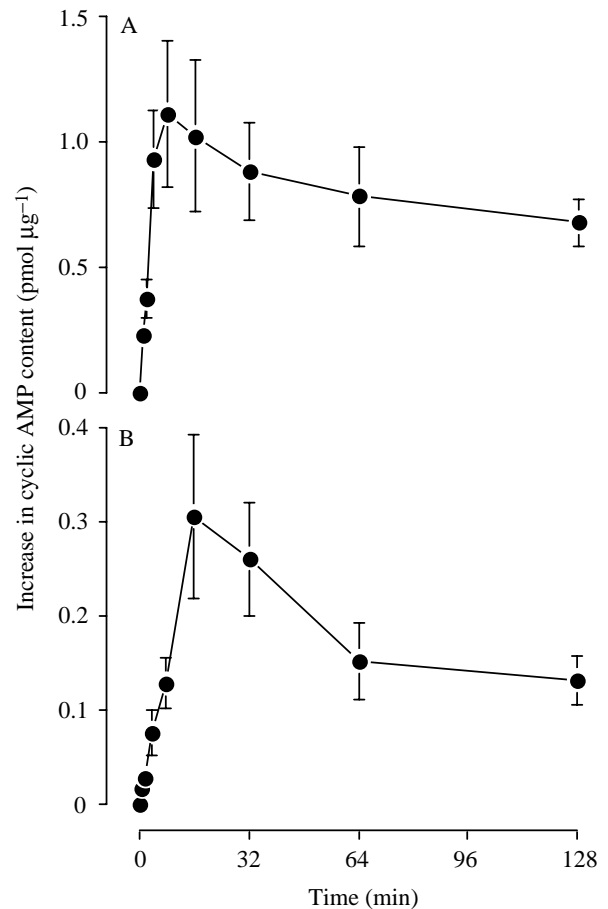

Fig. 3. Time courses showing the changes in cyclic AMP that occurred during exposure to (A) $100 \mu mol\,l^{-1}$ forskolin ($N=4$, basal cyclic AMP content $0.029\pm0.023\,pmol\,\mu g^{-1}$) and (B) $100 \mu mol\,l^{-1}$ ATP ($N=5$, basal cyclic AMP content $0.036\pm0.020\,pmol\,\mu g^{-1}$). Values are means ± S.E.M.

adrenaline, and the concentrations required to achieve half-maximal inhibition were estimated to be $1.2\pm0.2\,nmol\,l^{-1}$ for ICI-118,551 and $4.5\pm3 \mu mol\,l^{-1}$ for atenolol. The cells also accumulated cyclic AMP when exposed to forskolin or ATP. The response to forskolin peaked after 8 min, but had decayed by only $15.1\pm10.4\%$ after 32 min. Even after 128 min, cellular cyclic AMP levels had only declined by $39\pm8.2\%$ (Fig. 3A). The response to ATP peaked after 16 min and had decayed by $5.4\pm12.0\%$ after 32 min and by $56.7\pm8.3\%$ after 128 min (Fig. 3B). These responses were thus more sustained than the response to adrenaline.

Effects of IBMX

Basal cyclic AMP content was unaffected by $1\,mmol\,l^{-1}$ IBMX (control $0.032\pm0.017\,pmol\,\mu g^{-1}$, IBMX $0.026\pm0.006\,pmol\,\mu g^{-1}$, $N=4$) but elevated (control $0.022\pm0.006\,pmol\,\mu g^{-1}$, IBMX $0.090\pm0.027\,pmol\,\mu g^{-1}$, $P<0.05$, $N=4$) by a higher concentration ($5\,mmol\,l^{-1}$). Both concentrations potentiated the response to adrenaline by prolonging its rising phase, although this effect was more marked at the higher concentration (Table 1). However, once

Table 1. *Responses to 10 μmol l^{-1} adrenaline (4 min) were quantified in control cells and in cells that had been preincubated in 1 mmol l^{-1} or 5 mmol l^{-1} IBMX*

Time (min)	Increase in cyclic AMP content (pmol μg^{-1})		
	Control (N=8)	1 mmol l^{-1} IBMX (N=4)	5 mmol l^{-1} IBMX (N=4)
2	0.529±0.161	0.369±0.049	0.660±0.171
4	0.599±0.193	0.535±0.095	1.222±0.159*
8	0.512±0.110	0.582±0.103**	1.464±0.292*
16	0.249±0.111	0.470±0.060***	1.711±0.289**
32	0.070±0.045	0.158±0.029*	0.877±0.308*

Asterisks denote values that differed significantly from control (*$P<0.05$, **$P<0.02$, ***$P<0.01$).

the peak of the response to adrenaline had been reached, a clear fall in cellular cyclic AMP content occurred despite the presence of IBMX (Table 1).

Effects of preincubation in adrenaline

In initial experiments, cells were preincubated in 10 μmol l^{-1} adrenaline for 32 min and then allowed to recover for 2–42 min before basal cyclic AMP content and the response to a second exposure to 10 μmol l^{-1} adrenaline were measured. The data ($N=5$) suggested that preincubation in adrenaline evoked a fall in basal cyclic AMP content that occurred after 22 min (control 0.04±0.008 pmol μg^{-1}, adrenaline-preincubated 0.026±0.005 pmol μg^{-1}, $P<0.05$) and clearly showed that preincubation essentially abolished (desensitisation >95 %) the response to a subsequent application of adrenaline. Moreover, the cells did not recover their sensitivity to adrenaline within the time scale of this experiment. We therefore undertook further experiments in which the preincubated cells were allowed to recover for up to 56 h. This work did not confirm the fall in basal cyclic AMP levels described above, but verified the almost total loss of sensitivity to adrenaline. Moreover, these experiments showed that full recovery did occur (Fig. 4A) and that the half time for this process was 6.3±0.9 h (Fig. 4B). The properties of adrenaline-preincubated cells were further investigated in experiments in which adrenaline-preincubated cells were allowed to recover for 10 min or 10 h. As expected, the cells were essentially unresponsive to adrenaline after 10 min of recovery (Fig. 5A). However, these desensitised cells consistently showed enhanced responses to ATP, although the response to forskolin was normal (Fig. 5A). Earlier work established that cultured equine sweat gland cells express type P$_{2U}$ purine receptors, which allow certain nucleotides, including uridine triphosphate (UTP), to inhibit adrenaline-evoked cyclic AMP accumulation (Wilson *et al.* 1996). To explore the possibility that this inhibitory pathway may be modified by adrenaline, we undertook experiments ($N=4$) in which we measured the extent to which UTP (1 mmol l^{-1}) could inhibit forskolin-evoked (1 μmol l^{-1}, 16 min) cyclic AMP accumulation in saline-preincubated and adrenaline-preincubated (10 min recovery) cells. In these experiments, the cyclic AMP content of
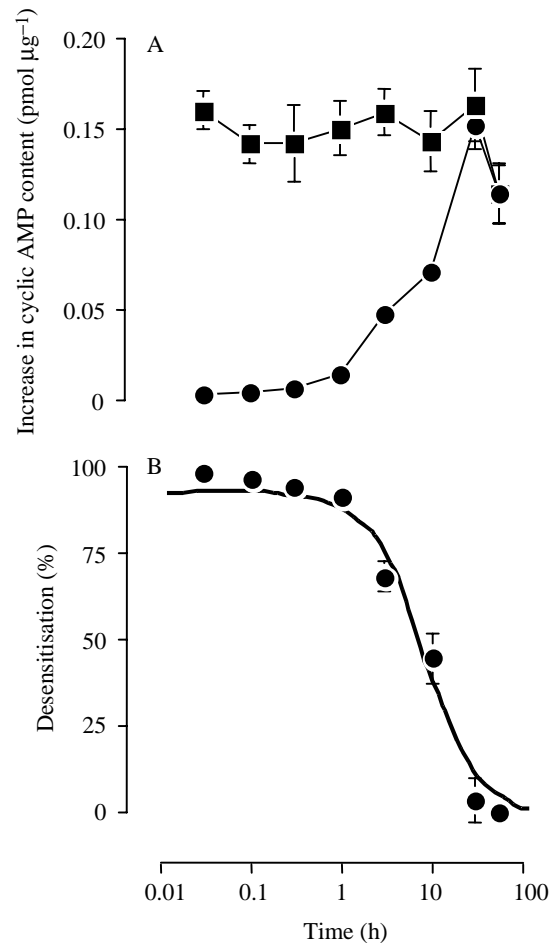

Fig. 4. The increases in cellular cyclic AMP content evoked by 10 μmol l^{-1} adrenaline (4 min incubation) were quantified in cells that had been preincubated (32 min, 37 °C) in physiological saline containing 10 μmol l^{-1} adrenaline, washed with control saline and then allowed to recover, under control conditions, for up to 56 h. The time course of recovery is shown in A, where data for preincubated cells are plotted together with the results of directly analogous experiments undertaken using cells that had been grown on the same plates but preincubated in control saline. Values are means ± S.E.M. Pooled data from 11 such paired experiments are shown (●, adrenaline-preincubated cells; ■, control cells). The basal cyclic AMP content of the control cells was initially 0.014±0.003 pmol μg^{-1} and that of the adrenaline-preincubated cells was initially 0.016±0.002 pmol μg^{-1}. These values did not change significantly throughout the experimental period. The effects of preincubation with adrenaline on the response to a subsequent adrenaline treatment are plotted against the recovery time in B.

unstimulated cells was 0.022±0.006 pmol μg^{-1} and this value was unaffected by preincubation in adrenaline (0.025±0.002 pmol μg^{-1}). As anticipated (see Fig. 5A), 1 μmol l^{-1} forskolin evoked essentially identical responses in saline-preincubated (0.179±0.036 pmol μg^{-1}) and adrenaline-preincubated (0.175±0.022 pmol μg^{-1}) cells. Moreover, preincubation in adrenaline did not affect the inhibitory action of UTP (control 31.1±10 %, preincubated 34.0±8.0 %, $N=5$).

Table 2. *Effects of IBMX upon adrenaline-evoked desensitisation*

	Control cells		IBMX-treated cells	
	Saline-preincubated	Adrenaline-preincubated	Saline-preincubated	Adrenaline-preincubated
10 min of recovery (N=5)				
Basal cyclic AMP content (pmol μg^{-1})	0.045±0.010	0.036±0.009	0.125±0.006†	0.142±0.033†
Response to adrenaline (pmol μg^{-1})	1.045±0.287	0.083±0.018*	1.390±0.298†	0.211±0.049**·††
Desensitisation (%)	–	90.4±1.8	–	85.6±3.0
10 h of recovery (N=5)				
Basal cyclic AMP content (pmol μg^{-1})	0.013±0.003	0.014±0.004	0.075±0.030†	0.083±0.027†
Response to adrenaline (pmol μg^{-1})	0.874±0.232	0.356±0.104*	1.177±0.289†	0.639±0.128*·††
Desensitisation (%)	–	60.7±8.0	–	40±12.2††

In each experiment, cells were preincubated (32 min, 37 °C) in physiological saline or 10 μmol l^{-1} adrenaline and allowed to recover for 10 min or 10 h before the cyclic AMP content of unstimulated cells and the rise in cyclic AMP content evoked by a second 4 min incubation (37 °C) in 10 μmol l^{-1} adrenaline were measured. These data are tabulated and were used to quantify the loss of sensitivity to adrenaline (% desensitisation).

For experiments involving 10 min of recovery, all measurements were made both for cells maintained under control conditions throughout the recovery period and for cells that had recovered in the presence of 5 mmol l^{-1} IBMX. In experiments involving 10 h of recovery, however, IBMX-treated cells were exposed to IBMX (5 mmol l^{-1}) for only the 10 min that immediately preceded the second exposure to adrenaline.

Experiments were undertaken using strictly paired protocols, and differences between mean values were evaluated using Student's paired *t*-test. Asterisks denote significant effects of preincubation in adrenaline (*$P<0.05$, **$P<0.02$), whilst daggers denote differences between control and IBMX-treated cells (†$P<0.05$, ††$P<0.02$).

Experiments in which adrenaline-preincubated cells were allowed to recover for 10 h showed that the cells had regained approximately 50 % of their sensitivity to adrenaline. However, in marked contrast to the data obtained after 10 min, these cells showed significantly reduced responses to ATP and forskolin (Fig. 5B).

Effects of IBMX upon adrenaline-evoked desensitisation

Adrenaline-preincubated cells were allowed to recover for 10 min before basal cyclic AMP levels and the response to a second, brief exposure (4 min) to 10 μmol l^{-1} adrenaline were quantified. Each such measurement was made both for cells that had been allowed to recover under control conditions and for cells that had been exposed to 5 mmol l^{-1} IBMX during the recovery period. The results of these experiments are presented in Table 2. The data confirm that IBMX raises basal cyclic AMP levels, both in saline-preincubated and in adrenaline-preincubated cells. Moreover, IBMX also potentiated the response to adrenaline in both groups of cells. However, the desensitisation seen in cells that had recovered under control conditions did not differ significantly from that seen in cells that had recovered in the presence of IBMX (Table 2). Table 2 also presents the results of experiments in which we explored
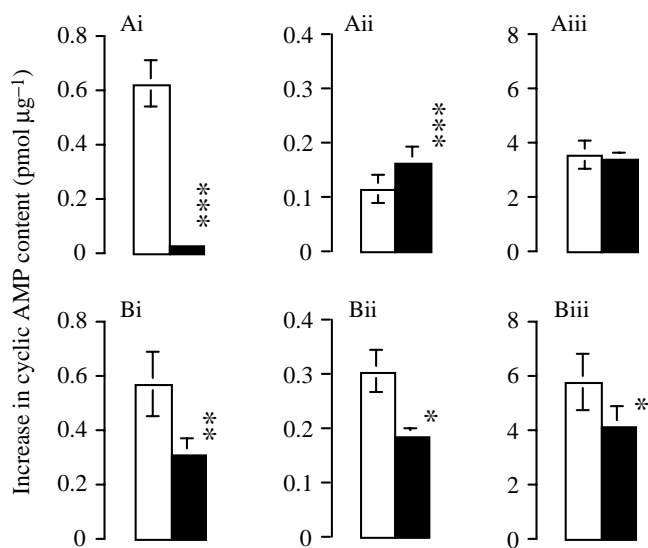

Fig. 5. Responses to 10 μmol l^{-1} adrenaline (i, 4 min incubation), 100 μmol l^{-1} ATP (ii, 16 min incubation) and 100 μmol l^{-1} forskolin (iii, 8 min incubation) were measured in cells that had been preincubated at 37 °C in physiological saline containing 10 μmol l^{-1} adrenaline for 32 min (filled columns) and in cells that had been grown on the same plates, cultured under identical conditions and preincubated in control saline (open columns). All cells were then washed with control saline and incubated under control conditions until the responses to the various test stimuli were quantified. The data presented in A were obtained from cells that had been allowed to recover for 10 min (N=5), whereas those in B were from cells that had been allowed to recover for 10 h (N=5). Basal cyclic AMP content was unaffected by preincubation in adrenaline both after 10 min (control 0.028±0.005 pmol μg^{-1}; pre-stimulated 0.021±0.008 pmol μg^{-1}) and after 10 h (control 0.030±0.009 pmol μg^{-1}; preincubated 0.035±0.016 pmol μg^{-1}) of recovery. Asterisks denote experiments in which the response of adrenaline-prestimulated cells differed significantly from that measured in control cells (*$P<0.05$, **$P<0.02$, ***$P<0.01$; Student's paired *t*-test). Values are means ± S.E.M.

the effects of IBMX upon the desensitisation that persisted after 10 h. In these experiments, the recovering cells were maintained in culture medium until 10 min before the end of the recovery period, when they were transferred either to control saline or to saline contining 5 mmol l^{-1} IBMX. Basal cyclic AMP levels and the response to adrenaline were, once again, elevated by IBMX (Table 2), but analysis of the data from these carefully paired experiments showed that the 10 min incubation in IBMX significantly attenuated the desensitisation that persisted after 10 h (Table 2).

Effects of preincubation in forskolin

To explore the role of cyclic AMP in the desensitisation process, cells were preincubated (32 min, 37 °C) in 100 μmol l^{-1} forskolin and allowed to recover for 10 min or 10 h before basal cyclic AMP levels and the responses to adrenaline (10 μmol l^{-1}, 4 min) and ATP (100 μmol l^{-1}, 16 min) were measured. After 10 min ($N=5$), basal cyclic AMP levels were elevated (control 0.013±0.002 pmol μg^{-1}; forskolin-stimulated 0.130±0.034 pmol μg^{-1}, $P<0.05$) and the responses to adrenaline (control 0.397±0.069 pmol μg^{-1}, preincubated 0.880±0.175 pmol μg^{-1}, $P<0.05$) and ATP (control 0.235±0.051 pmol μg^{-1}, preincubated 0.616±0.168 pmol μg^{-1}, $P<0.05$) were potentiated. After 10 h ($N=5$), however, basal cyclic AMP levels were normal (control 0.011±0.003 pmol μg^{-1}, forskolin-prestimulated 0.009±0.002 pmol μg^{-1}) and the responses to adrenaline (control 0.409±0.062 pmol μg^{-1}; forskolin-stimulated 0.312±0.052 pmol μg^{-1}, $P<0.01$) and ATP (control 0.242±0.049 pmol μg^{-1}; forskolin-stimulated 0.085±0.029 pmol μg^{-1}, $P<0.001$) were now reduced. Analysis of these data showed that preincubation in forskolin inhibited the response to ATP (67.8±6.3 %) more effectively ($P<0.001$) than the response to adrenaline (24.4±4.3 %).

Discussion

The response to adrenaline

The magnitude of the response to adrenaline was concentration-dependent and the EC$_{50}$ derived from the present data (approximately 35 nmol l^{-1}) was similar to the value obtained in an earlier study undertaken in the presence of a PDE inhibitor (approximately 45 nmol l^{-1}; Wilson et al. 1993). The cells responded to the selective β$_2$ adrenoceptor agonist salbutamol, although the maximal response was only approximately 35 % of the maximal response evoked by adrenaline, suggesting that salbutamol acts as a partial agonist. The limited sensitivity to salbutamol, which has been reported in other cell types (O'Donnell, 1972; Grove et al. 1995), thus supports our earlier suggestion (Wilson et al. 1993) that these cells express β$_2$ adrenoceptors. Further evidence for this came from the experiments using ICI-118,551 and atenolol. Both of these compounds could abolish the response to adrenaline, but ICI-118,551 was approximately 4000 times more potent. This compound preferentially blocks β$_2$ receptors whereas atenolol is primarily a β$_1$ antagonist, and so these data also indicate that

the cells express β$_2$ adrenoceptors. The present results, together with our earlier data (Wilson et al. 1993), therefore suggest that cultured equine sweat gland epithelial cells express the signal transduction pathway that allows adrenaline to control secretion in vivo (Robertshaw and Taylor, 1969; Snow, 1977; Johnson and Creed, 1982; Bijman and Quinton, 1984a,b).

The cells began to accumulate cyclic AMP as soon as they were exposed to adrenaline, but this initial rapid rise was followed by a decline that occurred despite the continued presence of agonist. The falling phase of this response was reduced, although not abolished, by the PDE-inhibitor IBMX. Studies of adipocytes (Pawlson et al. 1974) provided an early indication that cyclic-AMP-mobilising agonists may also activate PDE, and it is now clear that the type 3 isoform of PDE (PDE-3) is activated by cyclic-AMP-dependent protein kinases (PKA). Increased PDE activity therefore accompanies a rise in cyclic AMP content in any cell type that expresses PDE-3 (Beavo, 1995; Erdogan and Houslay, 1997). However, the present experiments showed that the responses to forskolin and ATP were far more sustained than the response to adrenaline. It is, therefore, perfectly clear that the cells are able to maintain elevated levels of cyclic AMP for long periods. Moreover, IBMX did not altogether abolish the falling phase of the response to adrenaline, indicating that cyclic-AMP-evoked activation of PDE cannot be the only mechanism that underlies the fall in cyclic AMP levels seen in adrenaline-stimulated cells.

Properties of adrenaline-preincubated cells

Cells that had been preincubated in adrenaline became essentially insensitive to this hormone, and experiments in which adrenaline-preincubated cells were allowed to recover for 10 min showed that, initially, this desensitisation was not accompanied by reduced sensitivity to other cyclic-AMP-mobilising agonists. Indeed, the cells became more sensitive to ATP. It is interesting, in this context, that the equine epithelial cells also express P$_{2U}$ purine receptors (Ko et al. 1994) that allow nucleotides to inhibit cyclic AMP accumulation (Sipma et al. 1994; Schulze-Lohoff et al. 1995; Wilson et al. 1996). As these P$_{2U}$ receptors are co-expressed with the receptors that allow ATP to increase cellular cyclic AMP content (Ko et al. 1994), the magnitude of the ATP-evoked rise in cyclic AMP content will reflect the balance between the stimulatory and inhibitory pathways. The augmented responses seen in the desensitised cells raised the possibility that the inhibitory pathway may become down-regulated in the adrenaline-preincubated cells, and this possibility was explored by measuring the extent to which UTP could inhibit the response to forskolin. UTP was used in these experiments as this nucleotide selectively activates the inhibitory pathway (Ko et al. 1994; Wilson et al. 1996). It was clear, however, that preincubation in adrenaline did not reduce the inhibitory effect of UTP, suggesting that an unknown mechanism enhances the effects of ATP upon the adrenaline-desensitised cells. The physiological basis of this novel effect is worthy of further study.

Effects of IBMX upon acutely desensitised cells

Experiments using adrenaline-preincubated cells that had been allowed to recover for 10 h showed that the persistent desensitisation was accompanied by reduced sensitivity to other cyclic-AMP-mobilising agonists. This suggested that the initial desensitisation and the persistent desensitisation had different physiological bases. Further evidence for this came from the experiments in which cells were preincubated in forskolin. This initially failed to reduce the responses to adrenaline and ATP; in fact, these responses were markedly potentiated. However, forskolin is a membrane-permeant agent that activates adenylate cyclase without activating cell surface receptors (Seamon and Daly, 1981), and so it may be difficult to wash the cells entirely free of this substance. The augmented responses could, therefore, reflect the continued presence of a small amount of forskolin, and the elevated basal cyclic AMP levels in forskolin-preincubated cells are consistent with this. The important point to emerge from this experiment was that forskolin failed to mimic the initial desensitisation seen in adrenaline-preincubated cells. After 10 h of recovery, however, forskolin-preincubated cells showed reduced responses to ATP and adrenaline. Increased levels of cyclic AMP thus appear to initiate a series of events leading, after 10 h, to reduced sensitivity to cyclic-AMP-mobilising agonists. Experiments using IBMX showed clearly that increased PDE activity did not contribute to the loss of sensitivity seen after 10 min of recovery, but suggested that these enzymes may contribute to the persistent desensitisation seen after 10 h.

Recent studies of Jurkat cells (Erdogan and Houslay, 1997) showed that forskolin initially evoked a small and transient rise in PDE activity caused by PKA-mediated activation of PDE-3. However, this small response was succeeded by a much larger increase that developed slowly and was due to increased expression of genes encoding the PDE-3 and PDE-4 isoforms. This became apparent after 2–5 h and reached a plateau after approximately 10 h (Erdogan and Houslay, 1997). Similar, although less well documented, effects have been reported for several other cell types (see Beavo, 1995; Giembycz, 1996), and these cyclic-AMP-dependent increases in PDE expression seem to be mediated by transcription factors that are activated by PKA (Beavo, 1995; Giembycz, 1996; Erdogan and Houslay, 1997). Moreover, agonist-evoked increases in PDE-4 expression can contribute to the desensitisation of the β_2 adrenoceptors in the immune and pro-inflammatory cells of the lung (see Giembycz, 1996).

Biological significance of the present findings

The present experiments showed that adrenaline-preincubated equine sweat gland epithelial cells became essentially insensitive to this hormone. Such desensitisation has been observed in many cell types and its mechanism extensively studied. It is now clear that an essentially ubiquitous enzyme, β-adrenoceptor kinase (β-ARK), selectively phosphorylates β-adrenoceptors that are occupied by agonist. Although these modified receptors can still bind adrenaline, the resultant hormone/receptor complex causes minimal activation of adenylate cyclase and so this phosphorylation process causes a progressive decline in adenylate cyclase activity during continuous stimulation. However, these modified receptors can be withdrawn from the plasma membrane by endocytosis, dephosphorylated and recycled to the cell surface by exocytosis. This allows desensitised cells to regain sensitivity once the desensitising stimulus is withdrawn. This recovery is usually complete within 20–40 min, and this capacity for rapid resensitisation is an important feature of β-ARK-mediated desensitisation (Su *et al.* 1980; Levitzki, 1988; Hausdorff *et al.* 1990; Yu *et al.* 1993). In the present study, however, several hours were required before the desensitised equine cells responded normally to adrenaline. These cells thus displayed unusually persistent desensitisation which was in excellent accord with the early observation made *in vivo* (Langley and Bennett, 1923). Moreover, the persistent desensitisation was, at least in part, sensitive to IBMX, suggesting a role for PDE, and this raises the possibility that cyclic-AMP-evoked increases in PDE expression (see Beavo, 1995; Giembycz, 1996; Erdogan and Houslay, 1997) may contribute to the persistent desensitisation seen in the equine sweat gland epithelium (Langley and Bennett, 1923; Evans and Smith, 1956; Evans *et al.* 1957).

References

BEAVO, J. A. (1995). Cyclic nucleotide phosphodiesterases: functional implications of multiple isoforms. *Physiol. Rev.* **75**, 725–748.

BIJMAN, J. AND QUINTON, P. M. (1984*a*). Predominantly β-adrenergic control of equine sweating. *Am. J. Physiol.* **246**, R349–R353.

BIJMAN, J. AND QUINTON, P. M. (1984*b*). Influence of calcium and cyclic nucleotides on β-adrenergic sweat secretion in equine sweat glands. *Am. J. Physiol.* **247**, C10–C13.

BONNER, J. (1994). They overheat horses, don't they? *New Scient.* **26 March**, 21–23.

CARLSON, G. P. (1994). Guest Editorial. Atlanta 1996 – heat, humidity and horses. *Br. vet. J.* **150**, 211–213.

ERDOGAN, S. AND HOUSLAY, M. D. (1997). Challenge of human Jurkat T-cells with the adenylate cyclase activator forskolin elicits major changes in cyclic AMP phoshodiesterase (PDE) expression by up-regulating PDE3 and inducing PDE4D1 and PDE4D2 splice variants as well as down regulating a novel PDE4A splice variant. *Biochem. J.* **321**, 165–175.

EVANS, C. L. AND SMITH, D. F. G. (1956). Sweating responses in the horse. *Proc. R. Soc. B* **145**, 61–83.

EVANS, C. L., SMITH, D. F. G., ROSS, K. A. AND WEIL-MALHERBE, H. (1957). Physiological factors in the condition of dry coat in horses. *Vet. Rec.* **69**, 1–9.

GIEMBYCZ, M. A. (1996). Phosphodiesterase 4 and tolerance to β_2-adrenoceptor agonists in asthma. *Trend. pharmac. Sci.* **17**, 331–336.

Grove, A., McFarlane, L. C. and Lipworth, B. J. (1995). Expression of the β2 adrenoceptor agonist/partial agonist activity of salbutamol in states of low and high adrenergic tone. *Thorax* **50**, 134–138.

Hausdorff, W. P., Caron, M. G. and Lefkowitz, R. J. (1990). Turning off the signal: desensitization of β-adrenergic receptor function. *FASEB J.* **4**, 2881–2889.

Hodgson, D. R., Davis, R. E. and McConaghy, F. F. (1994). Thermoregulation in the horse in response to exercise. *Br. vet. J.* **150**, 219–235.

Johnson, K. G. and Creed, K. E. (1982). Sweating in the intact horse and in isolated perfused horse skin. *Comp. Biochem. Physiol.* **73**C, 259–264.

Ko, W. H., O'Dowd, J. J. M., Pediani, J. D., Elder, H. Y., Bovell, D. L., Jenkinson, D. M. and Wilson, S. M. (1994). Extracellular ATP can activate autonomic signal transduction pathways in cultured equine sweat gland epithelial cells. *J. exp. Biol.* **190**, 239–252.

Langley, J. N. and Bennett, S. (1923). Action of pilocarpine, arecoline and adrenaline on sweating in the horse. *J. Physiol., Lond.* **57**, Plxxi–Plxxii.

Levitzki, A. (1988). From epinephrine to cyclic AMP. *Science* **241**, 800–806.

Pawlson, L. G., Lovell-Smith, C. J., Manganiello, V. C. and Vaughan, M. (1974). Effects of epinephrine adrenocorticotrophic hormone, and theophylline on adenosine-3′,5′-monophosphate phosphodiesterase activity in fat cells. *Proc. natn Acad. Sci. U.S.A.* **71**, 1639–1642.

O'Donnell, S. R. (1972). An examination of some β-adrenoceptor stimulants for selectivity using the isolated trachea and atria of the guinea pig. *Eur. J. Pharmac.* **19**, 371–379.

Robertshaw, D. and Taylor, C. R. (1969). Sweat gland function in the donkey (*Equus asinus*). *J. Physiol., Lond.* **205**, 79–89.

Schulze-Lohoff, E., Bitzer, M., Ogilvie, A. and Sterzel, R. B. (1995). P2U-purinergic receptor activation mediates inhibition of cyclic AMP accumulation in cultured renal mesangial cells. *Renal Physiol. Biochem.* **18**, 219–230.

Seamon, K. B. and Daly, J. W. (1981). Forskolin a unique diterpene activator of cyclic AMP generating systems. *J. cyclic Nucleotide Res.* **7**, 201–225.

Sipma, H., Den Hertog, A. and Nelemans, A. (1994). The phospholipase C activating P2U purinoceptor also inhibits cyclic AMP formation in DDT$_1$ MF-2 smooth muscle cells. *Eur. J. Pharmac.* **268**, 431–437.

Snow, D. H. (1977). Identification of the receptor involved in adrenaline mediated sweating in the horse. *Res. vet. Sci.* **23**, 246–247.

Su, Y. F., Harden, T. K. and Perkins, J. P. (1980). Catecholamine-specific desensitization of adenylate cyclase. Evidence for a multistep process. *J. biol. Chem.* **255**, 7410–7419.

Wilson, S. M., Pediani, J. D., Ko, W. H., Bovell, D. L., Kitson, S., Montgomery, I., Brown, U. M. O., Smith, G. L., Elder, H. Y. and Jenkinson, D. M. (1993). Investigation of stimulus–secretion coupling in equine sweat gland epithelia using cell culture techniques. *J. exp. Biol.* **183**, 179–199.

Wilson, S. M., Rakhit, S., Murdoch, R., Pediani, J. D., Elder, H. Y., Baines, D. L., Ko, W. H. and Wong, P. Y. D. (1996). Activation of apical P2U receptors permits inhibition of adrenaline-evoked cyclic AMP accumulation in cultured equine sweat gland epithelial cells. *J. exp. Biol.* **199**, 2153–2160.

Yu, S. S., Lefkowitz, R. J. and Hausdorff, W. P. (1993). β-Adrenergic receptor sequestration. A potential mechanism for receptor resensitization. *J. biol. Chem.* **268**, 337–341.