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Rapid non-genomic inhibition of ATP-induced Cl^- secretion by dexamethasone in human bronchial epithelium

V. Urbach, D. E. Walsh*, B. Mainprice, J. Bousquet and B. J. Harvey†

*INSERM U454, CHU A. de Villeneuve, 34295 Montpellier Cedex 05, France, *Wellcome Trust Cellular Physiology Research Unit, University College Cork, Cork and †Molecular Medicine, Royal College of Surgeons in Ireland, Dublin 2, Ireland*

A non-genomic antisecretory role for dexamethasone at low concentrations (0.1 nM to1 μM) is described in monolayers of human bronchial epithelial cells in primary culture and in a continuous cell line (16HBE14o- cells). Dexamethasone produced a rapid decrease of $[Ca^{2+}]_i$ (measured with fura-2 spectrofluorescence) to a new steady-state concentration. After 15 min exposure to dexamethasone (1 nM), $[Ca^{2+}]_i$ was reduced by 32 ± 11 nM ($n = 7$, $P < 0.0001$) from a basal value of 213 ± 36 nM ($n = 7$). We have shown previously that aldosterone (1 nM) also produces a rapid fall in $[Ca^{2+}]_i$; however, after the decrease in $[Ca^{2+}]_i$ induced by dexamethasone, subsequent addition of aldosterone did not produced any further lowering of $[Ca^{2+}]_i$. The rapid response to dexamethasone was insensitive to pretreatment with cycloheximide and unaffected by the glucocorticoid type II and mineralocorticoid receptor antagonists RU486 and spironolactone, respectively. The rapid $[Ca^{2+}]_i$ decrease induced by dexamethasone was inhibited by the Ca^{2+}-ATPase pump inhibitor thapsigargin (1 μM), the adenylate cyclase inhibitor MDL hydrochloride (500 μM) and the protein kinase A inhibitor Rp-adenosine 3′,5′-cyclic monophosphorothioate (200 μM), but was not affected by the protein kinase C inhibitor, chelerythrine chloride (0.1 μM). Treatment of 16HBE14o- cell monolayers with dexamethasone (1 nM) inhibited the large and transient $[Ca^{2+}]_i$ increase induced by apical exposure to ATP (10^{-4} M). Dexamethasone (1 nM) also reduced by 30 % the Ca^{2+}-dependant Cl^- secretion induced by apical exposure to ATP (measured as the Cl^--sensitive short-circuit current across monolayers mounted in Ussing chambers). Our results demonstrate, for the first time, that dexamethasone at low concentrations inhibits Cl^- secretion in human bronchial epithelial cells. The rapid inhibition of Cl^- secretion induced by the synthetic glucocorticoid is associated with a rapid decrease in $[Ca^{2+}]_i$ via a non-genomic mechanism that does not involve the classical glucocorticoid or mineralocorticoid receptor. Rather, it is a result of rapid non-genomic stimulation of thapsigargin-sensitive Ca^{2+}-ATPase, via adenylate cyclase and protein kinase A signalling.

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Corresponding author V. Urbach: INSERM U454, CHU A. de Villeneuve, 34295 Montpellier Cedex 05, France.
Email: urbach@montp.inserm.fr

In the present study, we investigated the rapid effect of dexamethasone on intracellular Ca^{2+} signalling and on the Cl^- secretion induced by apical ATP in cultured human bronchial epithelial cell monolayers. The glucocorticoids are an important class of drug for their anti-inflammatory and immunosupressive activity and are used clinically at various doses. Low doses are used for basal immuno-suppressive treatment, including the treatment of airway tract infections such as asthma and cystic fibrosis. High doses are used for the treatment of acute conditions such as inflammatory exacerbation in multiple sclerosis (Filipovic *et al.* 1997), acute spinal cord injury (Bracken *et al.* 1990) or severe attacks of asthma (Bousquet, 2000). It has been shown that glucocorticoids produce their pharmacological effects through a classical genomic pathway involving binding to a specific cytosolic receptor, translocation into the nucleus and subsequent activation or repression of protein synthesis. The genomic effects of steroids are characterised by a latency of onset lasting several hours. In contrast, many steroid hormones have been reported to induce rapid effects on various second messenger systems and ion transporters. These fast responses are incompatible with the involvement of the classical genomic pathway for steroid hormone action. The rapid effects of glucocorticoids have been described in several tissues such as endometrial cells (Koukouritaki *et al.* 1996), vascular smooth muscle (Steiner *et al.* 1988; Muto *et al.* 2000) and renal cortical collecting duct (Harvey & Higgins, 2000). However, the cellular mechanism and the physiological role of the non-genomic effects of glucocorticoids are not understood and have not been reported in human bronchial epithelia.

Modulation of $[Ca^{2+}]_i$ mediates different physiological responses to glucocorticoids (Lewis *et al.* 1986; Han *et al.* 1999), and opposing effects of glucocorticoids on $[Ca^{2+}]_i$ have been reported. An increase in $[Ca^{2+}]_i$ is induced by glucocorticoids in cortical collecting duct (Harvey & Higgins, 2000) and in vascular smooth muscle (Steiner *et al.* 1988). In contrast, a decrease of $[Ca^{2+}]_i$ following glucocorticoid treatment has been reported in other tissues such as rat thymocyte (Buttgereit *et al.* 1997), human leukocyte, airway smooth muscle (Chhabra *et al.* 1999) and in human lymphoblast (Gardner & Zhang, 1999).

Using Ca^{2+} imaging and short-circuit current (I_{SC}) experiments, we have investigated the effects of low concentrations (1 nM to 1 μM) of dexamethasone on $[Ca^{2+}]_i$ and the Ca^{2+}-dependent Cl^- secretion induced by apical ATP in human bronchial epithelial cells. Airway epithelial cells have been shown to respond to extracellular nucleotides including ATP by activation of a Cl^- secretory pathway (Willumsen & Boucher, 1989; Stutts *et al.* 1992) and inhibition of Na^+ absorption (Mall *et al.* 2000). More specifically, the stimulation of Cl^- secretion through airway epithelia in response to apical nucleotides has been described as being mediated by purinoreceptor activation and a rapid transient $[Ca^{2+}]_i$ increase (Mason *et al.* 1991; Walsh *et al.* 2000).

The findings reported here of rapid antisecretory responses to a glucocorticoid in human airway epithelial cells might have interesting clinical implications regarding the immediate consequences of corticotherapy. In addition, cystic fibrosis and asthma are two pathologies that are correlated with a dysfunction of the secretory properties of the airway epithelium that controls the fluid and electrolyte composition of the lumen. Cystic fibrosis is associated with a deficit of Cl^- secretion arising from the mutation of the gene coding for the cystic fibrosis conductance regulator (CFTR) protein, which functions as a Cl^- channel (Clarke *et al.* 1992). In asthma, it has been reported recently that mucus overproduction is associated with an increased expression of Ca^{2+}-activated Cl^- channels (Hoshino *et al.* 2002). The rapid antisecretory role of glucocorticoid is a newly discovered property of glucocorticoid activity in human bronchial epithelium, which may be distinct from its anti-inflammatory role, but is relevant to the treatment of airway infections.

METHODS

Cell culture

The human bronchial epithelial 16HBE14o- cell line is derived from the surface epithelium of mainstream, second-generation bronchi (Cozens *et al.* 1994) and form polarised monolayers with intact tight junctions, retain the Cl^- transport properties typical of freshly isolated surface airway epithelial cells and express other differentiated features characteristic of the native epithelium (Cozens *et al.* 1994). Cells were grown on a vitrogen/collagen/fibronectin coating, in Eagle's Minimal Essential Medium (EMEM, BioWhittaker) supplemented with 10 % fetal calf serum, 1 % penicillin G, 1 % streptomycin and 1 % L-glutamine, and incubated in a 37 °C, 5 % CO_2 atmosphere.

For cultures of native bronchus epithelium, primary cultures were grown from human bronchial epithelial cells obtained from bronchial tube surgery biopsy samples. The bronchial samples were taken from a normal area of bronchi removed from patients suffering from lung cancer but who had normal lung function, with their consent (approved by local medical ethics committee, Comité Consultatif de Protection des Personnes se prêtant à des Recherches Biomédicales (CCPPRB), Montpellier). After excision, the bronchial tubes were washed and incubated either overnight at 4 °C or for 2 h at 37 °C with 0.38 mg ml^{-1} hyaluronidase, 0.75 mg ml^{-1} collagenase, 1 mg ml^{-1} protease and 0.3 mg ml^{-1} DNAse in RPMI 1640 medium (Gibco, Grand Island, NY, USA) and then filtered through a 70 mm mesh nylon strainer. Pieces of epithelium retained on the strainer were washed and resuspended in phosphate-buffered saline (PBS). After centrifugation (1200 *g*, 5 min), epithelial cells were resuspended in small airway epithelium basal medium (Clonetics, BioWhittaker, San Diego, CA, USA) supplemented with 0.5 μg ml^{-1} human recombinant epidermal growth factors, 7.5 mg ml^{-1} bovine pituitary extract, 0.5 mg ml^{-1} adrenaline, 10 mg ml^{-1} transferrin, 5 mg ml^{-1} insulin, 0.1 μg ml^{-1} retinoic acid, 6.5 μg ml^{-1} triiodothyronine, 50 mg ml^{-1} gentamicin, 50 μg ml^{-1} amphotericin B and 50 mg ml^{-1} fatty-acid-free bovine serum albumin. The epithelial cell suspension was then transferred to 12 mm diameter permeable filters (Costar Snapwell-Clear 3801, 0.4 mm pore size) or onto a glass coverslip and incubated at 37 °C in a 5 % CO_2 humidified atmosphere.

Ca^{2+} imaging

$[Ca^{2+}]_i$ was determined in confluent monolayers of 16HBE14o- cells or primary cultures of human bronchial epithelial cells grown on fibronectin/collagen/BSA-coated glass coverslips, as described previously (Urbach & Harvey, 2001). The cells were loaded with 5 μM of the Ca^{2+}-sensitive fluorescent probe fura-2 acetoxymethyl ester (fura-2/AM) for 30 min, in the dark at room temperature (22 °C). Cells were then washed twice in Hepes-buffered Krebs-Heinseleit solution (NaCl 140 mM, KCl 5 mM, $CaCl_2$ 2 mM, $MgCl_2$ 1 mM, Hepes 10 mM, Tris-HCl 10 mM, glucose 10 mM, pH 7.4, 280–290 mosmol l^{-1}). The coverslip covered with a confluent monolayer of bronchial cells was mounted on the stage of an inverted microscope equipped for epifluorescence (Diaphot 200, Nikon, The Netherlands). The light from a xenon lamp (Osram, Germany) was filtered through alternating 340 and 380 nm filters (Nikon), which were mounted on a motorised chopper under computer control (Starwise Fluo system, Imstar, France). The emission fluorescence produced after fura-2/AM excitation was filtered at 510 nm. The transmitted light image was detected using an intensified CCD video camera (Darkstar, Photonics Sciences, UK) that was coupled to the microscope. The fluorescence obtained at each excitation wavelength (F_{340} and F_{380}) depends upon the level of Ca^{2+} binding to fura-2/AM, according to an *in vivo* calibration performed using a range of EGTA-buffered Ca^{2+} solutions of the fura-2 free acid. $[Ca^{2+}]_i$ was calculated automatically by a computer program (Starwise, Imstar) using the Grynkiewicz equation (Grynkiewicz *et al.* 1985).

Cells were exposed to various dexamethasone concentrations made up from a 10^{-2} M stock solution of hormone dissolved in methanol. We verified that 0.01 % methanol, corresponding to the maximum amount of solvent at which the cells were exposed,

had no effect on $[Ca^{2+}]_i$ (Urbach & Harvey, 2001). We also verified that the resting $[Ca^{2+}]_i$ levels of 16HBE14o- cell monolayers were not significantly altered 20 min after the beginning of the recording in non-treated cells (Urbach & Harvey, 2001).

I_{SC} measurement

16HBE14o- cells or human bronchial cells in primary culture were grown on permeable filters (Costar Snapwell-Clear 3801, 12 mm diameter, 0.4 mm pore size). Monolayers of 1.2 cm^2 exposed surface area were mounted in temperature-controlled horizontal Ussing chambers and bathed in Hepes-buffered Krebs-Heinseleit solution for between 0 and 3 days after the electrical resistance had reached a maximum value (800–1200 Ω). The I_{SC} experiments were usually performed at 37 °C. However, since the Ca^{2+}-imaging experiments were carried out at room temperature, we verified that at room temperature, ATP produced a similar I_{SC} response. The spontaneous transmembrane potential was measured using a voltage-clamp model amplifier (EVC 4000, World Precision Instruments) and clamped to 0 mV by application of a I_{SC}. The I_{SC} was sampled digitally into the computer at 10 s intervals. Under these conditions, the I_{SC} is a measure of electrogenic transepithelial ion transfer. The output from the amplifier was digitised using a PowerLab system (Chart for Windows v4.0, ADInstruments).

Chemicals

Most of the chemicals were provided by Sigma: dexamethasone, budesonide, triamcilone, hydrocortisone (10^{-2} M stock solution in methanol), RU486 (10^{-2} M stock solution in ethanol), cycloheximide (10^{-1} M in ethanol), spironolactone (10^{-1} M in chloroform), adenosine 3′, 5′–cyclic monophosphorothioate, Rp-isomer, triethylammonium salt (Rp-cAMP, 5×10^{-3} M stock in water), chelerythrine chloride (10^{-2} M stock in water), thapsigargin (10^{-2} M stock in DMSO). BAPTA-AM (10^{-2} M stock in DMSO) and MDL-12, 330A hydrochloride (MDL; 5×10^{-3} M stock in water) were provided by Calbiochem. Fura-2/AM (5×10^{-3} M stock in DMSO) and 4-bromo-A23187 (10^{-3} M stock solution in ethanol) were provided by Molecular Probes.

Data analysis

The mean $[Ca^{2+}]_i$ and I_{SC} variations given in the results section correspond to the $[Ca^{2+}]_i$ or I_{SC} variations between the mean $[Ca^{2+}]_i$ or I_{SC} measured during the 2 min prior to treatment with dexamethasone and the $[Ca^{2+}]_i$ or I_{SC} measured between 10 and 15 min after hormone treatment. In each experiment, the mean $[Ca^{2+}]_i$ was obtained from all cells in the microscope field, irrespective of their ability to respond. Data are shown as the means ± S.E.M. of n experiments. Measures of statistical significance were obtained using either Student's t tests for paired data, or one-way analysis of variance (ANOVA) for multiple testing. A P value of < 0.05 was deemed to be significant. All statistical operations were performed using Excel software (Microsoft, USA).

RESULTS

Rapid effect of dexamethasone on $[Ca^{2+}]_i$

Exposure of human bronchial epithelial cell monolayers to dexamethasone produced a rapid and significant decrease in $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ levels decreased within 30 s of exposure to dexamethasone (1 nM), and reached a new lower plateau value within 5 min (Fig. 1). Following 10 min exposure of the 16HBE14o- cell monolayers to dexamethasone (1 nM), $[Ca^{2+}]_i$ was reduced by 32 ± 11 nM ($n = 7$, $P < 0.0001$) from a basal value of 213 ± 36 nM ($n = 7$; Fig. 1*A*). In primary cultures of human bronchial epithelial cells, dexamethasone (1 nM) also produced a $[Ca^{2+}]_i$ decrease of 30 ± 12 nM ($n = 3$, $P < 0.001$; Fig. 1*B*). The lower $[Ca^{2+}]_i$ was sustained for at least 90 min post-dexamethasone exposure. As illustrated by the effect of three different concentrations of dexamethasone (10^{-9}, 10^{-7} and 10^{-6} M), the magnitude of the $[Ca^{2+}]_i$ decrease in 16HBE14o- cells, was dependent on the hormone concentration (Fig. 2). The effects of other glucocorticoids (budesonide, triamcilone acetonide and hydrocortisone) were also tested at different concentrations. These three glucocorticoids had a reduced potency in the stimulation of the rapid $[Ca^{2+}]_i$ decrease compared to dexamethasone. Budesonide did not produce any significant $[Ca^{2+}]_i$ change and triamcilone acetonide stimulated a $[Ca^{2+}]_i$ decrease only at the highest concentration tested (1 μM). As shown in Fig. 2, and as reported previously (Urbach & Harvey, 2001), aldosterone also produced a $[Ca^{2+}]_i$ decrease. However, after the $[Ca^{2+}]_i$ decrease induced by dexamethasone, aldosterone did not produce any further change in $[Ca^{2+}]_i$ (Fig. 1*C*).

Effect of RU486, spironolactone and cycloheximide

Since both dexamethasone and aldosterone stimulated a similar rapid $[Ca^{2+}]_i$ response in human bronchial epithelium, we also tested the eventual involvement of glucocorticoid and mineralocorticoid receptors in the rapid response to dexamethasone on intracellular $[Ca^{2+}]_i$, using two antagonists of these receptors, RU486 and

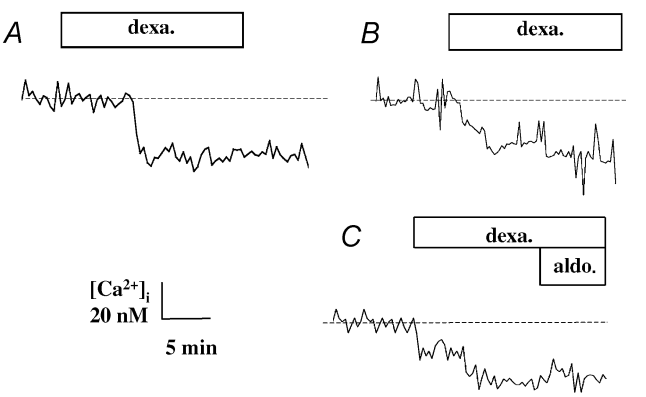

Figure 1. Dexamethasone effect on $[Ca^{2+}]_i$

A, typical $[Ca^{2+}]_i$ change upon apical exposure of monolayers of 16HBE14o- cells to dexamethasone (1 nM). *B*, typical $[Ca^{2+}]_i$ change upon exposure of primary culture of human bronchial epithelial cells to dexamethasone (dexa.; 1 nM). *C*, typical $[Ca^{2+}]_i$ response to apical exposure of monolayers of 16HBE14o- cells to dexamethasone (1 nM) followed by aldosterone (aldo; 1 nM).

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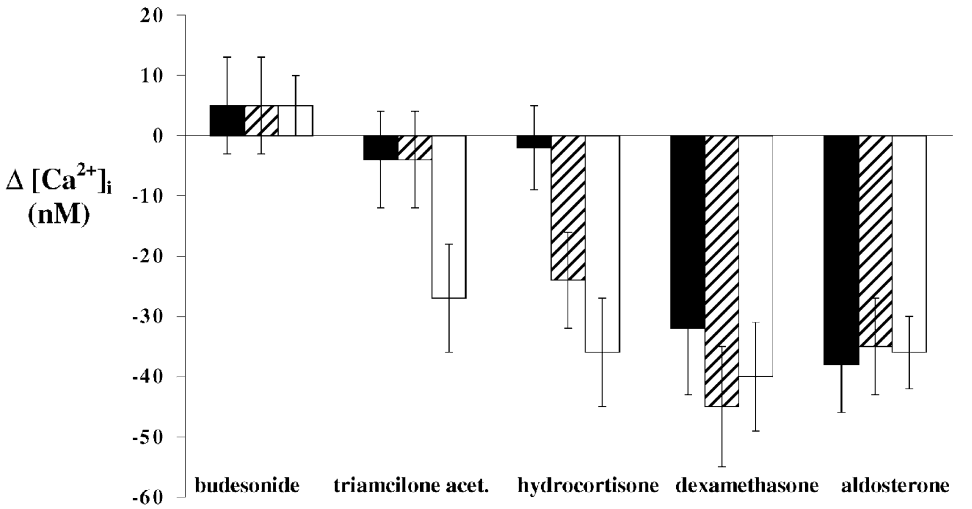

Figure 2. Effect of different concentrations of a variety of glucocorticoids on $[Ca^{2+}]_i$

Mean $[Ca^{2+}]_i$ variations in response to three different concentrations (10^{-6} M, □; 10^{-7} M, ▨; and 10^{-9} M, ■) of budesonide, triamcilone acetonide, hydrocortisone, dexamethasone and aldosterone in 16HBE14o- cell monolayers.

spironolactone, respectively (Fig. 3). The glucocorticoid receptor antagonist RU486 (1 μM) did not affect either the basal intracellular $[Ca^{2+}]_i$ or the $[Ca^{2+}]_i$ decrease induced by dexamethasone in 16HBE14o- cell monolayers (Fig. 3*A*). In the presence of RU486, dexamethasone (1 nM) still produced a $[Ca^{2+}]_i$ decrease by 29 ± 8 nM ($n = 6$, $P < 0.0001$). As shown in Fig. 3*B*, spironolactone (10 μM) alone produced a transient $[Ca^{2+}]_i$ increase by 22 ± 13 nM ($n = 6$, $P < 0.0001$) but did not affect the $[Ca^{2+}]_i$ decrease induced by dexamethasone stimulation ($\Delta[Ca^{2+}]_i = -25 \pm 13$ nM, $n = 6$, $P < 0.0001$).

The rapidity of the $[Ca^{2+}]_i$ decrease induced by dexamethasone and the insensitivity of the response to inhibitors of the classical gluco- or mineralocorticoid receptors suggests strongly that the response does not involve a transcriptional pathway. In order to verify this hypothesis, we tested the effect of cycloheximide, a protein synthesis inhibitor. As shown in Fig. 3*C*, cycloheximide (1 μM) did not affect the basal $[Ca^{2+}]_i$ or the $[Ca^{2+}]_i$ response to dexamethasone (1 nM) ($\Delta[Ca^{2+}]_i = -27 \pm 11$ nM, $n = 6$, $P < 0.0001$).

Intracellular signalling pathway involved in the rapid response

In order to identify the intracellular signalling mechanism involved in the rapid Ca^{2+} response to dexamethasone we tested the effect of several inhibitors of signal transduction. MDL (500 μM), an inhibitor of adenylate cyclase, did not affect the basal $[Ca^{2+}]_i$ but completely abolished the $[Ca^{2+}]_i$ response usually observed with dexamethasone (1 nM; $\Delta[Ca^{2+}]_i = 0 \pm 2$ nM, $n = 6$, $P < 0.0001$), suggesting a role for adenylate cyclase activity in the response to dexamethasone (Fig. 4*A*). In order to test the role of cAMP-dependant protein kinase (PKA), we used Rp-cAMP (200 μM) to inhibit this pathway. Rp-cAMP did not affect the basal $[Ca^{2+}]_i$, but completely abolished the response to dexamethasone ($\Delta[Ca^{2+}]_i = 0 \pm 3$ nM, $n = 6$, $P < 0.0001$; Fig. 4*B*). In addition, we used the permeant form of cAMP, 8-bromo cAMP (5 μM) to stimulate PKA activity. After the initial stimulation of the PKA activity using 8-bromo cAMP, dexamethasone did not produce a further Ca^{2+} response ($\Delta[Ca^{2+}]_i = 0 \pm 2$ nM, $n = 3$, $P < 0.05$). Since dexamethasone did not cause an additive Ca^{2+} response under these conditions, it is proposed that

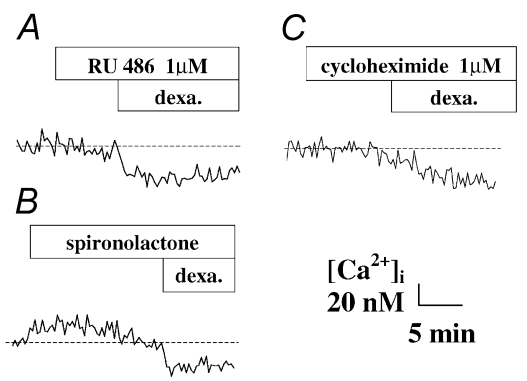

Figure 3. Effect of mineralocorticoid and glucocorticoid receptor antagonists and a protein synthesis inhibitor on the dexamethasone-induced decrease in $[Ca^{2+}]_i$

A and *B*, effect of the glucocorticoid receptor antagonist RU486 (*A*) and the mineralocorticoid receptor antagonist spironolactone (*B*) on the $[Ca^{2+}]_i$ decrease induced by dexamethasone (1 nM) in 16HBE14o- cell monolayers. *C*, effect of cycloheximide, an inhibitor of protein synthesis, on the $[Ca^{2+}]_i$ decrease induced by dexamethasone (1 nM) in 16HBE14o- cell monolayers.

the dexamethasone response occurs via cAMP. Taken together, these two results indicate a role for PKA activity in the response to dexamethasone.

Protein kinase C (PKC) does not appear to be involved in the signal transduction of the dexamethasone effect. The PKC inhibitor chelerythrine chloride (0.1 μM) did not affect the dexamethasone response ($\Delta[Ca^{2+}]_i = -23 \pm 9$ nM, $n = 6$, $P < 0.0001$), which was not significantly different from the dexamethasone-induced $[Ca^{2+}]_i$ decrease observed under control conditions ($P > 0.1$).

These latter results suggest that the rapid $[Ca^{2+}]_i$ response to dexamethasone may involve the same intracellular signalling pathway as for the $[Ca^{2+}]_i$ decrease induced by aldosterone (1 nM) in airway epithelial cells (Urbach & Harvey, 2001). Given these similarities, we tested the effects of thapsigargin, an intracellular Ca^{2+}-ATPase inhibitor, on the rapid response to dexamethasone. Thapsigargin (1 μM, in normal Ringer) produced a $[Ca^{2+}]_i$ increase of 95 ± 20 nM ($n = 6$) followed by a slow return to a plateau value that was higher than basal levels. After thapsigargin treatment, dexamethasone (1 nM) did not produce any subsequent change in $[Ca^{2+}]_i$, suggesting that the rapid effect of dexamethasone to lower cytosolic Ca^{2+} involves the stimulation of thapsigargin-sensitive Ca^{2+} pumps (Fig. 4*C*). This conclusion is strengthened by the finding that pretreatment (5 min) of the cells with dexamethasone (1 nM) enhanced the effect of thapsigargin to increase $[Ca^{2+}]$. Thapsigargin produced a $[Ca^{2+}]_i$ increase of 132 ± 20 nM ($n = 6$) after dexamethasone exposure, which is significantly higher compared to the thapsigargin response obtained without dexamethasone (97 ± 21 nM, $n = 6$, $P < 0.05$). This result is compatible with an increased Ca^{2+} store following dexamethasone and is not compatible with an interpretation that dexamethasone reduces leakage of Ca^{2+} from stores.

We further verified that the latter results could not be explained by the inhibitory effect of dexamethasone on leakage of Ca^{2+} into the cell by testing the effect of dexamethasone after treatment by the Ca^{2+} ionophore 4-bromo-A23187 (5 μM). The 16HBE14o- monolayers treated with 4-bromo-A23187 still exhibited a Ca^{2+} decrease by 20 ± 11 nM ($n = 3$). Taken together, these results suggest that dexamethasone activates Ca^{2+} uptake into stores and does not affect the leakage pathways.

Apical ATP effect on $[Ca^{2+}]_i$

Exposure of 16HBE14o- monolayers to apical ATP produced an immediate increase in $[Ca^{2+}]_i$ followed by a rapid decline towards a lower plateau value (Fig. 5). Upon apical addition of ATP (10^{-4} M), $[Ca^{2+}]_i$ increased by 377 ± 66 nM ($n = 7$) from a basal value of 112 ± 2 nM, with a return to a lower sustained value of 184 ± 8 nM ($n = 7$) within 5 min after ATP addition. As demonstrated in Fig. 5*B*, the $[Ca^{2+}]_i$ response to apical ATP was dependent on the external ATP concentration, with a maximum response obtained at 10^{-4} M ATP. Removal of external Ca^{2+}

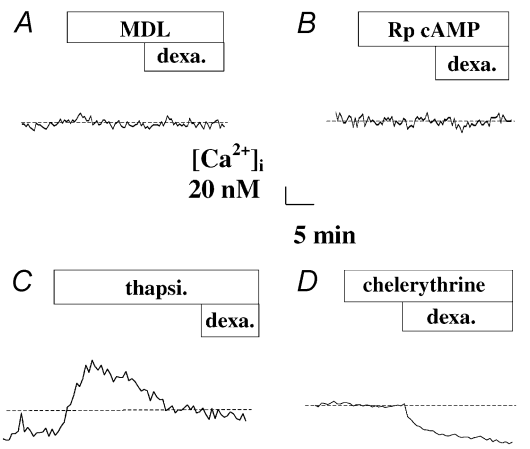

Figure 4. Effect of inhibitors of adenylate cyclase, protein kinase A and protein kinase C on the $[Ca^{2+}]_i$ decrease induced by dexamethasone in 16HBE14o- cell monolayers

Effect of (*A*) 500 μM MDL 12, 230 hydrochloride, an inhibitor of adenylate cyclase, (*B*) 200 μM Rp-adenosine 3′,5′-cyclic monophosphorothioate triethylammonium salt (Rp-cAMP), an inhibitor of protein kinase A, (*C*) 1 μM thapsigargin (thapsi), a Ca^{2+}-ATPase pump inhibitor, and (*D*) 0.1 μM chelerythrine chloride, an inhibitor of protein kinase C, on the $[Ca^{2+}]_i$ decrease induced by dexamethasone (1 nM) in 16HBE14o- cell monolayers.

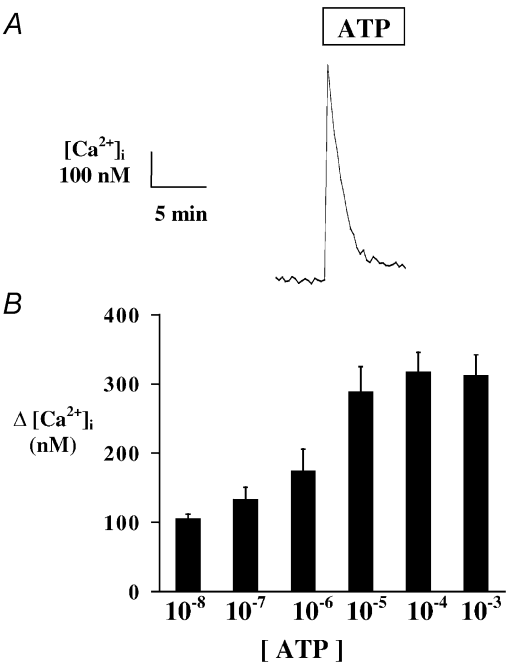

Figure 5. External ATP effect on $[Ca^{2+}]_i$

A, typical changes in $[Ca^{2+}]_i$ upon apical exposure of 16HBE14o- monolayers to ATP (10^{-4} M). *B*, concentration dependency of the $[Ca^{2+}]_i$ response to apical ATP.

did not affect the $[Ca^{2+}]_i$ response to apical ATP in 16HBE14o- monolayers. In the absence of external Ca^{2+}, apical ATP (10^{-4} M) produced a mean $[Ca^{2+}]_i$ increase of 369 ± 29 nM ($n = 6$), which was not significantly different from the ATP-induced $[Ca^{2+}]_i$ increase in the presence of external Ca^{2+}. Furthermore, incubation of 16HBE14o- monolayers with thapsigargin (1 μM), produced a transient increase in $[Ca^{2+}]_i$ of 89 ± 12 nM ($n = 19$), and completely abolished the $[Ca^{2+}]_i$ response to subsequent apical ATP exposure. $[Ca^{2+}]_i$ measured upon ATP (10^{-4} M) exposure 20 min post-thapsigargin treatment was 123 ± 25 nM, which was not significantly different from basal levels of $[Ca^{2+}]_i$ ($n = 19$, $P < 0.001$). Together, these results demonstrate that apical ATP stimulates a $[Ca^{2+}]_i$ increase in 16HBE14o- monolayers due mainly to Ca^{2+} release from thapsigargin-sensitive intracellular stores.

Dexamethasone effect on the ATP-stimulated $[Ca^{2+}]_i$ response

In addition to the decrease of basal $[Ca^{2+}]_i$, pre-exposure of 16HBE14o- cell monolayers to dexamethasone (1 nM) for < 15 min caused a reduction in the $[Ca^{2+}]_i$ response to apical ATP. As illustrated in Fig. 6, dexamethasone (1 nM) pretreatment largely reduced the amplitude of the ATP-induced $[Ca^{2+}]_i$ increase. Following dexamethasone treatment, apical ATP (0.1 mM) produced a mean increase in $[Ca^{2+}]_i$ of 214 ± 89 nM; ($n = 7$) from a basal value of 100 ± 13 nM. This response was significantly reduced compared to the ATP response produced in non-glucocorticoid-treated monolayers ($\Delta[Ca^{2+}]_i = 377 \pm 66$ nM; $n = 7$; $P < 0.02$). The duration of the dexamethasone exposure prior to ATP addition was between 5 and 15 min and the duration of the delay did not affect the amplitude of the inhibitory effect.

Stimulation of Cl^- secretion by apical ATP

The transepithelial I_{SC} measured across 16HBE14o- monolayers bathed in Krebs-Heinseleit solution on both sides of the epithelium, was 2.9 ± 1.6 μA cm^{-2} ($n = 38$). As shown in Fig. 7, apical application of ATP (10^{-4} M) evoked a rapid and transient increase in I_{SC} of 12.6 ± 3.8 μA cm^{-2} ($n = 34$). The I_{SC} increase started without measurable latency, the peak was reached within 2 min of nucleotide application and was then followed by a rapid decline to baseline values in the continuous presence of external ATP (Fig. 7*A*). Since the I_{SC} may be generated by either Na^+ absorption or Cl^- secretion (or both), we tested the effect of amiloride as an epithelial Na^+ channel inhibitor. Amiloride did not significantly alter the basal or the ATP-stimulated I_{SC} responses. In the presence of amiloride (10^{-4} M), basal I_{SC} was 3.23 ± 0.18 μA cm^{-2} ($n = 12$), and was not significantly different from the I_{SC} value obtained

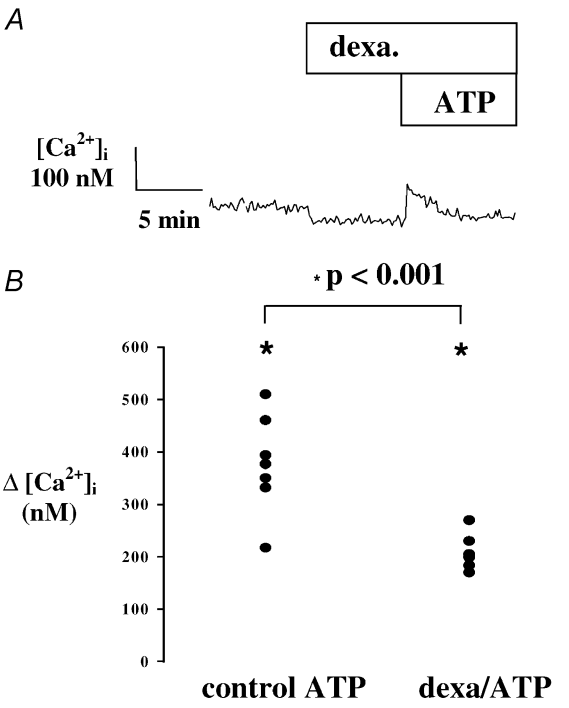

Figure 6. Dexamethasone effect on the $[Ca^{2+}]_i$ response to ATP

A, typical $[Ca^{2+}]_i$ response to ATP (10^{-4} M) following pretreatment with dexamethasone (10 nM). *B*, comparison of the $[Ca^{2+}]_i$ variation produced by ATP (10^{-4} M) under control conditions (control ATP 10^{-4} M), and following pre-incubation with dexamethasone (10 nM, 5–15 min; dexa/ATP).

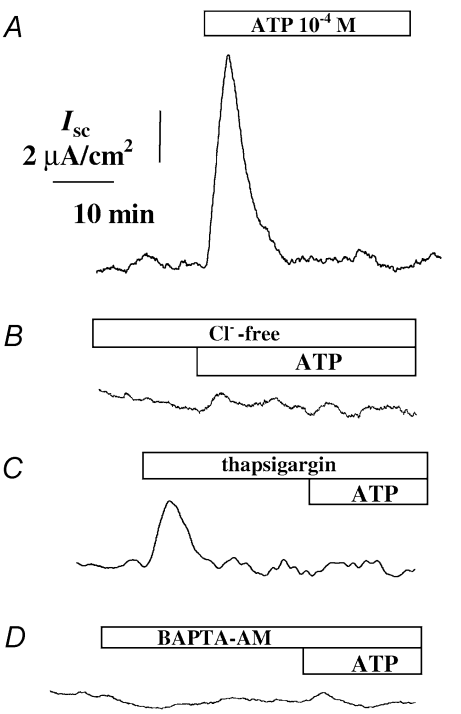

Figure 7. ATP induced Cl^- secretion

A, typical record of short-circuit current (I_{SC}) during apical ATP (10^{-4} M) exposure. *B*, inhibition of the I_{SC} response to ATP (10^{-4} M) in Cl^--free medium (Cl^--free). Inhibition of the I_{SC} response to ATP by pretreatment with either thapsigargin (1 μM) (*C*) or BATA-AM (50 μM; *D*).

in the absence of amiloride. During amiloride (10^{-4} M) treatment, apical ATP (10^{-4}M) exposure to 16HBE14o- cells, produced a I_{SC} increase of 12.3 ± 3.1 μA cm^{-2} ($n = 12$), which was not significantly different from the I_{SC} increase obtained in the absence of amiloride ($P > 0.32$).

We further demonstrated that the I_{SC} was primarily generated by Cl^- secretion. Direct replacement of basolateral Cl^- by gluconate completely abolished the ATP-induced I_{SC} increase ($\Delta I_{SC} = 0.2 \pm 0.1$ μA cm^{-2} $n = 7$, $P < 0.05$; Fig. 7*B*).

Role of $[Ca^{2+}]_i$ in the ATP-induced Cl^- secretion

Our previous results demonstrated that thapsigargin pretreatment completely abolished the ATP-induced $[Ca^{2+}]_i$ increase. The effect of thapsigargin on I_{SC} was recorded to determine the role of $[Ca^{2+}]_i$ in apical ATP-induced Cl^- secretion. Thapsigargin (1 μM) produced a transient increase in I_{SC} of 3.09 ± 1.6 μA cm^{-2} ($n = 6$), and completely abolished the I_{SC} response to subsequent apical ATP (10^{-4} M) exposure (Fig. 7*C*). This result indicates an intracellular Ca^{2+} dependency of the apical ATP-induced Cl^- secretion.

We further investigated the role of $[Ca^{2+}]_i$ in apical ATP-induced Cl^- secretion in 16HBE14o- monolayers, using the intracellular Ca^{2+} chelator BAPTA-AM. Following pretreatment of cells with BAPTA-AM (50 μM, 20 min), the I_{SC} response to ATP (10^{-4} M) was completely abolished ($\Delta I_{SC} = 0.5 \pm 0.3$ μA cm^{-2}, $P < 0.05$, $n = 4$; Fig. 7*D*).

Rapid effect of dexamethasone on ATP-induced Cl^- secretion

Since intracellular Ca^{2+} ions are a major signal transducer of secretion in epithelia, we tested the hypothesis that the reduction in $[Ca^{2+}]_i$ induced by dexamethasone may antagonise the secretory event. Dexamethasone (1 nM, 5–15 min), applied to the apical side of 16HBE14o- monolayers did not significantly alter basal I_{SC}. However, as illustrated Fig. 8, dexamethasone (1 nM), significantly inhibited apical ATP-induced Cl^- secretion in 16HBE14o- cell monolayers. Pre-incubation with dexamethasone (10^{-9} M, 10 min), reduced the mean ATP-induced I_{SC} response to 0.86 ± 1.5 μA cm^{-2} ($n = 7$), significantly smaller than the increase obtained with ATP (10^{-4} M) alone ($\Delta I_{SC} = 15.2 \pm 5.6$ μA cm^{-2}, $n = 7$).

DISCUSSION

Taken together, our results provide evidence for a rapid non-genomic antisecretory effect of dexamethasone at low concentration (1 nM) involving an inhibition of intracellular Ca^{2+} signalling in the human airway epithelium. In a recent study, we provided evidence for a rapid decrease of $[Ca^{2+}]_i$ induced by aldosterone (1 nM) in 16HBE14o- monolayers. This was the first report of a non-genomic effect of steroid hormones in human bronchial epithelium (Urbach & Harvey, 2001). Similarly to aldosterone, we show in the present paper that low concentrations (1 nM) of the glucocorticoid hormone dexamethasone, rapidly decreases basal $[Ca^{2+}]_i$ in the immortalised 16HBE14o- cell line and in primary cultures of human bronchial epithelial cell monolayers An effect of steroid hormones (including glucocorticoids) on $[Ca^{2+}]_i$ has previously been reported in different tissues. In some tissues, such as cortical collecting duct (Harvey & Higgins, 2000), proximal tubule (Han *et al.* 1999) and vascular smooth muscle (Steiner *et al.* 1988), glucocorticoids produce a rapid $[Ca^{2+}]_i$ increase. In contrast, glucocorticoid treatment causes a decrease in Ca^{2+} influx via a non-genomic mechanism in myocytes (Passaquin *et al.* 1998), thymocytes (Buttgereit *et al.* 1997), human lymphoblast (Gardner & Zhang, 1999), human leukocytes and airway smooth muscle (Chhabra *et al.* 1999). We also reported previously that the glucocorticoids triamcilone acetonide and hydrocortisone, at high concentrations (10^{-6} M) produce a rapid of $[Ca^{2+}]_i$ in 16HBE14o- cells (Urbach & Harvey, 2001).

The fast (30 s) onset and the insensitivity of the dexamethasone-induced Ca^{2+} response to cycloheximide reported in the present study indicate a non-genomic mechanism. The nature of the eventual receptor(s) involved in the non-genomic effects of steroid hormone is still not clear. It has been suggested that at least some of the

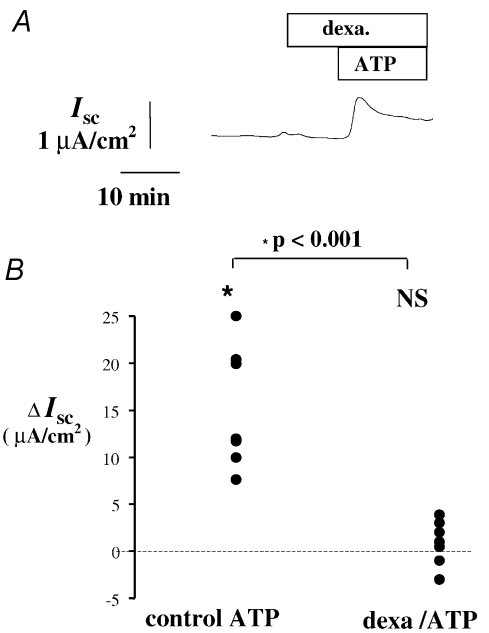

Figure 8. Dexamethasone effect on the Cl^- secretory response to ATP

A, typical Cl^- secretory response to ATP (10^{-4} M) following pretreatment with dexamethasone (10 nM). *B*, comparison of the I_{SC} variation produced by ATP (10^{-4} M) under control conditions (control ATP 10^{-4} M), and following pre-incubation with dexamethasone (10 nM, 5–15 min; dexa/ATP). NS = not significant.

non-genomic effects of steroids are mediated by binding to a membrane receptor. Several membrane receptors or membrane binding sites for glucocorticoids have been described in various tissues. A receptor to cortisol has been characterised in neuronal membranes of the amphibian *Tarisha granulosa* (Moore *et al.* 1995). However, aldosterone and dexamethasone do not display a high affinity for this membrane receptor. Other putative membrane binding sites for glucocorticoids have been detected in mouse and rat liver, rat kidney, rat brain and calf adrenal cortex (Ibarrola *et al.* 1991; Trueba *et al.* 1991; Guo *et al.* 1995; Andres *et al.* 1997).

In our study, the similarity between the $[Ca^{2+}]_i$ decrease induced by aldosterone in 16HBE14o- cells and the response to dexamethasone in the same cell preparation, suggest that dexamethasone acts as a mineralocorticoid-like agonist. In addition, the fact that subsequent addition of aldosterone did not stimulate a further response after the initial rapid response to dexamethasone suggests strongly that both hormones share a common receptor to produce the rapid response. However the insensitivity of the dexamethasone-induced $[Ca^{2+}]_i$ decrease to spironolatone or RU486, indicates that the non-genomic effect of dexamethasone is not mediated via the classical mineralocorticoid or glucocorticoid receptor. In addition, we investigated the non-genomic effect of other glucocorticoids having a higher relative binding affinity than dexamethasone for the glucocorticoid receptor (Brattsand & Axelsson, 1997). Triamcylone acetonide only produced a detectable $[Ca^{2+}]_i$ decrease at high pharmacological concentrations (10^{-6} M) and budesonide did not produce any change in $[Ca^{2+}]_i$. These results support our conclusion of a non-genomic mechanism that is not mediated by the classical glucocorticoid receptor.

The difference in ability for the different glucocorticoids to produce the non-genomic response may be explained by their relative lipophilicity. The lipophilicity of dexamethasone is lower than that of triamcylone acetonide and budesonide, which may suggest that the non-genomic effect is the result of a direct interaction within the membrane. In addition, the rapidity of the onset of the $[Ca^{2+}]_i$ decrease according to Buttgereit's classification, also suggests a non-specific binding of the hormone at the membrane (Buttgereit *et al.* 1999). It has been reported that the non-specific and non-genomic effects of glucocorticoids occur at high concentrations, $> 10^{-4}$ M (Buttgereit *et al.* 1999); however, we observed the rapid Ca^{2+} response to occur at low concentrations (10^{-9} M) of dexamethasone.

The inhibition of the rapid response to dexamethasone by antagonists of adenylate cyclase and PKA suggests that the dexamethasone response occurs through PKA stimulation. The inhibition of the response to dexamethasone by an antagonist of Ca^{2+}-ATPase and the stimulation by dexamethasone of the Ca^{2+} release following thapsigargin also suggests that dexamethasone acts to lower cytosolic Ca^{2+} via stimulation of the Ca^{2+}-ATPase of intracellular stores. In addition, dexamethasone still produced an increase in $[Ca^{2+}]_i$ after treatment with a Ca^{2+} ionophore, indicating that dexamethasone is not affecting the membrane Ca^{2+} leak pathways. There is evidence that Ca^{2+} ionophores also perforate intracellular organelles (Tarran *et al.* 2002). However, under our experimental conditions, such a possibility is incompatible with the dexamethasone effect of lowering cytosolic Ca^{2+} in the presence of the ionophore A23187. Other studies have also shown that very high concentrations of steroids decrease $[Ca^{2+}]_i$ via stimulation of Ca^{2+}-ATPase activity by affecting membrane fluidity (Massa *et al.* 1975; Whiting *et al.* 2000). The similarity of the aldosterone and glucocorticoid responses and the absence of an additive effect of the two hormones is most probably due to the sharing of an intracellular Ca^{2+} signalling pathway.

Extracellular nucleotides exert significant biological actions on different cell types in the upper airways and in the lung, and there are several reports of ATP release from epithelial tissues by physiological stimuli that are associated with the secretory event (Grygorzyk & Hanrahan, 1997; Sorensen & Novak, 2001). At least some of these diverse biological effects are mediated via cell-surface purino receptors that activate inositol 1,4,5-trisphosphate hydrolysis and Ca^{2+} mobilisation. Many studies substantiate the concept that ATP is a potent agonist with multiple receptor subtypes expressed ubiquitously on the cell surface, which are linked to diverse intracellular signal transduction pathways (Stutts *et al.* 1994). Evidence supporting the existence of a $P2Y_2$ receptor in airway epithelia has emerged from functional studies of 16HBE14o- cells (Koslowsky *et al.* 1994), human tracheal epithelial cells (Yamaya *et al.* 1996), nasal epithelial cells (Mason *et al.* 1991), alveolar type II cells (Rice *et al.* 1995), CF/T43 cells (Brown *et al.* 1991), A549 cells (Clunes & Kemp, 1996) and rat tracheal epithelial cells (Hwang *et al.* 1996). Here we show that external ATP causes a transient increase in Ca^{2+} and I_{SC} when added to the apical surface of 16HBE14o- cell monolayers. We showed the transient I_{SC} increase induced by apical ATP was completely abolished in the absence of basolateral Cl^-. We have also demonstrated that the source of the Ca^{2+} rise induced by apical ATP occurs from the mobilisation of Ca^{2+} sequestered within thapsigargin-sensitive intracellular stores. Finally, the Ca^{2+} dependency of the Cl^- secretion induced by apical ATP was further verified by the inhibitory effect of thapsigargin and BAPTA-AM. These results support the conclusion that the apical ATP-induced Cl^- secretory response in 16HBE14o- cells is mediated by cell-surface purinoceptors that respond by elevating $[Ca^{2+}]_i$ and activating Ca^{2+}-dependent Cl^- channels.

In this study, we tested the hypothesis that the dexamethasone effect on $[Ca^{2+}]_i$ might generate a rapid antisecretory effect. Dexamethasone applied to the apical side of the 16HBE14o- monolayer did not produce any significant effect on basal I_{SC}. However, after exposure to dexamethasone, subsequent exposure to ATP resulted in a reduced Cl^- secretory effect. In other tissues, dexamethasone has been reported to modulate Ca^{2+}-dependent secretory mechanisms. Dexamethasone inhibited the nicotine-induced secretion of catecholamines in porcine adrenal medulla (Wagner *et al.* 1999) and the corticotropin-releasing-factor-induced ACTH secretion in rat (Hinz & Hirschelmann, 2000). Steroid hormones have also been described to exert an antisecretory effect on cAMP-dependent Cl^- secretion. Inhibition of the cAMP-dependent Cl^- secretion by 17β-oestradiol has also been reported to occur in T84 cells (Singh *et al.* 2000) and in rat distal colonic epithelium (Condliffe *et al.* 2001). In nasal epithelial cells, it has been reported that 4 days of treatment (for 30 min every 12 h) with the glucocorticoid fluticasone propionate stimulates an amiloride-sensitive I_{SC} current and partially decreases cAMP-dependent Cl^- secretion (Jepsen *et al.* 2000). Our study is the first report of a rapid response to glucocorticoid on Ca^{2+}-dependent Cl^- secretion.

Glucocorticoids are potent anti-inflammatory and immunosuppressive compounds that are used via systemic or inhaled delivery for the treatment of numerous diseases. The steroids are usually used at low doses for basal immunosuppressive treatment and their effects are not observed until after a few hours or days of treatment. It has been observed that a high-dose and short-term intravenous glucocorticoid therapy improves symptoms in acute exacerbation of multiple sclerosis or acute spinal cord injury (Bracken *et al.* 1990). It has been proposed that in some acute clinical situations the additional benefit of the use of high doses of glucocorticoids is due to their non-genomic effects (Buttgereit *et al.* 1997). The rapid non-genomic effect of glucocorticoids in human bronchial epithelia, as reported in this study, may have important clinical implications in the treatment of airway infections such as rhinitis, asthma and cystic fibrosis. In particular, the antisecretory effect of glucocorticoids used as anti-inflammatory compounds in the treatment of cystic fibrosis, may exacerbate the deficit in Cl^- secretion associated with the disease.

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