Alveolar Epithelial Ion and Fluid Transport
β-Adrenoceptor-mediated control of apical membrane conductive properties in fetal distal lung epithelia

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Collett, A., S. J. Ramminger, R. E. Olver, and S. M. Wilson. β-Adrenoceptor-mediated control of apical membrane conductive properties in fetal distal lung epithelia. Am J Physiol Lung Cell Mol Physiol 282: L621–L630, 2002.—Distal lung epithelial cells isolated from fetal rats were cultured (48 h) on permeable supports so that transepithelial ion transport could be quantified electrometrically. Unstimulated cells generated a short-circuit current (I_{sc}) that was inhibited (~80%) by apical amiloride. The current is thus due, predominantly, to the absorption of Na⁺ from the apical solution. Isoproterenol increased the amiloride-sensitive I_{sc} about twofold. Experiments in which apical membrane Na⁺ currents were monitored in basolaterally permeabilized cells showed that this was accompanied by a rise in apical Na⁺ conductance (G_{Na⁺}). Isoproterenol also increased apical Cl⁻ conductance (G_{Cl⁻}) by activating an anion channel species sensitive to glibenclamide but unaffected by 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS). The isoproterenol-evoked changes in G_{Na⁺} and G_{Cl⁻} could account for the changes in I_{sc} observed in intact cells. Glibenclamide had no effect upon the isoproterenol-evoked stimulation of I_{sc} or G_{Na⁺}, demonstrating that the rise in G_{Cl⁻} is not essential to the stimulation of Na⁺ transport.

alveolar ion transport; Ussing chambers; permeabilized epithelia

THROUGHOUT FETAL LIFE the distal lung epithelium secretes fluid into the developing air spaces (31) and establishes a distending pressure that is crucial to lung morphogenesis (11). However, this liquid must be removed from the lungs if the newborn infant is to breathe at birth. The absorption of this liquid occurs during the final stages of gestation and is dependent upon the active withdrawal of Na⁺ from the lung lumen, a process that can be controlled via β-adrenoceptors (23, 30, 36–38). The means by which this control is achieved are not fully understood, but there is evidence that the process involves a rise in apical Na⁺ conductance (G_{Na⁺}) (15, 20). It is also known that β-adrenoceptor agonists can increase apical Cl⁻ conductance (G_{Cl⁻}) in these cells, and it has been suggested that this may facilitate Na⁺ transport by increasing the driving force for Na⁺ entry (16, 29). It is, however, difficult to see how the ionic gradients that normally prevail in epithelial cells could allow Na⁺ transport to be controlled in this way (see Refs. 20, 44). To clarify the means by which β-adrenoceptor agonists can control Na⁺ absorption in the distal lung epithelium, we now explore the effects of isoproterenol upon the conductive properties of the apical membrane.

METHODS

Isolation of culture of rat fetal distal lung epithelial cells. Fetuses removed from anesthetized (3% halothane), 20-day pregnant (term = 22 days) rats were immediately decapitated, and their lung tissue was collected into ice-cold, Ca²⁺- and Mg²⁺-free Hanks’ balanced salt solution. The anesthetized animals were then killed (cervical dislocation/exsanguination) before regaining consciousness. The fetal lung tissue was chopped into pieces (<0.5 mm) and disaggregated using 0.2% trypsin/0.012% DNase (2 × 20 min, 37°C) followed by 0.1% collagenase/0.012% DNase [15 min, 37°C, both in Dulbecco’s modified Eagle’s medium (DMEM)]. Cells pelleted from the resultant digest were resuspended in DMEM containing 10% fetal calf serum and then incubated (1 h, 37°C) in a 75-cm² culture flask. The supernatant was then gently decanted to separate nonadherent epithelial cells from fibroblasts and smooth muscle cells. After a second such fractionation, the nonadherent cells were resuspended in culture medium, plated (1.5 × 10⁶ cells/cm²) onto Transwell-Col

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membranes (Costar, High Wycomb, UK) and incubated (37°C) in an atmosphere of water-saturated room air containing 5% CO₂ and sufficient N₂ to reduce partial pressure of oxygen (PO₂) to the level found in the adult alveolar region (100 mmHg). Cells were incubated in PC-1 medium unless otherwise stated. This is a serum-free medium that contains defined amounts of the hormones/growth factors found in fetal calf serum, which is almost invariably added to media used to maintain epithelial cells in primary culture. Its exact composition, however, is regarded as commercially sensitive. After 24 h, we changed the medium and removed nonviable cells by gently washing each culture. The cells were then incubated for a further 24 h before being used in experiments. By this time, the cells had almost invariably (>90% of cell preparations) become integrated into epithelial sheets with transepithelial resistances (Rt) >200 Ωcm² (see also Refs. 1, 37). Although the cells are isolated from fetal animals, previously published work (1, 33, 36) shows that maintaining these cells in an atmosphere that mimics the PO₂ of the postnatal alveolar region (~100 mmHg) causes the development of a Na⁺-absorbing phenotype typical of the neonatal, rather than the fetal lung.

Measurement of Isc and the conductive properties of the apical membrane. Cultured epithelia were mounted in Ussing chambers where they were bathed with bicarbonate-buffered physiological salt solution (composition given in Solutions and chemicals) that was continually circulated with a gas mixture identical to that in which the epithelia had been incubated (PO₂ = 100 mmHg). Initially, the cells were maintained under open-circuit conditions until the transepithelial potential difference (Vt) had stabilized (30–40 min). Vt was then clamped to 0 mV, and the current required to hold this potential [short-circuit current (Isc)] was digitized (4 Hz) and displayed on a computer screen while simultaneously being recorded to computer disk using a PowerLab computer interface and associated software (ADI Instruments, Hastings, UK). In some experiments, Rt was also monitored by observing the currents flowing in response to repeated, 1-mV excursions in Vt. The conductive properties of the apical membrane were explored by using polyelectrolyte antibiotics (nystatin or amphotericin B) to permeabilize the basolateral plasma membrane. These compounds form pores in cholesterol-containing membranes that are permeable to Na⁺, K⁺, and Cl⁻ but not to divalent cations or highermolecular-weight substances and thus allow experimental control over the cytoplasmic [Na⁺], [K⁺], and [Cl⁻], whereas intracellular Ca²⁺ is regulated by the normal, physiological mechanisms. Because the pores formed by amphotericin B have a higher Cl⁻ conductance than do those formed by nystatin (13, 21), the former compound was used to make measurements of GCl⁻.

To determine apical GNa⁺, cells bathed with the cytoplasm-like solution (Table 1) were exposed to basolateral nystatin (75 μM) to permeabilize this membrane. An inwardly directed Na⁺ gradient was then imposed upon the permeabilized preparations by selectively modifying the composition of the apical solution (Table 1). Under these conditions, the driving force for Na⁺ entry (VNa⁺) is determined by the difference between Vt (0 mV) and the equilibrium potential for Na⁺ (E<sub>Na⁺</sub>, 41.8 mV). GNa⁺ can thus be calculated using the expression GNa⁺ = I<sub>in</sub>/V<sub>Na⁺</sub>, in which I<sub>in</sub> is the change in apical membrane current (I<sub>ap</sub>) elicited by apical amiloride (10 μM). The apical membranes' GCl⁻ was measured using an analogous approach in which the I<sub>ap</sub> was evoked by imposing an outwardly directed Cl⁻ gradient (Table 1) in amphotericin B (100 μM)-permeabilized epithelia.

| Table 1. Final concentrations (in mM) of the principal anions and cations in the solutions used to determine G<sub>Na⁺</sub> and G<sub>Cl⁻</sub>. |
|------------------|------------------|------------------|
| **Cytoplasm-like** | **Apical Solution Used to Measure G<sub>Na⁺</sub>** | **Basolateral Solution Used to Measure G<sub>Cl⁻</sub>** |
| Na⁺ | K⁺ | Cl⁻ | Gluconate |
| 11.5 | 135.3 | 10.3 | 122.0 |
| 55.0 | 92.2 | 10.3 | 122.0 |
| 11.5 | 135.3 | 49.1 | 81.3 |

Initially, most preparations were bathed symmetrically with standard physiological saline. Three aliquots of saline were then withdrawn from the apical and basolateral baths, and each was replaced successively with an equal volume of the K⁺-glucosolate solution. In this way the standard solution was diluted with K⁺-glucosolate solution (8.1:91.9) to make up the cytoplasm-like solution whose composition is tabulated. In some experiments, however, cells were bathed with an inwardly-directed Na⁺ gradient, a 5-ml aliquot of the cytoplasm-like solution was withdrawn from the apical bath and replaced with 5 ml of a separate solution prepared by diluting (8.1:91.9) the standard salt solution with K⁺-glucosolate solution. Apical [Na⁺] was thus raised to 55 mM by isosmotically replacing K⁺ while the concentrations of other ions remained constant. To impose an outwardly-directed Cl⁻ gradient, a 5-ml aliquot of the cytoplasm-like solution was withdrawn from the basolateral bath and replaced with an equal volume of a solution prepared by diluting (8.1:91.9) the standard physiological saline with K⁺-Cl⁻ solution. Basolateral [Cl⁻] was thus raised to 49 mM by isosmotically replacing gluconate without affecting the concentrations of other ions present. G<sub>Na⁺</sub> and G<sub>Cl⁻</sub>, apical Na⁺ and Cl⁻ conductance, respectively.

The use of such pore-forming agents can be complicated by the fact that they can promote cell swelling under certain conditions. This problem can be particularly severe when external [Cl⁻] is high, as the pore-forming agent will allow internal [Cl⁻] to reach a level greater than that found in intact cells. This will promote cell swelling by providing an osmotic driving force for water entry, and this, in turn, could activate other membrane conductances. Moreover, as most intracellular anions are essentially immobile, exposing permeabilized cells to high external [Cl⁻] can allow a Donnan potential to be established across the permeabilized membrane, which will affect the driving force for ion movement across the intact side of the epithelium. The design of the present study was thus influenced by a need to prevent internal [Cl⁻] rising outside its normal, physiological range, and so in all experiments involving permeabilized epithelia, most Cl⁻ in the standard solution was isosmotically replaced by gluconate, a nominally impermeant anion (Table 1). Although we could not measure cell volume in our system, preliminary experiments showed that no spontaneous changes in G<sub>Cl⁻</sub> conductance occurred over the time scale of the present experiments. We have thus assumed that the present measurements were not complicated by the activation of volume-sensitive conductances.

The technique used to measure G<sub>Na⁺</sub> and G<sub>Cl⁻</sub> relies upon exposing the cultured epithelia to asymmetrical ionic conditions, and, by definition, this will establish a liquid junction potential that, if uncompensated, would provide a driving force for the movement of ions. Immediately before each experiment, we therefore monitored the effects of imposing the appropriate ionic gradient upon the potential difference across a culture membrane bearing no cells. The adjustments needed to offset this potential were noted and applied during the subsequent experiments.
Quantification of Na$^+$ pump capacity. The method used to determine the capacity of the basolateral Na$^+$ pump is described elsewhere (21, 36), and so only brief details are presented here. Epithelial monolayers were mounted in Ussing chambers, bathed with the standard physiological solution (i.e., high Na$^+$), and treated with apical amiloride (10 μM) to block the Na$^+$ channels in this membrane, which was then permeabilized using nystatin (75 μM). This elicited a slowly developing rise in $I_{sc}$ attributed to the activity of the basolaterally located Na$^+$ pump. The fall in $I_{sc}$ evoked by subsequently adding ouabain (1 mM) to the basolateral solution ($I_{pump}$) was then measured to provide an indicator of the Na$^+$ extrusion capacity of this pump. Our previously published work (36) shows that 1 mM ouabain never entirely abolished the $I_{sc}$ recorded from nystatin-permeabilized cells, and it is therefore possible that the method may slightly underestimate the capacity of the basolateral pump. However, we are forced to accept this limitation as higher concentrations of ouabain caused loss of epithelial integrity.

Data analysis and experimental design. Data are presented as means ± SE, and values of $n$ refer to the number of times a protocol was repeated using cells prepared from different litters. The control current was defined as that measured under basal conditions at the onset of the experiment, and control and experimental cells were age matched and derived from the same litters. The statistical significance of differences between mean values was assessed using Student’s $t$-test. In studies of intact cells, positive $I_{sc}$ was defined as the current carried by cations moving from the apical to the basolateral compartments, whereas, in basolaterally permeabilized preparations, positive $I_{lp}$ is that carried by cations leaving the cytoplasm. These are standard electrophysiological conventions.

Solutions and chemicals. The standard physiological salt solution contained (in mM) 117 NaCl, 25 NaHCO$_3$, 4.7 KCl, 1.2 MgSO$_4$, 1.2 KH$_2$PO$_4$, 2.5 CaCl$_2$, and 11 d-glucose, pH 7.3–7.4 when bubbled with 5% CO$_2$. The K$^+$-gluconate solution was prepared by isosmotically replacing Cl$^-$ in this standard solution with gluconate, whereas the K$^+$-Cl$^-$ solution was prepared by replacing Na$^+$ with K$^+$. Both ionic substitutions were made in the K$^+$-gluconate solution. The amount of calcium gluconate added to gluconate-containing solutions was raised to 11.5 mM to maintain Ca$^{2+}$ activity despite gluconate’s capacity to bind this cation. All solutions were bicarbonate buffered and continually bubbled with 5% CO$_2$ to maintain pH. The minimal defined-composition serum-free (MDSF) medium used in some experiments consisted of a mixture (1:1) of DMEM/Ham’s F-12 nutrient mix that contained bovine serum albumin (1.25 mg/ml), L-glutamine (2 mM), and nonessential amino acids (0.1%). Amiloride and isoprenaline were freshly prepared (10 mM in distilled water) on each experimental day, whereas stock solutions (10 mM) of ethylisopropyl amiloride (EIPA; RBI International, Gillingham, UK) and glibenclamide (Tocris, St. Albans, UK) were prepared in dimethyl sulfoxide, and benzamil (50 mM; Molecular Probes) was dissolved in distilled water containing 20% (vol/vol) methanol. These solutions were divided into aliquots and stored at −20°C. Appropriate experiments showed that the solvent vehicles had no effect upon the parameters under study. Cell culture reagents were purchased from Paisley Life Technologies (Paisley, UK), and general laboratory reagents were from Sigma Chemical (Poole, UK).

RESULTS

Properties of intact epithelia. Apical amiloride (10 μM) caused the spontaneous $I_{sc}$ to fall rapidly (20–40 s) to a value that was 23.1 ± 6.6% ($n = 7$) of the control level, and the concentration required to exert half-maximal inhibition (IC$_{50}$) was 0.47 ± 0.03 μM (Fig. 1A). EIPA and benzamil also caused ~80% inhibition
of the basal $I_{sc}$, and the IC<sub>50</sub> values for these compounds were 3.3 ± 0.8 μM and 10.8 ± 1.8 nM, respectively (Fig. 1A). The rank order of potency amongst these Na<sup>+</sup> channel antagonists was thus benzamil > amiloride > EIPA, a profile suggesting the involvement of selective Na<sup>+</sup> channels in the generation of the basal $I_{sc}$ (4). These experiments also revealed an amiloride-resistant component to the spontaneous $I_{sc}$, and previous work has shown that subsequent application of bumetanide causes very little further fall in the spontaneous current; the ionic basis of this current is thus unknown (34). Basolateral isoprenaline (10 μM) caused a slowly developing rise in $I_{sc}$, although an initial, more rapid component to this response was usually evident (Fig. 1B, see also Refs. 36, 37). After 30–40 min stimulation with this drug, $I_{sc}$ had risen to a value that was 173.2 ± 10.3% of the control level. Subsequent addition of apical amiloride (10 μM) caused a rapid fall to a value that was only 34.1 ± 3.8% of the control current measured at the onset of the experiment (Fig. 1B). Further experiments were undertaken in which amiloride-pretreated cells were exposed to isoprenaline. Examination of the currents recorded at the onset of these experiments confirmed that amiloride caused ~80% inhibition of basal $I_{sc}$, and, as anticipated, the response to isoprenaline was essentially abolished (94.7 ± 1.3% inhibition) by this drug (Fig. 1B).

**Effects of basolateral Ba<sup>2+</sup>**. We studied the effects of basolateral Ba<sup>2+</sup>, a cation that blocks epithelial K<sup>+</sup> channels, in an attempt to explore the role of these channels in the response to β-adrenoceptor agonists. The application of Ba<sup>2+</sup> to unstimulated cells caused a rapid fall in spontaneous $I_{sc}$, but this effect was transient, and $I_{sc}$ returned to its basal value within 10 min (Fig. 2). Subsequent application of isoprenaline also caused a small fall in $I_{sc}$, which contrasted with the initial rapid rise seen under control conditions. Although the recorded current returned to its basal value after ~5 min, $I_{sc}$ failed to rise above this level during 30–40 min of exposure to isoprenaline (Fig. 2). Ba<sup>2+</sup> thus abolishes the isoprenaline-evoked rise in $I_{sc}$. Subsequent experiments in which we monitored Na<sup>+</sup> pump capacity (see METHODS) in control and Ba<sup>2+</sup>-treated cells showed that this cation caused 40.2 ± 6.5% inhibition of $I_{pump}$ (control, 10.9 ± 1.4 μA/cm<sup>2</sup>, Ba<sup>2+</sup>-treated, 6.5 ± 1.1 μA/cm<sup>2</sup>, $P < 0.01$, Student’s paired t-test), indicating that Ba<sup>2+</sup>, a well-known inhibitor of epithelial K<sup>+</sup> channels, causes substantial loss of Na<sup>+</sup> pump capacity.

**Regulation of G<sub>Cl</sub>**. Imposing an outwardly directed Cl<sup>-</sup> gradient upon basolaterally permeabilized cells evoked only small (1–2 μA/cm<sup>2</sup>) currents, demonstrating that G<sub>Cl</sub> is normally low (~30 μS/cm<sup>2</sup>). Application of DIDS or glibenclamide (both 100 μM) reduced $I_{ap}$, establishing that this small conductance is due to the efflux of Cl<sup>-</sup> through channels sensitive to these compounds. Analysis of these data showed that the DIDS-sensitive and glibenclamide-sensitive components of G<sub>Cl</sub> were 10.7 ± 1.7 μS/cm<sup>2</sup> (n = 4) and 11.8 ± 4.0 μS/cm<sup>2</sup> (n = 4), respectively. Basolateral isoprenaline (10 μM) evoked a rise in $I_{ap}$ that occurred with no discernible latency, reaching a level that was ~15-fold greater than the basal value after ~20 s. $I_{ap}$ then fell to a plateau value two- to threefold above control (Fig. 3A). The application of glibenclamide (100 μM) to isoprenaline-stimulated preparations caused a rapid fall in $I_{ap}$, and subsequent application of DIDS (100 μM) caused a further small fall (Fig. 3A). Analysis of these data indicated that isoprenaline augmented the glibenclamide-sensitive component of G<sub>Cl</sub> approximately fivefold (55.7 ± 7.2 μS/cm<sup>2</sup>, P < 0.05 vs. control data presented above) but had no significant effect upon the DIDS-sensitive component (17.1 ± 1.9 μS/cm<sup>2</sup>). Experiments in which cells were exposed to these Cl<sup>-</sup>-gradient upon basolaterally permeabilized cells (n = 4) showed that basolateral isoprenaline had no discernible effect upon $I_{ap}$ when both apical and basolateral [Cl<sup>-</sup>] were maintained at 10.3 mM. The isoprenaline-evoked changes in $I_{ap}$ are thus dependent upon the presence of the Cl<sup>-</sup> gradient and so reflect an increase in G<sub>Cl</sub> that would facilitate the flow of Cl<sup>-</sup> down its electrochemical gradient. To establish the extent to which this response is maintained during prolonged stimulation, cells were first exposed to isoprenaline while bathed with standard physiological salt solution, and the basolateral membrane was then permeabilized to allow the glibenclamide-sensitive component of G<sub>Cl</sub> to be measured. These experiments revealed an approximately fourfold stimulation of glibenclamide-sensitive G<sub>Cl</sub> after 30–40 min of stimulation (control; 8.5 ± 2.4 μS/cm<sup>2</sup>, isoprenaline-stimulated; 34.3 ± 4.5 μS/cm<sup>2</sup>, n = 4, P < 0.01). Forskolin (100 μM), a drug that directly activates adenylyl cyclase (40), caused changes in G<sub>Cl</sub> essentially identical to those seen during stimulation with isoprenaline (n = 4), and this response, in common with the response to isoprenaline, was inhibited by glibenclamide but unaffected by DIDS (Fig. 3D).
Regulation of $G_{Na^+}$. Experiments in which $I_{ap}$ was recorded from basolaterally permeabilized cells exposed to an inwardly directed $Na^+$ gradient showed that isoprenaline had no effect over a 10- to 15-min period ($n = 4$). However, the isoprenaline-evoked rise in $I_{sc}$ develops over 30–40 min (Fig. 2), and so further experiments were undertaken in which cells were permeabilized and $G_{Na^+}$ was quantified once this response had become fully established. The data derived from intact cells (Fig. 4B) confirmed that isoprenaline evoked a slowly developing rise in $I_{sc}$, and the corresponding measurements of $G_{Na^+}$ (Fig. 4, Aii and Bii) showed that this was accompanied by a rise (approximately twofold) in $G_{Na^+}$ (control: $60.0 \pm 9.9 \mu S/cm^2$, $n = 6$; isoprenaline-stimulated: $138 \pm 12.0 \mu S/cm^2$, $n = 4$, $P < 0.01$). We studied the effects of glibenclamide upon this response to explore the role of the cAMP-activated anion channels (16, 29). These studies showed that apical glibenclamide had no significant effect upon basal $I_{sc}$ (control $I_{sc}$: $7.4 \pm 1.4 \mu A/cm^2$; postglibenclamide $I_{sc}$: $7.4 \pm 1.7 \mu A/cm^2$, $n = 4$). Moreover, application of basolateral isoprenaline evoked a rise in $I_{sc}$ ($3.5 \pm 1.5 \mu A/cm^2$, $n = 4$) in the glibenclamide-treated cells that did not differ significantly from that seen in age-matched control cells at identical passage ($3.6 \pm 0.94 \mu A/cm^2$, $n = 5$). Once these responses to isoprenaline had become established, the control and glibenclamide-treated cells were basolaterally permeabilized so that $G_{Na^+}$ could be measured. This analysis showed that glibenclamide had no statistically significant effect upon this parameter (control: $138.4 \pm 12.0 \mu S/cm^2$, $n = 5$; glibenclamide-treated, $143.1 \pm 26.4 \mu S/cm^2$, $n = 4$). Thus glibenclamide, at a concentration that can cause >90% inhibition of the cAMP-evoked rise in $G_{Na^+}$, has no significant effect upon the overall response to isoprenaline.

Responses to isoprenaline in cells maintained in MDSF medium. Experiments in which the standard culture medium (PC-1) was replaced with the MDSF medium (see METHODS) used in a previous study of alveolar epithelial cells isolated from adult animals (16) showed that the cells still became incorporated into epithelial layers ($R_t = 510 \pm 27 \Omega cm^2$) under these conditions. These cultured epithelia generated a spontaneous $I_{sc}$ ($10.4 \pm 0.5 \mu A/cm^2$) similar to that recorded from cells maintained in PC-1 medium. Apical amiloride (10 $\mu M$) elicited an immediate inhibition of this spontaneous current ($83.4 \pm 1.5\% \ n = 6$). Further studies of cells cultured under these conditions ($n = 5$; spontaneous $I_{sc} = 7.5 \pm 0.6 \mu A/cm^2$; $R_t = 389 \pm 81 \Omega cm^2$) showed that basolateral isoprenaline (10 $\mu M$) elicited only a barely discernible ($\Delta I_{sc} < 0.5 \mu A/cm^2$), transient fall in $I_{sc}$, so that the current measured after 20–30 min exposure to this drug ($7.5 \pm 1.4 \mu A/cm^2$) did
Fig. 4. Effects of Iso upon Na⁺ conductance (G_{Na⁺}). Cultured distal lung epithelial cells were initially bathed with the standard physiological salt solution, and I_{sc} was monitored. Although V_i was normally held at 0 mV, brief excursions (1 mV) from this holding potential were regularly imposed so that R_i could be monitored. At the points indicated by the arrows [K⁺-glucuronate solution (K-Gluc.)], aliquots of salt solution were withdrawn from the apical and basolateral baths and replaced with K-Gluc. to adjust [Na⁺], [Cl⁻], and [K⁺] to 11.5 and 10.3 mM, respectively. The basolateral membrane was then permeabilized by adding nystatin (Nys) to the salt solution, bathing this side of the cell layer, and the [Na⁺] was then raised to 55 mM to establish driving force for Na⁺ influx. The cells were then exposed to apical amiloride (10 μM) as indicated. A: representative currents recorded from unstimulated cells. B: representative currents recorded during stimulation with basolateral isoprenaline (10 μM). Each major panel is subdivided to include records of I_{sc} derived from intact cells (i), and I_{sc}, which were obtained from the same cells after basolateral permeabilizations (ii).

not differ significantly from the initial control value. Although cells maintained in MDSF medium thus generate a spontaneous I_{sc} that appears to be due largely to electrogenic Na⁺ absorption, this transport process does not appear to be subject to β-adrenoceptor-mediated control under these conditions. This contrasts markedly with the consistent stimulation of I_{sc} seen in cells maintained in medium PC-1 (Fig. 1).

DISCUSSION

Properties of intact cells. Data obtained from cells maintained in medium PC-1 accord with the view that cultured distal lung epithelial cells spontaneously absorb Na⁺ from the apical solution and show that cAMP-coupled agonists elicit a slowly developing but substantial rise in the rate of transepithelial ion transport. This response was due to a stimulation of amiloride-sensitive I_{sc}, and so these data confirm that the Na⁺ transport process underlying the basal I_{sc} is subject to β-adrenoceptor-mediated control (see also Refs. 3, 23, 26, 36–38). However, net Na⁺ absorption is not a feature of the fetal lung where the dominant ion transport process is the secretion of anions into the lung lumen (31). This discrepancy between the present observations and those made in the intact fetal lung almost certainly reflects the fact that the present study was undertaken using cells cultured at adult alveolar PO₂ (100 mmHg) rather than at fetal PO₂ (~23 mmHg). We (1, 36) and others (2, 33) have recently shown that this causes the development of the Na⁺-absorbing phenotype characteristic of the adult lung. Although the present study was undertaken using cells isolated from fetal animals, we believe that these cells expressed an adult phenotype when used in the experiments but cannot formally exclude the possibility that they may have adopted an intermediate phenotype.

Ba²⁺-evoked changes in I_{sc}. It is well documented that basolateral K⁺ channels, which can be blocked by Ba²⁺, make an important contribution to the maintenance of membrane potential (V_m) in epithelial cells (32), and such channels could thus play an important role in epithelial Na⁺ transport by maintaining the driving force for Na⁺ entry (25, 27). The present study, in common with earlier data (27), showed clearly that Ba²⁺ abolishes the response to isoprenaline, but, in our hands, this cation caused only transient inhibition of basal I_{sc}. This result is difficult to reconcile with the view that Ba²⁺ acts by selectively inhibiting K⁺ channels, and subsequent experiments showed that it also reduced the activity of the basolateral Na⁺ pump. Although this provides an alternative explanation for the inhibition of the response to isoprenaline, it cannot account for the transient inhibition of the basal I_{sc}. High concentrations of Ba²⁺, in common with other alkaline earth ions, have the potential to interfere with the control of the internal free Ca²⁺ concentration ([Ca²⁺]_i) (18, 19), and it is interesting, in this context, that Marunaka and colleagues (22) attribute the isoprenaline-evoked increase in I_{sc} to a cAMP-evoked increase in intracellular [Ca²⁺]_i. The inhibitory effect of
Ba\textsuperscript{2+} that we now describe could, therefore, also involve a direct interaction with the \(\beta\)-adrenoceptor-activated signal transduction pathway. The important point to emerge from these experiments is that Ba\textsuperscript{2+} exerts a number of effects under the present conditions, and so this cation cannot be used as a selective inhibitor of the K\textsuperscript{+} channels found in Na\textsuperscript{+}-absorbing epithelia.

**Conductive properties of the apical membrane.** The small \(G_{Cl}\) measured in unstimulated cells appears to be due to the presence of anion channels sensitive to DIDS and glibenclamide. Isoprenaline and forskolin elicited clear increases in \(G_{Cl}\), and these responses were inhibited by glibenclamide but not DIDS (see also Ref. 16), suggesting (see for example Refs. 10, 24, 41) that the currents elicited by cAMP-coupled agonists reflect the activation of anion channels formed by the cystic fibrosis transmembrane conductance regulator (CFTR), the gene product that is defective in cystic fibrosis (CF) (43). Support for this view came from parallel studies (O. G. Best, A. Collett, S. M. Wilson, and A. Mehta, unpublished data) in which apical membrane Cl\textsuperscript{−} currents were characterized in two human airway epithelial cell lines. In wild-type cells (16HBE14o−), forskolin evoked changes in \(G_{Cl}\) that were qualitatively similar to those described here. However, this response was absent from cells expressing a CF phenotype (CFBE41D\textsuperscript{−}), which have only minimal levels of functional CFTR in the apical membrane. Our findings thus accord with the results of earlier, more detailed studies of cells isolated from adult animals that identified CFTR-like anion channels in distal lung epithelial cells (16, 29). However, in our hands, the cAMP-evoked increase in \(G_{Cl}\) consisted of an initial peak followed by a slower, more sustained phase, whereas previous studies of anion currents flowing through CFTR-like channels have reported that cAMP causes a much more sustained response (12, 16). The reason for this discrepancy between these two studies is unknown but may reflect the different origin of the cells used in the two studies. However, CFTR can be subject to relatively rapid “rundown” in many experimental situations, and it has become clear that this process is controlled, at least in part, by Ca\textsuperscript{2+}-dependent protein kinases (45). The cells used in the present study were cultured under conditions different to those used by Jiang et al. (16), and so it is therefore possible that the variations between the observed time courses may reflect slight differences in the basal level of protein kinase C activity. However, it is also possible that the increase in \(G_{Cl}\) that we report may involve the activation of other anion channel species. It is interesting, in this context, that activation of CFTR has been shown to trigger the increased activity of other anion channels in epithelial cells and that this downstream effect is abolished when CFTR is blocked (9, 39). It is therefore possible that the ionic movements underlying the cAMP-evoked Cl\textsuperscript{−} currents that we now describe may not occur exclusively via CFTR.

\(G_{Na}\) was normally ~60 \(\mu A/cm^2\), and previous work has shown that this conductance reflects the presence of channels that discriminate clearly between Na\textsuperscript{+} and K\textsuperscript{+} (1), a situation very similar to that documented by Jiang et al.’s (16) study of cells isolated from adult animals. The channels underlying this apical conduc-
tance almost certainly correspond to the selective epithelial Na\textsuperscript{+} channels (ENaC) formed by the proteins encoded by the \(\alpha\), \(\beta\), and \(\gamma\)-ENaC genes (5, 6, 14, 23). Experiments in which nystatin-treated cells were exposed to isoprenaline, in common with data presented by Jiang et al. (16), failed to provide evidence that these channels were subject to acute control via \(\beta\)-adrenoceptor agonists. We found this surprising, because our studies of intact cells showed that the isoprenaline-evoked increase in \(I_{sc}\) is evident within ~10 min and because a number of previous studies have suggested that cAMP-dependent agonists can increase \(G_{Na}\) in distal lung epithelia (15, 20). To resolve this apparent contradiction, we undertook further experiments in which cells were stimulated with isoprenaline before the basolateral membrane was permeabilized. These studies showed clearly that \(G_{Na}\) was higher than normal after stimulation with this drug. It is thus clear that cAMP-dependent agonists increase both \(G_{Na}\) and \(G_{Cl}\), but, in the present study, the rise in \(G_{Cl}\) was a much more rapid phenomenon than the rise in \(G_{Na}\). It is possible, however, that rapid changes in \(G_{Na}\) might occur in intact cells, since permeabilizing the basolateral membrane could allow the loss of a cytoplasmic component essential to the regulation of \(G_{Na}\). Indeed, recent work has shown that ENaC can be rapidly controlled via changes in internal [Na\textsuperscript{+}] [41] and [Cl\textsuperscript{−}] (8, 22), and it is very unlikely that such ionic control of \(G_{Na}\) [(8, 22) would be detected in studies of basolater-
ally permeabilized cells. In this experimental situation, transient changes in internal ionic composition may occur as apical conductances are activated, but the relatively large volume of the external solution implies that such changes would be rapidly compensated for by ions entering/leaving the cytoplasm via the permeabilized membrane (21). The present estimates of \(G_{Na}\) were thus made under relatively constant ionic conditions.

**Physiological effects of the changes in \(G_{Na}\) and \(G_{Cl}\).** The present estimates of \(G_{Cl}\) and \(G_{Na}\) were combined with previously reported values of \(V_m\) and internal [Cl\textsuperscript{−}] and [Na\textsuperscript{+}] to allow us to predict the Cl\textsuperscript{−} and Na\textsuperscript{+} currents flowing across the apical membrane under basal conditions and after prolonged stimulation with isoprenaline. Although there is a driving force for Cl\textsuperscript{−} entry in unstimulated cells, \(G_{Cl}\) is so low that only a negligible (<0.5 \(\mu A/cm^2\)) Cl\textsuperscript{−} current can flow (Table 2). However, our analysis suggests that this would be accompanied by a substantial (~7 \(\mu A/cm^2\)) inward Na\textsuperscript{+} current (Table 2) and thus predicts that Na\textsuperscript{+} absorption is the dominant electrogenic transport process in unstimulated cells. Stimulation with isoprenaline reverses the driving force for Cl\textsuperscript{−} movement, suggesting that this drug may evoke the secretion of this anion, and so our analysis thus predicts a rapid but transient rise in \(I_{sc}\) due to the secretion of Cl\textsuperscript{−}, although it is difficult to anticipate the magnitude of this early cur-
Table 2. Values of $V_m$, $V_{Na}^+$, $V_{Cl}^-$ and estimates of the appropriate apical membrane currents for unstimulated and isoprenaline-stimulated cells

<table>
<thead>
<tr>
<th></th>
<th>$V_m$, mV</th>
<th>$V_{Na}^+$, mV</th>
<th>$V_{Cl}^-$, mV</th>
<th>$I_{Na}$, $\mu$A/cm$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting cells</td>
<td>$-40$</td>
<td>$-107$</td>
<td>$+13$</td>
<td>$-6.4$</td>
</tr>
<tr>
<td>Isoprenaline-stimulated</td>
<td>$-18$</td>
<td>$-85$</td>
<td>$-22$</td>
<td>$-12.3$</td>
</tr>
</tbody>
</table>

The values of $V_m$ are derived from data presented by Marunaka and colleagues (20), who measured reversal potentials for nonselective cation channels ($V_{Na}^+$) in control and isoprenaline-stimulated distal lung epithelial cells using the cell-attached recording mode. Under the conditions of these experiments, $V_m$ was $V_m = -2$ mV (22, 42). The tabulated values of $V_{Na}^+$, $V_{Cl}^-$ were obtained using the expression $V_{ion} = V_m - E_{ion}$. In this equation, the values of $E_{Na}^+$ and $E_{Cl}^-$ were calculated using the Nernst equation and previously published estimates (28, 42) of internal $[Cl^-]$ and $[Na^+]$ for resting ([Cl$^-$], 45 mM; [Na$^+$], 12 mM) and isoprenaline-stimulated ([Cl$^-$], 27 mM; [Na$^+$], 12 mM) distal lung epithelial cells. The predicted values of apical membrane currents ($I_{Na}$) for each ion were obtained by solving Ohm’s Law using the values of $G_{Na}^+$ and $G_{Cl}^-$ obtained in the present study. The total current is the sum of all ionic currents. Glib., glibenclamide.

Recent as $V_m$ and internal $[Cl^-]$ are both subject to rapid change at this time (22, 42). During prolonged stimulation, however, $G_{Cl}^-$ remains relatively low, and so the total, inwardly directed $Cl^-$ current would amount to only $\sim 1$ $\mu$A cm$^{-2}$ (Table 2). Most importantly, our model predicts that this initial transient would be followed by a slowly developing but persistent rise in $I_{Na}$ that is due, very largely (>90%), to increased $Na^+$ absorption. There is thus excellent agreement between the observed and predicted responses, and so the changes in $G_{Cl}^-$ and $G_{Na}^-$ that we now report can explain the well-documented effects of isoprenaline upon these cells (23, 30, 36–38). Moreover, in A6 cells, a $Na^+$-absorbing cell line derived from the amphibian kidney, $cAMP$-dependent agonists have been reported to elicit a biphasic response similar to that described above (7). This pattern of response may thus be a generalized feature of salt-absorbing epithelia.

Comparison with previous work. When stimulated with $cAMP$-coupled agonists, the adult cells used by Jiang et al. (16) displayed a rapid fall in $I_{sc}$ followed by a slow recovery toward the basal level consistent with a rapid stimulation of $Cl^-$ absorption superimposed upon a slowly developing stimulation of $Na^+$ transport, a finding that accords with data from earlier isotope flux studies of such cells (17). The fetally derived cells used in the present study, however, responded to isoprenaline with a clear stimulation of $Na^+$ transport that appeared to be accompanied by only a very small stimulation of $Cl^-$ secretion. As anion secretion is an important physiological feature of the fetal lung (31), it could be argued that the presence of this small secretory response implies that the cells have retained some features of the fetal lung.

There are thus clear physiological differences between the adult cells used by Jiang et al. (16) and the fetally derived cells used in the present study. However, the cells’ origin is not the only difference between the two studies. It may also be relevant that the cells used by Jiang et al. (16) were cultured on permeable supports for 5–7 days in a simple, serum-free medium devoid of hormones/growth factors, whereas our cells were normally cultured for only 48 h in a medium containing many of the hormones and growth factors present in serum. The present study shows clearly that the isoprenaline-evoked stimulation of amiloride-sensitive $I_{Na}$ is suppressed when fetally derived cells are maintained in the medium used by Jiang et al. It is therefore possible that their failure to detect a rise in $G_{Na}^-$ may be due, at least in part, to the fact that the culture medium used lacks a component needed for $\beta$-adrenoceptor-mediated control of alveolar $Na^+$ transport.

Possible role of the $CFTR$-like channels. The present study shows that prolonged stimulation with $cAMP$-coupled agonists leads to a clear rise in $G_{Na}^-$, which implies increased $Na^+$ entry despite a fall in $V_{Na}^-$. (Table 2). Because Jiang et al. (16) did not detect such control over $G_{Na}^-$, they concluded that $cAMP$-dependent agonists do not activate $Na^+$ channels in alveolar epithelia. Instead, they suggested that such drugs might stimulate $Na^+$ transport by activating the $CFTR$-like anion channels, hyperpolarizing the cell and thus increasing $V_{Na}^-$. (16). There are, however, conceptual problems with this model, some of which have been discussed by Widdicombe (44). For example, the analysis presented here suggests that $V_{Cl}^-$ is negative under resting conditions, and, rather than hyperpolarizing the cell, a rise in $G_{Cl}^-$ would initially favor depolarization and a fall in $V_{Na}^-$. (Table 2).

Difficulties remain even if the estimates of $V_m$ and $[Cl^-]$; used here are rejected to allow the premise that increased $G_{Cl}^-$ might hyperpolarize the cell (16, 29). If it is accepted that $[Na^+]_i$ normally lies between 12 and 25 mM, then $E_{Na}^-$ must be between 46 and 65 mV, and, so, in resting cells, $V_{Na}^-$ will be between 80 and 100 mV. There is thus a substantial driving force for $Na^+$ influx under resting conditions. If the rate of $Na^+$ entry is to double with no change in $G_{Na}^-$, then $V_{Na}^-$ must rise to at least 160 mV, which will require a hyperpolarization of at least 80 mV. Changes in $G_{Cl}^-$ cannot be transduced into such large changes in $V_m$ unless $E_{Cl}^-$ is at least 80 mV more negative than the resting $V_m$ (i.e., approximately $-120$ mV). Examination of this problem using the Nernst equation shows that this condition can be satisfied only if internal $[Cl^-]$ is $\sim 1$ mM, a value at least one order of magnitude lower than the values typically reported for mammalian epithelia. Moreover, the effect of selectively increasing $G_{Cl}^-$ upon $V_m$ will be dependent upon the contribution that $G_{Cl}^-$ makes to the membrane’s total ionic conductance. If the plasma membrane has substantial conductances to
Na\(^+\) and K\(^+\), then the effect of changing \(G_{\text{Cl}}\) will be smaller than described above. This implies that internal Cl\(^–\) must be even lower (i.e., the \(E_{\text{Cl}}\) must be even more negative) to allow Na\(^+\) transport to be controlled in the manner predicted by Jiang et al. (16).

In summary, the present study shows that, in cells derived from fetal animals but maintained under conditions that favor the development of an adult phenotype, the cAMP-dependent stimulation of Na\(^+\) transport reflects a rise in \(G_{\text{Na}}\) rather than the parallel stimulation of \(G_{\text{Cl}}\). Nevertheless, the studies of adult cells (16) suggest that this response is dependent, in some way, upon the presence of external Cl\(^–\). It is interesting, in this context, that studies of the fertilally derived cells have also shown that the isoprenaline-evoked stimulation of \(I_{\text{sc}}\) is abolished if Cl\(^–\) transport is blocked with a combination of bumetanide and DPC (35). In both experimental systems, the transport of Na\(^+\) and the transport of Cl\(^–\) thus seem to be linked in some way. Interestingly, changes in [Cl\(^–\)] have recently been shown to influence epithelial Na\(^+\) channel activity in some systems (8, 22). It is therefore possible that the apparent coupling between Na\(^+\) and Cl\(^–\)-transport (16, 29) may, at least in part, be due to [Cl\(^–\)]-dependent control of \(G_{\text{Na}}\).

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REFERENCES


40. **Seamon KB and Daly JW.** Forskolin a unique diterpene activator of cyclic AMP generating systems. *J Cyclic Nucleotide Res* 7: 201–225, 1981.


