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Iontophoretic β-adrenergic stimulation of human sweat glands: possible assay for cystic fibrosis transmembrane conductance regulator activity *in vivo*

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With the advent of numerous candidate drugs for therapy in cystic fibrosis (CF), there is an urgent need for easily interpretable assays for testing their therapeutic value. Defects in the cystic fibrosis transmembrane conductance regulator (CFTR) abolished β-adrenergic but not cholinergic sweating in CF. Therefore, the β-adrenergic response of the sweat gland may serve both as an *in vivo* diagnostic tool for CF and as a quantitative assay for testing the efficacy of new drugs designed to restore CFTR function in CF. Hence, with the objective of defining optimal conditions for stimulating β-adrenergic sweating, we have investigated the components and pharmacology of sweat secretion using cell cultures and intact sweat glands. We studied the electrical responses and ionic mechanisms involved in β-adrenergic and cholinergic sweating. We also tested the efficacy of different β-adrenergic agonists. Our results indicated that in normal subjects the cholinergic secretory response is mediated by activation of Ca^{2+}-dependent Cl^- conductance as well as K^+ conductances. In contrast, the β-adrenergic secretory response is mediated exclusively by activation of a cAMP-dependent CFTR Cl^- conductance without a concurrent activation of a K^+ conductance. Thus, the electrochemical driving forces generated by β-adrenergic agonists are significantly smaller compared with those generated by cholinergic agonists, which in turn reflects in smaller β-adrenergic secretory responses compared with cholinergic secretory responses. Furthermore, the β-adrenergic agonists, isoproprenaline and salbutamol, induced sweat secretion only when applied in combination with an adenylyl cyclase activator (forskolin) or a phosphodiesterase inhibitor (3-isobutyl-1-methylxanthine, aminophylline or theophylline). We surmise that to obtain consistent β-adrenergic sweat responses, levels of intracellular cAMP above that achievable with a β-adrenergic agonist alone are essential. β-Adrenergic secretion can be stimulated *in vivo* by concurrent iontophoresis of these drugs in normal, but not in CF, subjects.

Both cholinergic and β-adrenergic agents can evoke sweat secretory responses from glands independently, but the magnitudes of the secretory responses are significantly different (Sato *et al.* 1989). The maximal secretory rates after cholinergic agonists are several-fold larger than sweat rates induced by β-adrenergic agonists. The lack of sweating in cystic fibrosis (CF) homozygotes and the reduced sweating of CF heterozygotes reflects reduced levels of functional gene product that are rate limiting for β-adrenergically stimulated sweat secretion (Sato &

Sato, 1984; Behm *et al.* 1987; Davis & Byard, 1989; Johnson *et al.* 1991). These observations indicate that these secretogogues have different mechanisms of secretion (Sato, 1984; Sato & Sato, 1984) and suggest that the β-adrenergically stimulated sweat response could serve not only as an *in vivo* diagnostic tool for CF, but also as an assay for therapeutic corrections of dysfunction (Best & Quinton, 2005). The recent advent of several candidate drugs for therapy in CF will probably require improved, more easily interpretable assays of their effects

on cystic fibrosis transmembrane conductance regulator (CFTR) function *in vivo* (Welsh, 1994). Hence, we sought to define conditions for eliciting consistent β-adrenergic responses *ex vivo* by using isolated human sweat glands in skin biopsies, where drug concentrations and combinations are readily controlled. We first examined the electrophysiological driving forces during stimulus–secretion coupling with β-adrenergic and cholinergic stimulation of primary cultures of sweat gland secretory cells from normal and CF subjects and of microperfused tubules from sweat gland secretory coils. These *in vitro* studies provide insights for potentially managing cellular mechanisms to ensure that selective β-adrenergic stimulation is consistently effective *in vivo*.

Earlier work on β-adrenergic stimulation of sweat glands *in vivo* applied a combination of isoproprenaline (IPR) and theophylline administered by subcutaneous injection (Sato & Sato, 1984; Behm *et al.* 1987) to raise intracellular cAMP levels. However, IPR has broad β-adrenergic effects involving both β_1- and β_2-adrenergic receptors, which can induce undesirable systemic side-effects (tachycardia, anxiety and papillary dilatation), and subcutaneous injection is fraught with disadvantages that can be avoided by local iontophoresis, which is preferred for stimulating sweat locally. Unfortunately, none of the commonly used phosphodiesterase inhibitors, forskolin (Fsk), 3-isobutyl-1-methylxanthine (IBMX) or theophylline, can be iontophoresed because they are not charged. Moreover, only theophylline is Food and Drug Administration (FDA) approved for use in humans. Thus, we investigated the efficacy of FDA-approved and commonly available pharmaceutical drugs, including the selective β_2-adrenergic agonist, salbutamol (Sal), and the phosphodiesterase inhibitor, aminophylline, to elicit consistent β-adrenergic secretory responses from sweat glands of normal subjects that would enable comparisons with the CF gland response for diagnosing CF or for assessing the therapeutic value of putative CF drugs. We found that a β_2-adrenergic agonist (such as Sal) must be combined with a phosphodiesterase inhibitor (such as aminophylline) for effective β-adrenergic stimulation when using iontophoresis to apply drugs to stimulate local sweating.

Methods

Tissue collection

After obtaining written informed consent, three or four biopsy specimens were quickly removed from the area over the scapula with an electric rotating punch (4 mm in diameter) from 29 normal young adult male volunteers. No anaesthetic was administered prior to biopsy. All wounds were closed with a suture and protected with a dry dressing. Sutures were removed after one week. Biopsied

tissue was immediately immersed in Ringer solution, stored at 4°C and used within 36 h. All protocols were approved by the UCSD Internal Review Board for human subjects and complied with the principles outlined in the Declaration of Helsinki.

Cell cultures

The primary cells from both normal and CF sweat secretory coils were grown as previously described in detail (Reddy & Bell, 1996). In short, isolated secretory coils were plated in a small culture dish containing droplets of medium. The secretory coil fragment was drawn out of droplet and attached by surface tension to the plastic before being quickly covered with medium from the surrounding droplets (Petersen, 1980). The explants were then left undisturbed for at least 12 h at 37°C in a humidified 5% CO_2 −95% air incubator. The next day, 1.5 ml of fresh medium was added, and subsequently replaced every 2 days. After explanting the isolated secretory coils, cells started proliferating in 2–3 days. We studied cells in culture from 5 to 23 days.

To investigate the electrophysiological properties of the cultured secretory coil cells, a plastic rectangle (6 × 12 mm) bearing the culture cells was cut from the dish and transferred to an experimental chamber where the cells were observed with an inverted microscope and perfused with Ringer solution at 37°C as described previously (Reddy & Quinton, 1992*a*). Microelectrodes were pulled from glass capillaries using a microelectrode puller (model P-80/PC; Sutter Instrument Co., Novato, CA, USA) and were filled with 3 M potassium acetate. Electrode resistance was 50–100 MΩ, and tip potentials were < 4 mV. A piezoelectric hybrid manipulator (PM500-20; FrankenBerger, Munich, Germany) mounted on an *X–Y* manipulating base was used to impale the cells. Cell membrane potentials were measured using a Dagon single-electrode voltage-clamp amplifier (Minneapolis, USA) referenced to bath through an agar bridge electrode. In general, the criteria for accepting the results from the impaled cells are the same as defined previously (Reddy *et al.* 1992; Reddy & Quinton, 1992*a*).

Isolated secretory tubules

Individual sweat glands from the skin biopsy were isolated from the skin in chilled Ringer solution by dissection with fine-tipped tweezers visualized at a magnification of ×80. The secretory coil was transferred to a perfusion chamber containing Ringer solution at 35 ± 2°C and microperfused (Quinton & Reddy, 1992; Reddy *et al.* 1999). Transepithelial potential (V_t) was measured using the perfusion pipette as the recording electrode (Quinton, 1986; Reddy & Quinton, 1992*b*) referenced to the bath via an agar bridge.

Intact glands *ex vivo*

Each skin plug (biopsy) was gently pushed through a 3.5 mm hole in a small Teflon wafer. The wafer was supported in a chamber so that the epidermal tissue was covered with mineral oil saturated with water while the subdermis was bathed in Ringer solution below (Prompt & Quinton, 1978; Quinton, 1981). Sweat secretions were either stimulated by agonists and/or inhibited by blockers added to the bathing solution. With the aid of a dissecting microscope, sweat secretion from individual glands was observed on the surface of the skin as discrete droplets of aqueous fluid in the oil. Sweat droplets were collected intermittently in constant-bore glass capillaries between oil blocks. Rates of secretion were calculated by dividing the volume of collected fluid by the time between collections. First we identified and determined the basal rate of secretion of each sweat gland in the bath during an initial period of at least 10 min, after which we washed out the tissue with fresh Ringer solution for about 15 min. Then we added drugs as required. Each drug tested was followed by a 15 min wash-out with fresh Ringer solution rinses. We collected sweat from single glands at approximately 2 min intervals. The experiments were conducted at $35 \pm 2°C$.

In vivo sweat stimulation

In order to avoid invasive subcutaneous injection of solutions *in vivo* we elected to iontophoresis drugs through the skin. A small area of skin on the volar surface of the forearm was gently cleaned with alcohol sponge wipes. A reference electrode consisting of a 3 cm × 4 cm flat metal electrode was attached over a saline-wetted cotton sponge to the dorsal surface of the forearm and connected to the negative terminal of the current source. The tip of a fire-polished glass-pipette (i.d. 1.1 mm) was placed in the centre of the previously cleaned skin surface. For cholinergic sweat stimulation, the pipette was filled with 1% pilocarpine and connected to the positive terminal of the current source (variable DC current battery source) via a silver electrode inserted inside the pipette. Direct current was gradually increased to 20 μA and applied for a period of 5 min. For *β*-adrenergic stimulation, three separate but adjacent pipettes were used for three different drugs. The centre pipette was filled with 1% atropine and 20 μA current was passed for a period of 5 min to inhibit of cholinergic secretion. The two other pipettes were filled with 1% Sal and 1% aminophylline, respectively, and connected to two separate current sources adjusted to pass the same amount of current (20 μA) for 5 min after the atropine iontophoresis. After stimulation, the electrodes were removed and the area was gently wiped with cotton sponges, painted with 2% iodine solution in ethanol and blown dry with air. After a prescribed period of spot stimulation, a small square of white typing paper was gently pressed over the coated area for ~1 min so that black dots appeared on the paper due to the starch/iodine reaction with sweat water (Randall, 1946; Shamsuddin & Togawa, 2000). Dots reflect the size and position of the sweat droplets on the skin.

Solutions and drugs

Incubation media contained the following (in mM l^{-1}): NaCl, 125; NaHCO$_3$, 25; Ca(C$_2$H$_3$O)$_2$, 1; MgSO$_4$, 1; glucose, 10; KH$_2$PO$_4$, 0.38; and K$_2$HPO$_4$, 2.13 and were bubbled with 5% CO$_2$ and 95% O$_2$ at 35°C to maintain pH at 7.4. Hepes Ringer solution with BaCl$_2$ contained the following (in mM l^{-1}): NaCl, 150; Hepes, 10; Ca(C$_2$H$_3$O)$_2$, 1; MgSO$_4$, 1; BaCl$_2$, 5; and glucose, 10 (pH adjusted to 7.4 with isotonic NaOH). For Ca^{2+}-free Ringer solution, 0 mmol l^{-1} Ca^{2+} and 2 mmol l^{-1} EGTA were used.

We added methacholine, a stable analogue of acetylcholine (MCh, 1 μM), IPR (10 μM), Sal (10 μM), IBMX (100 μM), Fsk (10 μM), aminophylline (100 μM), theophylline (100 μM), 5,6-dichloro-1-ethyl-1,3-dihydro-2H-benzimidazol-2-one (DCEBIO, 10 μM), chromanol 293B (10 μM), tetrapentylammonium (TPeA, 10 μM), tolbutamide (100 μM), tetraethylammonium (TEA, 5 mM) and Ba^{2+} (5 mM) to the Ringer solution either separately or in combination as needed. Atropine (1 μM) was added to all solutions containing *β*-adrenergic agonists to avoid any possibility of cross-stimulating cholinergic sweating with adrenergic agonists. All chemicals were obtained from Sigma Chemical Co. (St Louis, MO, USA).

Data analysis

The data are presented as means $\pm$ s.e.m. Statistical significance was determined on the basis of Student's paired *t* test, and $P < 0.05$ was taken to be significantly different. For graphic comparisons of the different effects (separately or in combination), we selected a representative gland from each plug.

Results

Secretory rates

Cholinergic stimulation of intact glands consistently produces several-fold greater secretory rates than *β*-adrenergic stimulation of same glands (Fig. 1*A* and *B*). The difference in rates is probably due to different ionic mechanisms or degrees of their activation by these agonists that determine the electrochemical driving forces that control secretion. We therefore first examined the electrical

responses of cultured secretory cells from normal and CF glands.

Intracellular potentials (V_m) in cultured cells

Cholinergic responses of V_m. In normal cells, methacholine (MCh) hyperpolarized the cell membrane potentials (V_m) by 19.7 ± 5.1 mV (from -61.5 ± 2.7 to -81.2 ± 4.1 mV, $n = 6$; Fig. 2*A*). After removing MCh, V_m depolarized immediately, with an overshoot beyond the resting membrane potential (Fig. 2*A*). The recovery from the overshoot of V_m required 3–5 min.

Application of MCh to the CF cultured cells also hyperpolarized the V_m, by 11.5 ± 4.1 mV (from -60.8 ± 4.6 to -72.3 ± 3.9 mV, $n = 6$). Following wash-out of MCh, V_m values depolarized almost immediately, with an overshoot beyond the resting membrane potential (Fig. 2*B*).

β-Adrenergic responses of V_m. Cholinergic stimulation always resulted in an initial hyperpolarization followed by sustained secondary depolarization of V_m of both normal and CF cells (Fig. 2*A* and 2*B*). In contrast, addition of the β-adrenergic agonist IPR always resulted in monophasic depolarization of V_m (from -64.7 ± 4.1 to -50.3 ± 1.9 mV, $n = 6$) in normal cells without hyperpolarization (Fig. 2*C*; Reddy & Bell, 1996; Reddy *et al.* 1997), while CF cells failed to respond to β-adrenergic stimulation (Fig. 2*D*).

Potassium conductance. Elevation of extracellular $[K^+]$ from 5 to 50 mM resulted in a significant depolarization of V_m from -65.0 ± 6.5 to -35.0 ± 1.9 mV, i.e. a change of 30.0 ± 8.4 mV, $n = 6$, in normal cultured cells and from -50.7 ± 8.2 to -27.8 ± 5.2 mV, i.e. a change of 22.8 ± 3.6 mV, $n = 3$, in CF culture cells (Fig. 3*A*, *B* and *C*). After wash-out of high $[K^+]$, the V_m returned to baseline with an undershoot that was consistent with the activation of the Na^+–K^+ pump when the extracellular $[K^+]$ is elevated (Reddy & Quinton, 1991, 2006).

Application of K^+ channel inhibitor, Ba^{2+}, significantly depolarized the V_m of both normal and CF cells in primary culture (Fig. 3*D*, *E* and *F*) by 27.3 ± 3.9 mV, $n = 5$ (from 56.3 ± 11.1 to -29.0 ± 7.2 mV), and 24.0 ± 5.5 mV, $n = 3$ (from 50.7 ± 8.2 to -26.7 ± 5.1 mV), respectively. There was no apparent difference between normal and CF cell responses to K^+ and Ba^{2+}.

Electrophysiological responses of secretory cell V_m in primary culture to cholinergic stimulation were insensitive to 5 mM TEA (K^+ channel blocker; Fig. 4).
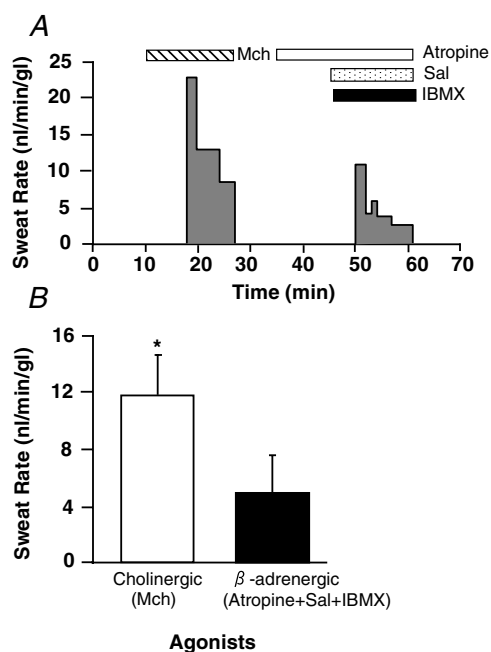
Transepithelial potentials (V_t) of isolated secretory coils

The magnitudes of V_t induced by cholinergic and β-adrenergic agonists are significantly different. The V_t of the microperfused secretory coil was hyperpolarized by -25 and -11 mV when stimulated with the cholinergic agonist MCh and the β-adrenergic agonist IPR, respectively, in the presence of an imposed transepithelial Cl^- gradient (150 mM NaCl in the bath and 150 mM sodium gluconate in the lumen) as shown in Fig. 5*A* and *B*.

Intact glands *ex vivo*

Potassium conductance. As shown in Fig. 6*A* neither adding Sal alone nor adding the cAMP-dependent K^+_{cAMP} channel activator, DCEBIO, to increase the driving force for Cl^- secretion (Singh *et al.* 2001) stimulated secretion in intact glands. However, after adding IBMX in the presence of Sal, secretion started within 5 min (also see Fig. 10*E*). These data suggested that sweat secretion is not dependent on a DCEBIO-inducible K^+ channel.

The K^+_{cAMP} channel blocker, chromanol 293B, also had no effect on Fsk + IBMX + Sal-induced sweating in intact glands. After removing atropine and other cAMP-mediated agonists, the cholinergic agonist MCh also induced secretion in the presence of chromanol 293B. These results suggested that the K^+_{cAMP} channel does not

Figure 1. A comparison of secretory responses of intact glands induced by cholinergic and β-adrenergic agonists
A, a representative example showing the time course of sweating response with the cholinergic agonist, methacholine (MCh), and the β-adrenergic agonists, salbutamol and IBMX. *B*, summary of the data comparing the sweating responses induced by cholinergic and β-adrenergic agonists. We alternated the order of adding drugs to help compensate for any time-related decreases in responses.

play a significant role in either β-adrenergic or cholinergic secretory responses (Fig. 6*B*).

The β-adrenergic and cholinergic sweating responses were attenuated in the presence of the non-specific K^+ channel blocker, Ba^{2+}. The effect of Ba^{2+} was reversible, since the secretory response of β-adrenergic and cholinergic agonist returned to normal levels following wash-out. These results suggested that Ba^{2+} partly, but not completely, blocked both the β-adrenergic and the cholinergic responses (Fig. 7*A*, *B* and *C*). The results are consistent with the presence of the Ba^{2+}-sensitive K^+ conductance noted in cultured sweat gland secretory cells shown in Fig. 3*A–F* (Reddy & Bell, 1996).

The β-adrenergic sweat secretory responses of intact glands were not affected by other K^+ channel blockers, namely, tolbutamide (100 μM) and TPeA (10 μM), as shown in Fig. 8*A*, even when applied together during either cholinergic or β-adrenergic sweat secretion (Fig. 8*A–C*).

Furthermore, TEA (Ca^{2+}-activated K^+ channel blocker, 5 mM) did not have any effect even on cholinergic secretory responses of intact glands (data not shown).

Calcium dependence. Sweat secretion in intact sweat glands showed that cholinergic, but not β-adrenergic, sweat secretory responses were abolished by removing Ca^{2+} from the medium (Fig. 9). The results are consistent with previous findings that both the apical Cl^- and basolateral K^+ conductances (Reddy & Bell, 1996) activated by cholinergic agonists are Ca^{2+} mediated.

β-Adrenergic stimulation. Isoprenaline alone failed to stimulate secretion. The secretory response to the cholinergic agonist, MCh, was completely blocked by atropine, but was restored following the wash-out of the inhibitor (Fig. 10*A*), and after blocking the cholinergic
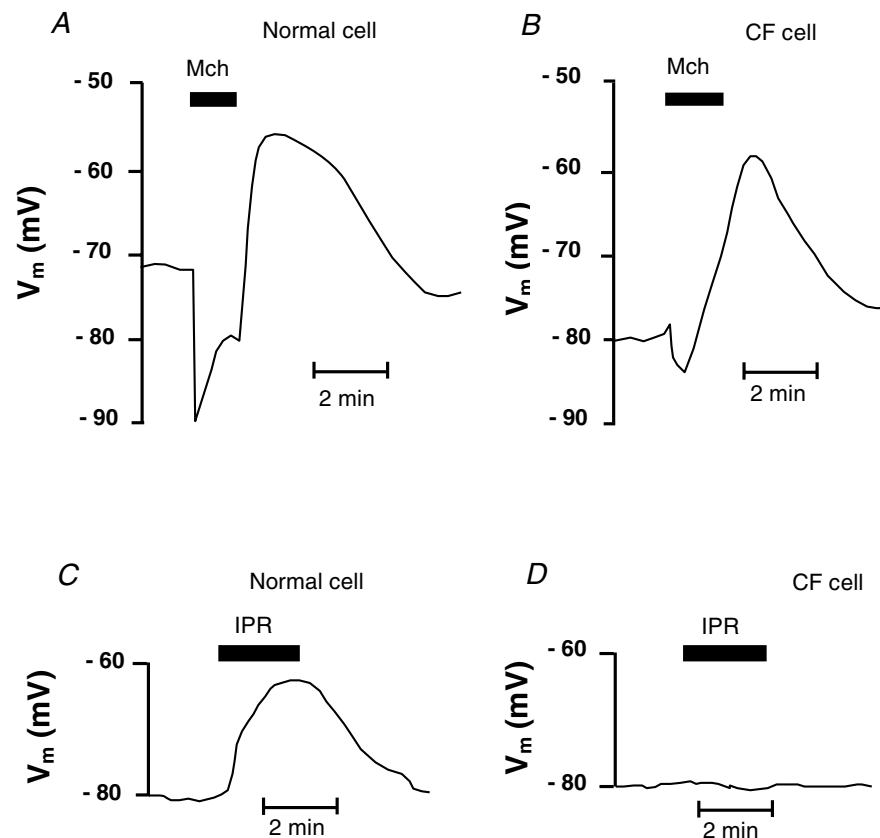

Figure 2. Electrophysiological responses of secretory cells in primary culture to cholinergic and β-adrenergic stimulation
A and *B* represent the cell membrane potential (V_m) responses induced by cholinergic stimulation of normal and CF cells, respectively. Notice that cholinergic stimulation induced a biphasic electrical response from both normal and CF cells; that is, an initial hyperpolarization, which is consistent with activation of a K^+ conductance, and a secondary depolarization, which is consistent with activation of a Cl^- conductance (please see Discussion for details). *C* and *D* represent the cell membrane potential responses induced by β-adrenergic stimulation of normal and CF cells, respectively. β-Adrenergic stimulation induced a monophasic depolarization of a normal cell (*C*), but had no effect on the CF cell (*D*).
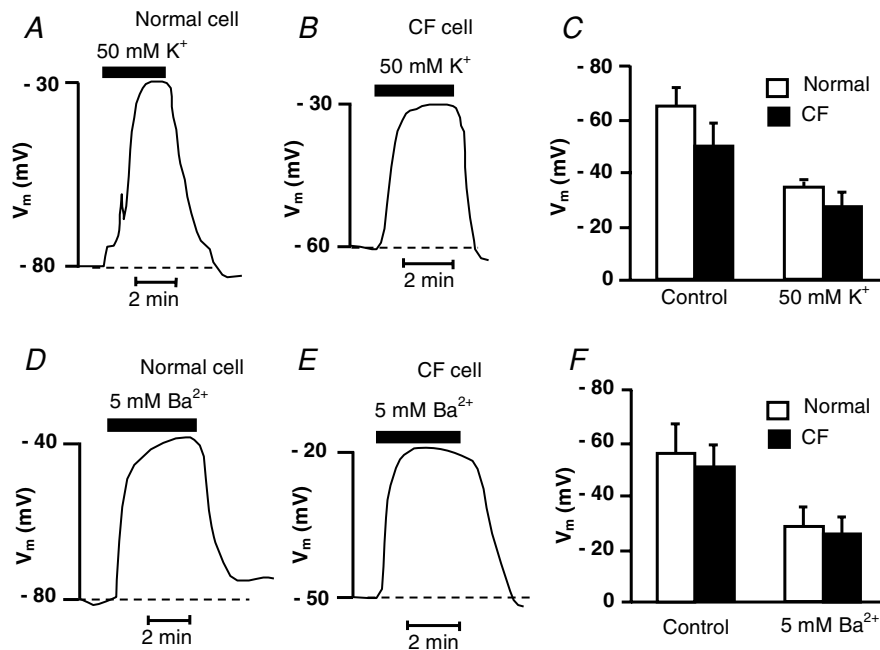
Figure 3. K$^+$ conductance of normal and CF cells
Effect of elevating bath K$^+$ on the V_m of normal and CF secretory cells in culture. Raising extracellular K$^+$ by 10-fold resulted in a sub-Nernstian response (<60 mV) of both normal (*A*) and CF cells (*B*), and the effects of 50 mM K$^+$ on membrane potential (V_m) of normal and CF cells were not significantly different (*C*). Effect of Ba^{2+} on the V_m of normal (*D*) and CF secretory cells (*E*). Barium ions partly blocked a 'resting' K$^+$ conductance of cells that resulted in depolarization of both normal and CF cell membrane potentials and was not significantly different between normal and CF cells (*F*).

response with atropine, the β-adrenergic agonist IPR alone also had no effect even after 20 min; however, adding the cAMP-elevating agents, IBMX (phosphodiesterase inhibitor) and Fsk (adenylyl cyclase activator), in the presence of IPR induced secretion within 10 min.

3-Isobutyl-1-methylxanthine alone failed to stimulate secretion. We added IBMX (phosphodiesterase inhibitor) in the bath after blocking the cholinergic response with atropine. The results showed that IBMX alone had no effect even after 30 min, but secretion started after adding

Figure 4. Response of V_m of a secretory cell in culture to cholinergic stimulation is insensitive to another K$^+$ channel blocker, TEA (5 mM)

the β_2-selective adrenergic agonist, Sal (Fig. 10*B*). After applying IBMX and Sal, adding the cyclic AMP-elevating agent, Fsk, appeared to further enhance secretory activity. Figure 10*C* summarizes the experiments shown in Fig. 10*A* and *B*, comparing the sweat responses induced by different agonists when applied separately and in combination.

Forskolin alone failed to stimulate secretion. We added Fsk only to the bath after blocking the cholinergic response with atropine. The result showed that Fsk alone had no effect even after 20 min, but induced significant secretion when added in combination with the β_2-adrenergic agonist, Sal (Fig. 10*D*). The secretory response to the cholinergic agonist, MCh, when added alone after washing out atropine showed the continued viability and responsiveness of the tissue (Fig. 10*D*).

Singularly, Sal did not activate secretion; however, adding IBMX to Sal stimulated secretion (Fig. 10*E* and *F*). The response to the combination seemed significantly delayed, which might be due to the time required to increase intracellular levels of cAMP after inhibiting the phosphodiesterase (Fig. 10*E*). A summary of sweat responses to the application of these agonists either alone or in combination is presented in Fig. 10*F*. Overall, the data show that a combined application of Sal with Fsk or IBMX is required to stimulate a sweating response ($P = 0.03$), whereas no sweating was induced by any agonist when applied singularly.
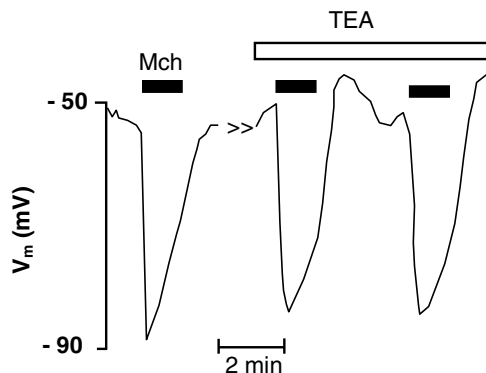
We examined the effect of other phosphodiesterase inhibitors. Since we cannot iontophorese theophylline, we evaluated the effects of its ionic analogue, aminophylline. Theophylline alone had no effect after 20 min of application, but adding Sal induced secretion within 5 min (Fig. 11*A*). Aminophylline gave similar results (Fig. 11*B* and *C*), suggesting that both of the FDA-approved drugs, aminophylline and theophylline, are equally effective.

Sweat glands *in vivo*

Figure 12 shows the application of these *in vitro* findings to stimulation *in vivo*. Spot iontophoresis of 1% pilocarpine or 1% Sal plus 1% aminophylline was done after 1% atropine was applied for 5 min with 20 μA current on the volar surface of the forearm. Subsequently, sweat detected by the starch/iodine test showed that normal subjects (numbers 1–3) responded to both cholinergic and *β*-adrenergic stimulation, but that the CF subject (number 4) subject responded only to cholinergic stimulation.

Discussion

One of the most consistent characteristics of pathophysiology in CF is the failure of *β*-adrenergically mediated secretion (Sato & Sato, 1984; Behm *et al.* 1987; Johnson *et al.* 1991). The defect was first described in the sweat gland (Sato & Sato, 1984) and has been confirmed repetitively since (Behm *et al.* 1987; Johnson *et al.* 1991; Best & Quinton, 2005). However, other than refractory responses of electrical potential differences in the nose (Knowles *et al.* 1981, 1983; Delmarco *et al.* 1997; Rodgers & Knox, 1999; Standaert *et al.* 2004), there has been little practical application of this property. In addition, the fact that there is a growing number of potential drugs that are being introduced into clinical trials as therapy for CF has stimulated interest in developing simple, straightforward assays that will provide rapid, accurate
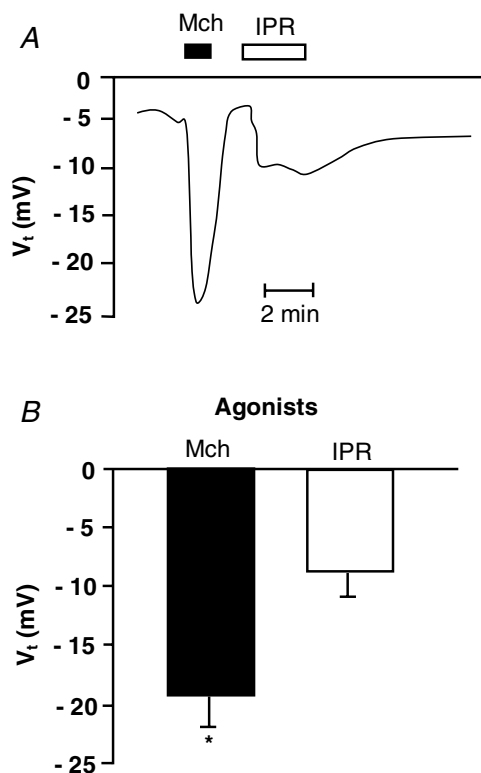

Figure 5. Transepithelial electrical potential (V_t) responses of intact secretory coil to cholinergic and *β*-adrenergic agonist stimulation

A, the magnitude of V_t hyperpolarization in response to cholinergic stimulation is larger compared with that induced by *β*-adrenergic agonist stimulation in the presence of an imposed tranepithelial Cl⁻ gradient (150 mM NaCl, bath and 150 mM sodium gluconate in the lumen). *B*, summary of experiments such as that shown in *A*. The cholinergic response is significantly larger than the *β*-adrenergic stimulation response ($P < 0.05$, $n = 3$).
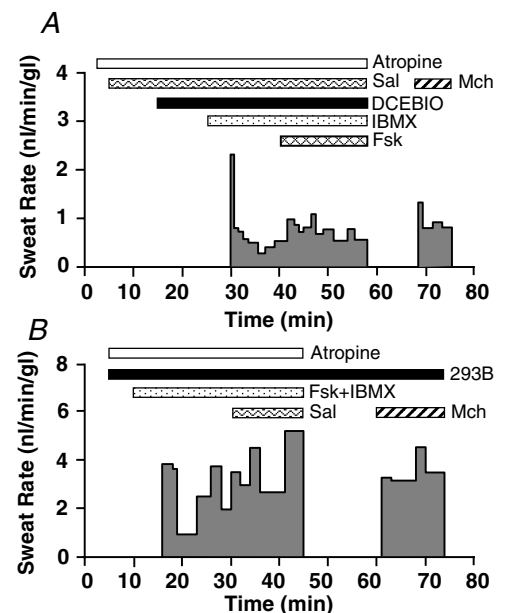

Figure 6. Role of K⁺ channels in sweat secretion

A, lack of effect of DCEBIO, a cAMP-dependent K⁺ (K^+_{cAMP}) channel activator, on $β_2$-adrenergic stimulation in intact glands. Salbutamol alone had no effect within 10 min or after another 10 min, after adding DCEBIO to increase the driving force for Cl⁻ secretion. After adding IBMX, however, secretion started within 5 min. These preliminary data suggested that sweat secretion is not limited by DCEBIO-inducible K⁺ channels. *B*, lack of effect of the K^+_{cAMP} channel blocker, chromanol 293B, on $β$-adrenergic and cholinergic secretion. In the presence of atropine and chromanol 293B, Fsk- and IBMX-induced sweating was enhanced by the $β_2$-adrenergic agonist, salbutamol. After removing atropine and other cAMP-mediated agonists, the cholinergic agonist, MCh, also induced secretion in the presence of chromanol 293B. These results suggested that K^+_{cAMP} channels may not have a role in *β*-adrenergic or cholinergic secretory responses.

assessment of drug effects on *in vivo* function. To that end, we have focused on the possible use of the pronounced differences between β-adrenergically stimulated sweat in CF homogyzotes (virtually no response), obligate heterozygotes (intermediate response) and homozygote normal subjects ('full' response). The approach is not without problems, however.

One of the difficulties in using β-adrenergic sweating arises from the fact that purely β-adrenergic sweating even in normal subjects is weak and relatively small compared with cholinergically stimulated sweating (Fig. 1; Sato & Sato, 1984; Behm *et al.* 1987). Normal thermoregulatory sweating is mediated via autonomic cholinergic receptors (Cummings, 1960; Nakazato *et al.* 2005; Shibasaki *et al.* 2006). There is no known function of the β-adrenergically

stimulated component (Gordon *et al.* 1987), but it is recognized that stimulating with a β-agonist alone induces cross-over cholinergic stimulation. Previously, in order to reliably obtain β-adrenergic sweating *in vivo*, investigators stimulated local sweating with a cocktail of isoprenaline, theophylline and atropine injected subcutaneously (Sato & Sato, 1984; Behm *et al.* 1987). Isoprenaline was used to stimulate the intracellular synthesis of cyclic AMP from ATP via adenylyl cyclase. Theophylline was used to block the degradation of cAMP via phosphodiesterase and thereby enhance its build-up, and atropine was used to prevent any neuronal cross-talk between adrenergic and cholinergic innervation.

While drug injections are not formidable impediments, they are invasive and do introduce potential variability in delivery as well as generally requiring licensed personnel to administer injections. In contrast, the Quantitative Pilocarpine Iontophoresis Test (QPIT) or 'sweat test',
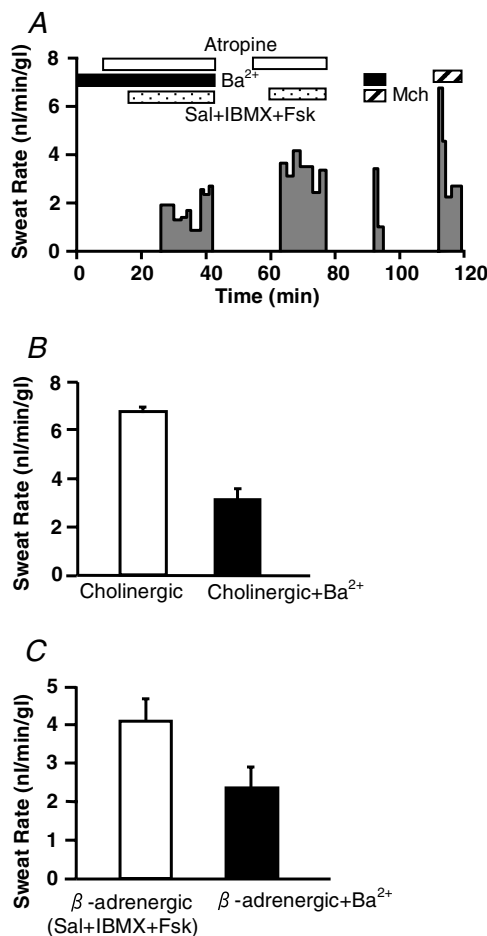

Figure 7. Effect of Ba^{2+} on β-adrenergic and cholinergic sweat secretion
A, the effect of the K^{+} channel blocker, Ba^{2+}, on β-adrenergic and cholinergic responses in intact glands. The promiscuous K^{+} channel blocker, Ba^{2+} attenuated β-adrenergic and cholinergic responses. After removing Ba^{2+}, the β-adrenergic and cholinergic agonist-induced secretions increased. Comparison of cholinergic secretion (*B*) and β-adrenergic secretion (*C*) without and with Ba^{2+}. These results suggested that Ba^{2+} partly blocked both β-adrenergic and cholinergic responses.
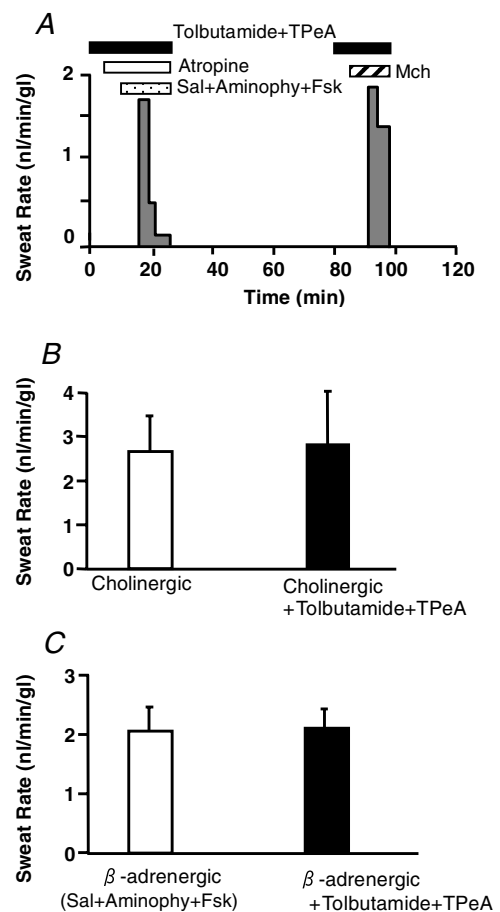

Figure 8. No effect of tolbutamide or TPeA on sweat secretion
Lack of effect of combined application of K^{+} channel blockers, tolbutamide (100 μM) and tetrapentylammonium (TPeA, 10 μM), on β-adrenergic and cholinergic secretions in intact glands (*A*). Comparison of cholinergic secretion (*B*) and β-adrenergic secretion (*C*) with the combined application of K^{+} channel blockers, tolbutamide (100 μM) and tetrapentylammonium (TPeA, 10 μM).
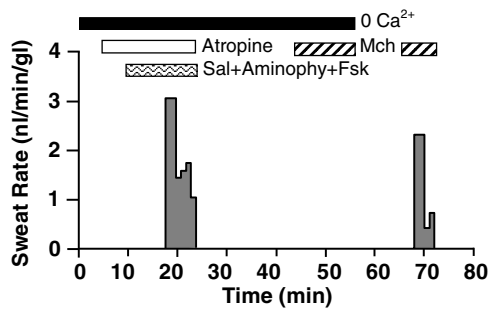
Figure 9. Roles of Ca²⁺ in cholinergic and β-adrenergic stimulation of intact glands

Cholinergic but not β-adrenergic sweat secretory responses were abolished by removing Ca²⁺ from the medium. These observations indicated that either or both the apical Cl⁻ and/or basolateral K⁺ conductances activated by cholinergic agonists are strictly mediated by Ca²⁺.

as conventionally carried out, has been employed in the diagnosis of CF for more than four decades and avoids some of these problems. Iontophoresis is a simple, non-invasive, reproducible, easily controlled method for administering small amounts of drug locally. Iontophoresis uses electrical voltage to force an ionized form of a drug across a barrier, in this case the skin, as a current that can be easily adjusted and monitored for control of drug delivery. In this context, we sought to define some of the parameters that seemed significant for selectively stimulating β-adrenergic sweating via iontophoretic drug administration.

We first evaluated the ion conductances thought to be required for β-adrenergic secretion and then sought forms of drugs amenable to iontophoretic use. In response to cholinergic stimulation (MCh), primary
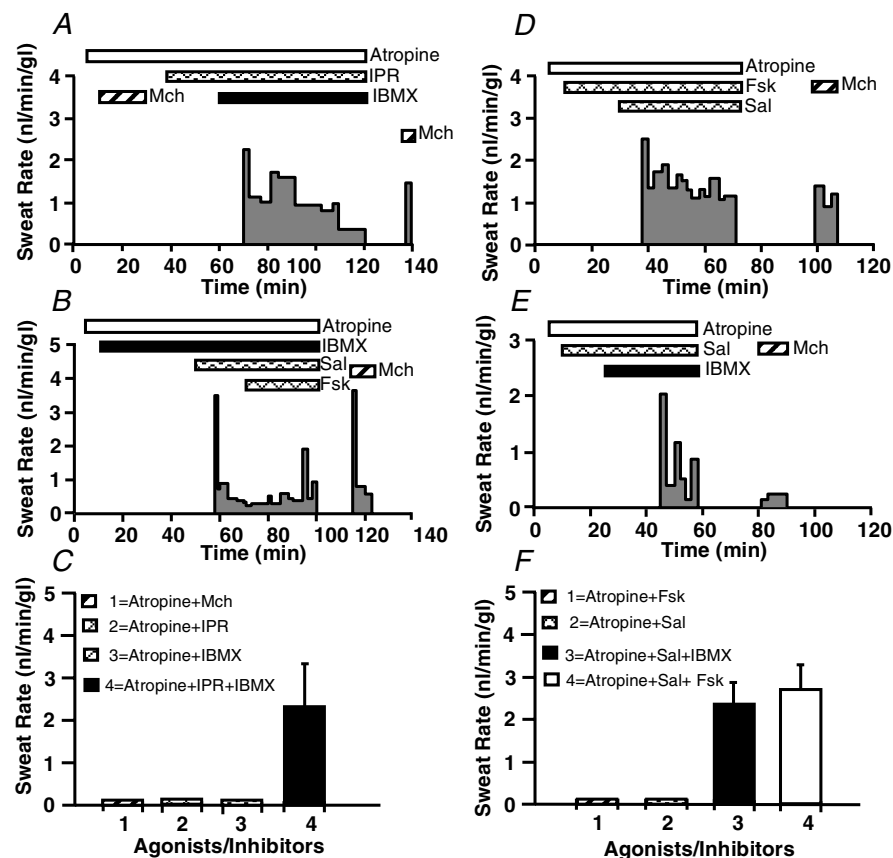

Figure 10. β-adrenergic sweat secretion requires multiple agonists

A, lack of effect of β-adrenergic agonist, isoprenaline (IPR), alone, but activated after adding phosphodiesterase inhibitor, IBMX, in intact glands. *B*, lack of effect of phosphodiesterase inhibitor, IBMX, alone, but activated after adding salbutamol in intact glands. Adding adenylyl cyclase activator, Fsk, to elevate cAMP maximally further enhanced secretory activity. *C*, summary of the experiments similar to those shown in *A* and *B*, which compares the sweating responses induced by different agonists. *D*, lack of effect of elevating cAMP by Fsk alone, but showing activation after adding the β₂-adrenergic agonist, salbutamol, in intact glands. *E*, lack of effect of salbutamol alone, but showing stimulation after adding IBMX in intact glands. *F*, summary of the experiments that are similar to those shown in *D* and *E*, which compares the sweating responses induced by different agonists.

cultured secretory cells from both control and CF subjects showed immediate cell membrane potential hyperpolarization with a rebound depolarization (Fig. 2*A* and *B*). However, with β-adrenergic stimulation the membrane only depolarized, but not in non-CF cells (Fig. 2*C* and *D*). These results seem consistent with an initial cholinergically activated K$^+$ conductance (G_{K+}) that immediately hyperpolarized the cell owing to increased K$^+$ loss followed by activation of a Cl$^-$ conductance (G_{Cl^-}) that depolarized V_m as Cl$^-$ conductance increased. When the agonist was removed, G_{K+} deactivated and the V_m depolarized further until the Cl$^-$ conductance also apparently deactivated over the subsequent brief period (Fig. 2). The fact that only depolarization was seen in β-adrenergically stimulated non-CF cells strongly suggests that only a G_{Cl^-} was activated.

Since the electrical driving force that supports Cl$^-$ secretion across the apical membrane is due to the K$^+$-dependent hyperpolarization of V_m as a function of activating a G_{K+} in the basolateral membrane, we attempted to characterize the G_{K+}. Classical responses to increased extracellular K$^+$ and Ba^{2+} were clearly present (Fig. 3) even though hyperpolarization was only partly blocked by Ba^{2+} and appeared insensitive to the Ca^{2+}-activated K$^+$ channel blocker, TEA (Fig. 4).

Since β-adrenergic secretion is mediated via increased levels of intracellular cAMP, it seemed reasonable to expect that a cAMP-dependent G_{K+} (K$^+_{cAMP}$) might increase the driving force and enhance β-adrenergic sweating. We tested the effects of DCEBIO (an activator of the K$^+_{cAMP}$ KvLQT1 channel) and its corresponding inhibitor (chromanol 293B) on intact gland secretion *ex vivo* in skin biopsy plugs. The DCEBIO did not produce any marked effect on β-adrenergic sweating, and chromanol 293B did not inhibit cholinergic or adrenergic sweat secretion (Fig. 6*A* and *B*), suggesting that the K$^+_{cAMP}$ channel (KvLQT1) is not involved in either form of sweat secretion. Similarly, even combining the ATP-dependent K$^+$ channel blocker, tolbutamide, with the Ca^{2+}-activated K$^+$ channel blocker, TPeA, did not block *ex vivo* non-CF gland secretions, suggesting that these channels probably do not support either β-adrenergic or cholinergic secretion
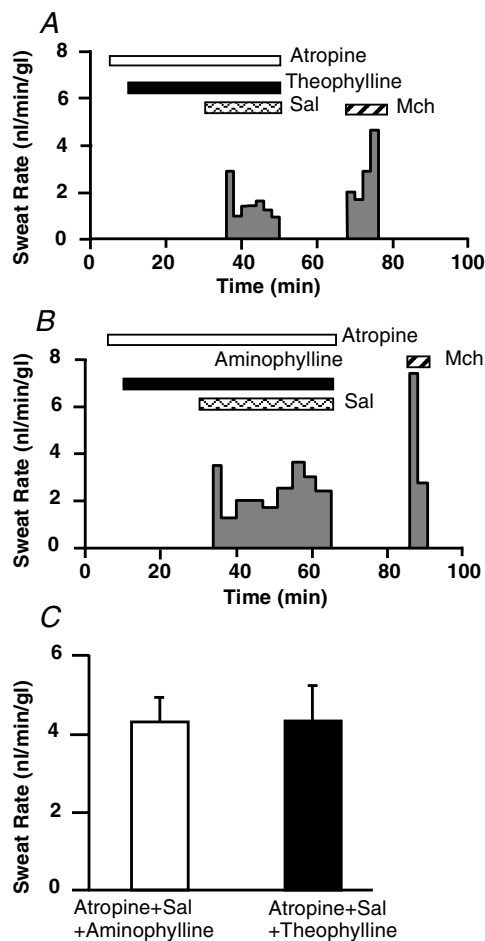

Figure 11. Aminophylline and theophylline are equally effective agonists in intact glands
Neither aminophylline nor theophylline alone stimulated sweating, but both stimulated secretion when combined with salbutamol. *A*, a representative example of the time course of sweating response with salbutamol and theophylline. *B*, a representative example of the time course of sweating response with salbutamol and aminophylline. *C*, summary of results computed from the experiments such as those illustrated in *A* and *B*, which compares sweating responses induced by aminophylline and theophylline.
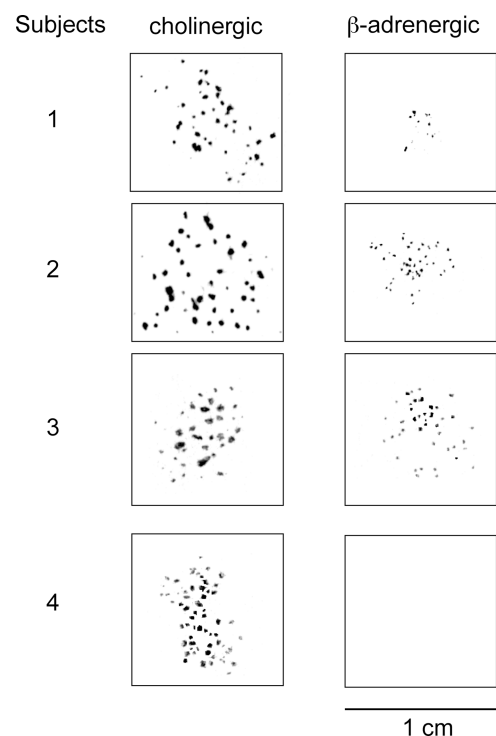

Figure 12. Detection of sweat secretion by starch/iodine method after cholinergic and β-adrenergic iontophoretic stimulation *in vivo* in control subjects (subjects 1–3) and a CF subject (subject 4)
See Methods for technical details.

(Fig. 8*A*). Thus, we were unable to detect a K$^+$ channel that might be specifically manipulated in β-adrenergic sweating. Only the non-specific K$^+$ channel inhibitor, Ba^{2+}, reduced but did not abolish secretion, consistent with its effect on V_m in cultured secretory cells mentioned above. These results may suggest that β-adrenergic secretion is driven only by the electrochemical potential of K$^+$ expressed through a non-excitable, possibly a 'house-keeping', K$^+$ channel in the basolateral membrane [e.g. voltage-gated potassium channel encoded by KCNQ genes (KCNQ); Boucherot *et al.* 2001].

We then turned to requirements for selectively activating the CFTR Cl$^-$ conductance. We found that in the non-CF secretory tubule microperfused in the presence and absence of an inward Cl$^-$ gradient, the lumen hyperpolarized more when stimulated cholinergically than adrenergically (Fig. 5*A* and *B*), suggesting either that the former is a more potent secretogogue than the latter, or that two different channels are involved. It is virtually certain that two distinct channels are present since, even without functional CFTR, CF patients sweat normally during cholinergically mediated thermal stress. Experimentally, cholinergic secretion is probably supported by a calcium-activated Cl$^-$ (CAC) channel, since removing calcium from the bathing solution completely inhibits cholinergic stimulation of *ex vivo* intact glands (Fig. 9; Prompt & Quinton, 1978; Sato & Sato, 1981) and electrical responses of cultured secretory cells (Reddy & Bell, 1996) while β-adrenergic stimulation is not affected. Since Sal is much more selective for β$_2$-adrenergic stimulation and avoids possible β$_1$-mediated side-effects, we elected to use this drug as a principal cAMP stimulating agonist. We found that β-adrenergic sweating could not be stimulated *ex vivo* in intact glands by any of these three classes of cAMP agonist acting singularly. Sweating was not induced by Sal (Figs 6 and 10*E*), IPR (Fig. 10*A*), Fsk (Fig. 10*D*) or IBMX (Fig. 10*B*) when applied in the absence of other agonists (Fig. 10*C*). That is, elevation of cAMP by: (1) activating β-adrenergic receptor with IPR; (2) activating adenylyl cyclase directly with Fsk; or (3) blocking the breakdown of cAMP by inhibiting phosphodiesterase with IBMX was not sufficient, in and of itself, to support sweat secretion.

These results may be surprising, since the cell membrane potential seemed more negative (Fig. 2) with respect to the more positive Cl$^-$ equilibrium potentials (about −20 to −30 mV) generally observed in epithelial cells (Reddy & Quinton, 1994). These observations are intriguing because the β-adrenergic agonist, IPR, stimulated cultured secretory cells (Fig. 2*C*; Reddy *et al.* 1992) and isolated native secretory coil (Reddy & Quinton, 1992*b*). We speculate that application of a single cAMP agonist fails to increase intracellular cAMP sufficiently in sweat glands *in situ* in skin to support secretion. For example, perhaps the production of prostanoids associated with dissection

trauma or culture conditions may alter the sensitivity to or production of cAMP of cells under *in vitro* conditions (Downie & Karmazyn, 1984).

However, any two of the three agonists acting together were sufficient to consistently stimulate secretion (Figs 6*B* and 10*A–F*). *In vitro*, the combination of Fsk and IBMX is probably the most common approach to reliably elevating intracellular [cAMP]. Unfortunately, both Fsk and IBMX are uncharged molecules and not suitable for iontophoresis, and neither drug is approved for human use. Therefore, we tested and compared the effects of FDA-approved theophylline with aminophylline. Theophylline has been used successfully in the past in combination with IPR to stimulate β-adrenergic sweating (Sato & Sato, 1984; Behm *et al.* 1987); however, like IBMX, it is uncharged. Aminophylline is theophylline combined with ethylenediamine to increase solubility by adding positive charge. We found that both drugs appear to be equally effective in combination with Sal to stimulate β-adrenergic secretion *ex vivo* in glands (Fig. 11).

We then tested whether these two drugs could be used to stimulate sweating *in vivo* when administered iontophoretically. In these preliminary studies, we first iontophoresed a 1% atropine solution through a small glass capillary into the skin to block cholinergic receptors (see Methods). Immediately thereafter, we iontophoresed 1% solutions of Sal and of aminophylline through separate capillaries, immediately adjacent to or over the same spot where atropine was applied. This approach avoided the possibility of damaging more than a single gland or so by the passage of current through the skin under the capillary, but allowed stimulation of glands surrounding the focal point of the current by diffusion of the drugs in the subdermal region. Separate iontophoresis of the drug avoided the confounding effects of disparate transfer numbers and helped to ensure delivery of similar doses. Figure 12 shows that the technique, as assayed by a modified starch/iodine test (Randall, 1946; Shamsuddin & Togawa, 2000), was feasible and successful. Three non-CF control subjects all responded to β-adrenergic stimulation with clearly detectable sweating, although with markedly less sweating than with cholinergic stimulation, while the CF subject clearly responded to cholinergic but not to β-adrenergic stimulation (Fig. 12.).

We suggest that this approach or similar protocols may be appropriate and useful as a diagnostic aid and as an assay for evaluating the effects of putative 'correctors' and 'potentiators' when used therapeutically in human subjects to improve the function of CFTR *in vivo*. For diagnosis, it could be used as simply and effectively as the QPIT, but without the requirement for sophisticated laboratory analytical devices (i.e. microbalances, chloridometers or flame photometers for measurements of Cl$^-$ or Na$^+$). The iontophoresis unit is easily constructed from a dry cell battery, a potentiometer,

capillary tubes and any inexpensive, commonly available multimeter. The diagnosis would be made easily on the basis of the presence of small dark dots on white copy paper, representing stimulated sweating activity, that are apparent to the eye, but very easily seen with low-power magnification and can be quantified by digital scan (see Fig. 12). The absence or clear reduction of such dots would support a diagnosis of CF. As an assay for therapeutic drugs, this approach seems minimally time consuming and relatively straightforward. The protocol could be applied before, during and after drug trials as a marker for progress and efficacy. Both inter- and intrasubject comparisons are possible, since each subject can serve as his own control. That is, not only could the presence of β-adrenergically stimulated sweating be followed, but it could also be quantified as a function of the number of glands and sweat volume stimulated. Furthermore, these parameters could be normalized relative to the subject's response to cholinergic sweating monitored concurrently. While further work will be required for validation, it seems likely that responses can be more accurately quantified by densitometry of computer scans of the test papers.

In summary, we present evidence that β-adrenergically stimulated sweating is probably weaker than cholinergically stimulated sweating because the former does not activate a G_{K^+} to enhance the driving force for Cl^- exit (secretion) during concurrent activation of CFTR. We were unable to identify a G_{K^+} that might be manipulated to enhance this secretion, but we found that a combination of at least two of three of the means of elevating intracellular cAMP seem essential to activate β-adrenergic CFTR-dependent secretion. We show that cationic forms of agonists such as Sal and aminophylline can be applied iontophoretically to selectively stimulate β-adrenergic sweating. We suggest the feasibility of using this protocol as a tool in the diagnosis of CF and as an assay to monitor the progress and efficacy of drugs in trials for potentiating or correcting the function of defective CFTR in cystic fibrosis patients.

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