# cAMP does not regulate [Ca$^{2+}$]$_i$ in human tracheal epithelial cells in primary culture

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## SUMMARY

**Human tracheal epithelial cells in primary culture respond to different receptor agonists with different peak intracellular calcium concentrations. From resting concentration 138±13 nM, bradykinin (0.1 μM) produces an increase to a maximum of 835±195 nM, histamine (10 μM) to 352±51 nM, and ATP (5-500 μM) to more than 1500 nM. Nine of 14 cultures also responded to isoproterenol (10 μM), though with a smaller increase, to 210±29 nM. A response was observed with isoproterenol, and epinephrine, but not norepinephrine, phenylephrine or methoxamine, was inhibited by propranolol but not phentolamine, and so this appeared to be a β-adrenergic response. However, no response could be detected to adenosine, prostaglandin E$_2$ or forskolin, agents that activate adenylate cyclase, or to permeant analogs of cAMP (CPT-cAMP or db-cAMP). The intracellular calcium response to isoproterenol did not follow either the time-course or the desensitization pattern of the cAMP response. Thus, this response to isoproterenol is not mediated by cAMP. No relation was demonstrated between cAMP production by other agonists and the response of intracellular calcium. Pretreatment with agents that increase cAMP did not affect the calcium responses to ATP or bradykinin. Thus, cAMP does not regulate intracellular calcium concentration in human tracheal epithelial cells. The variation in peak intracellular calcium responses to various agonists may be explained by the presence of multiple second messengers (other than cAMP), multiple intracellular pools of calcium, or cell heterogeneity. The agonists tested had the same relative potency in cells from patients with cystic fibrosis as in non-cystic fibrosis cells.**

Key words: cystic fibrosis, cAMP, β-adrenergic receptor, purinergic receptor, histamine, bradykinin, intracellular calcium concentration

## INTRODUCTION

In human airway epithelial cells, an increase in intracellular free calcium ([Ca$^{2+}$]$_i$) causes basolateral potassium channels and apical chloride channels to open (McCann and Welsh, 1990; Welsh and Liedtke, 1986; Widdicombe, 1986; Willumsen and Boucher, 1989; Yamaya et al., 1993). Regulation of these channels is critical for the epithelium in the regulation of the ionic milieu in the airway. Several receptors that regulate [Ca$^{2+}$]$_i$ in airway epithelial cells have been identified (Yamaya et al., 1993; Boucher et al., 1989; McCann et al., 1989; Murphy et al., 1988; Reinlib et al., 1992; Mason et al., 1991). These receptors, when stimulated, produce different maximal responses of [Ca$^{2+}$]$_i$, suggesting that either the cell population under study is heterogeneous and the cells in it express different receptor complements, or the different receptors use different signalling mechanisms that entrain different responses of [Ca$^{2+}$]$_i$, or the [Ca$^{2+}$]$_i$ response can be modulated in these cells by other second messengers (or some combination of these possibilities).

In canine airway epithelial cells, both inositol phosphates and cAMP stimulate increases in [Ca$^{2+}$]$_i$ (McCann et al., 1989). It is not clear whether this dual regulation also occurs in human airway epithelial cells. Studies of chloride transport in human airway epithelial cells suggest that cAMP does not regulate chloride transport through the calcium-regulated chloride channel (Boucher et al., 1991), but these data are indirect. In the present study, we test whether cAMP can regulate [Ca$^{2+}$]$_i$ in human airway epithelial cells in culture. We studied human tracheal epithelial (HTE) cells grown in monolayer sheets on flexible plastic squares, loaded with the calcium-sensitive probe fura-2, and wedged into a spectrofluorimeter. These measurements permit detection of small responses in only a few cells in the culture, without sampling error. These studies were complemented by cell-by-cell studies in a video-enhanced fluorescence microscope to assess the heterogeneity of cellular response. In addition, we were able to compare the response of [Ca$^{2+}$]$_i$ to receptor agonists in cells from patients with cystic fibrosis (CF) with those from cells from normal subjects. Such comparisons are of interest because activation of the calcium-regulated chloride channel in patients with CF has been proposed for therapeutic purposes (Knowles et al., 1991), and some investigators have reported significant differences between CF and normal airway epithelial cells in their [Ca$^{2+}$]$_i$ response to agonists (Reinlib et al., 1992).

## MATERIALS AND METHODS

### Cell culture

Human tracheal epithelial (HTE) cells were recovered from necropsy specimens of both CF and non-CF patients as previously described (Davis et al., 1990; Kercsmar et al., 1990). Nasal polyps were recovered during surgery from CF patients, and epithelial cells separated from them as previously described (Welsh and Liedtke, 1986). For studies of $[Ca^{2+}]_i$, cells were plated either on squares (about 1.5 cm × 1.5 cm) (for the spectrofluorimeter), or on circles about 25 mm in diameter (for the video-enhanced fluorescence microscope), of Aclar plastic (Allied Engineered Plastics, Pittsburgh, PA) that had been treated previously with detergent and nitric acid washes, and coated with Vitrogen. For studies of cAMP metabolism, they were plated on Vitrogen-coated plastic in Costar 24- or 48-well plates. Otherwise, culture conditions were identical to those previously described (Davis et al., 1990; Kercsmar et al., 1990). These cells were identified as epithelial by their positive staining for cytokeratins using antikeratin antisera and appropriate morphology under phase-contrast microscopy and electron microscopy as previously described (Davis et al., 1990; Kercsmar et al., 1990).

### Spectrofluorimetry

The Vitrogen-coated squares on which cells were growing were washed with Ham's F-12 medium and then exposed to F-12 medium containing 0.1% bovine serum albumin (BSA) and the acetoxymethylester of fura-2 (2 µM) for 60 minutes at room temperature. At the conclusion of the loading period, the cells were washed in F-12 medium containing 0.1% BSA, placed on ice, and studied within the next 30 minutes. Some experiments were conducted with cyclooxygenase inhibitors (ibuprofen, 50 µg/ml, or indomethacin, 5 or 50 µg/ml). These inhibitors were added during the fura-2 loading step and were included in all the subsequent buffers.

The squares were removed from the dish and wedged diagonally into a quartz cuvette in buffer containing 126 mM NaCl, 5 mM KCl, 20 mM HEPES, 1 mg/ml glucose, 1 mM MgCl$_2$, 0.1% BSA, and either 1.5 µM CaCl$_2$ or 1.5 mM CaCl$_2$. The Aclar squares, which are flexible, were cut slightly larger than the diagonal of the cuvette so that a slightly crescent-shaped curve resulted when the coverslip was wedged in the cuvette. The square was positioned a few millimetres from the bottom of the cuvette to allow clearance of the stirrer bar, with the cells on the inner curve facing the beam at an angle of about 45˚. The cuvette was placed in a spectrofluorimeter in a thermostated cell (maintained at 37˚C), with constant stirring. Fluorescence at 510 nm was recorded; the excitation wavelength was 340 nm. After thermal equilibration had occurred and the fluorescence (F) at 510 nm was stable, the cells were exposed to various agonists, which were added (1000-fold concentrated) by rapid pipetting into the cuvettes. $F_{max}$ was determined by addition of 7.5 to 10 µM ionomycin. Subsequent additions of ionomycin failed to elicit further increases in fluorescence. $F_{min}$ was determined by addition of an excess of EGTA. The intracellular calcium concentration was determined as follows: each individual trace was scanned using the Image-In Color software, V3.0 (Image-In, Inc., Minneapolis, MN) and a UMAX scanner, model UC630 Max Color (UMAX Technologies, Inc.) connected to a 486-66 mHZ PC-based computer. Images were scanned as line art with a resolution of 300 dpi and saved as PCX images. The scanned images were then digitized using the Un-Scan-It software, V2.0 (Silk Scientific, Inc., Orem, UT). The resulting data ($y(F)$ = relative fluorescence at 340 nm) was fitted to the following equation:

$$[Ca^{2+}] = K*(F - F_{min})/(F_{max} - F)$$

using the SigmaPlot for Windows, V1.0 software (Jandel Scientific, San Rafael, CA). $F_{min}$ and $F_{max}$ were determined as indicated above and $K$, determined as described by Tsien (1989) and Grynkiewicz et al. (1985), was taken to be 224 nM (at 37˚C).

For most cell samples from the same trachea, studied on the same day, results were consistent from Aclar square to Aclar square, and $F_{max} - F_{min}$ for each sample differed from the mean by 10% or less. When this criterion was met, data from different Aclar squares plated with cells from the same trachea were sometimes shown on the same axis in the figures.

### Video-enhanced fluorescence microscopy

The Vitrogen-coated circles on which cells were growing were washed twice with HEPES-buffered medium containing 0.1% bovine serum albumin (BSA) and 0.1% glucose (126 mM NaCl, 5 mM KCl, 1 mM MgCl$_2$, 1.5 mM CaCl$_2$) and then exposed to the same medium containing the acetoxymethylester of fura-2 (2 µM) for 30 minutes at room temperature. fura-2 AM was washed away and cells were incubated at 37˚C for another 30 minutes. Cells were then kept on ice until they were used.

The round Aclar coverslips were placed upside-down in a modified Sykes-Moore chamber (Bellco Biotechnology, Vineland, NJ) and perfused continuously by gravity feed at a rate of 3-4 ml/min with the HEPES-buffered solution mentioned above. Experiments were performed at 37˚C and analyzed cell-by-cell with the aid of an upright Zeiss epifluorescence microscope. Fluorescence was excited alternatively at 340 and 380 nm by a 75 W xenon lamp (Omega, Brattleboro, VT). Excitation light was reflected by a 400 nm fused silica dichroic mirror (Omega, Brattleboro, VT) and illuminated the cells through a 20× objective, NA 0.75, working distance 0.66 mm (Nikon, Garden City, NY). Emitted light was filtered by a 510 nm lens and detected by a microchannel plate image intensifier, model KS-1381 in series with a CCD-200 solid state video camera (Video Scope International, Ltd, Washington, DC). The images were quantified with a model MVP-AT frame grabber board (Matrox, Quebec, Canada) mounted internally in a host 386 computer and processed with the Image-1 Fl software (Universal Imaging, West Chester, PA). Every image taken represents the average of 16 images (~0.26 seconds per wavelength). Successive images were taken at a rate of ~1 every 5 seconds. The 'blank field' obtained by closing the camera shutter was used as the background image and it was subtracted prior to the acquisition of the images. fura-2 ratio values were converted to $[Ca^{2+}]_i$ by in vitro calibration and the use of the following equation:

$$[Ca^{2+}] = K_D \frac{R - R_{min}}{R_{max} - R} \cdot \frac{S_{f2}}{S_{b2}} \ .$$

$R_{max}$ and $R_{min}$ are the ratios measured, under the same experimental conditions, in the presence of either 2 mM Ca$^{2+}$ (max) or 3 mM EGTA (min), 150 mM KCl, 2 M glucose (to correct for intracellular viscosity) and 2 µM fura-2. $S_{f2}$ and $S_{b2}$ are the fluorescence intensity values of the 380 nm wavelength in the absence or presence of calcium, respectively. $K_D$ for fura-2 under our experimental conditions was determined to be 223 nM, a value identical to the one previously reported (Grynkiewicz et al., 1985). Solutions used to determine the $K_D$ contained (in mM): KCl (48), K$_2$HPO$_4$ (45), creatine phosphate (sodium salt) (10), Na$_2$ATP (5), MgCl$_2$ (1), and 2 M sucrose, pH 7.1, 37˚C. The total amount of CaCl$_2$ necessary to give the desired free Ca$^{2+}$ was calculated with the program presented by Fabiato (1988).

### cAMP measurements

Stimulation of intracellular cAMP synthesis was performed as previously described (Davis et al., 1990; Kercsmar et al., 1990). Cultures were pre-incubated with 0.25 mM isobutylmethylxanthine (IBMX) in all cases except for the time-course experiments and the desensitization experiments. For the desensitization experiments, cells were exposed to 10 µM isoproterenol for 15 minutes at 37˚C, thoroughly washed, preincubated with 0.25 mM IBMX for 20 minutes, and then exposed to isoproterenol again and processed for cAMP determination. Intracellular cAMP content was measured by radioimmunoassay as previously described (Davis et al., 1990; Kercsmar et al., 1990).

## Chemical sources

fura-2/AM and fura-2 were obtained from Molecular Probes, Inc. Eugene, OR; ionomycin, albumin and forskolin were from Calbiochem, San Diego, CA. Ibuprofen was a gift from Upjohn Co., Kalamazoo, MI. All other chemicals were obtained from Sigma Chemical Co., St Louis, MO.

## Statistics

Data are reported as mean ± s.e.m. Comparisons were made by the paired *t*-test for samples from the same trachea and the unpaired *t*-test under other circumstances. Values of *P*<0.05 were considered significant.

## RESULTS

### Variation in peak [Ca$^{2+}$]$_i$

We determined the peak [Ca$^{2+}$]$_i$ responses to various agonists. Basal [Ca$^{2+}$]$_i$ in HTE cells in culture was 138±30 nM (*n*=5) in buffer containing 1.5 μM CaCl$_2$ (designated 'low calcium'), and 138±13 nM (*n*=25) in buffer containing 1.5 mM CaCl$_2$ (designated 'high calcium'). Addition of agonist was followed in most cases by a rapid (<30 second) rise in [Ca$^{2+}$]$_i$ to a peak value, which then declined either to basal levels or to some stable elevated resting level, called a 'plateau'. Peak values were comparable for all agonists reported in high and low calcium buffers (see below), but the plateau values were higher in the high calcium buffers. Unless specified otherwise, measurements were made in the high calcium buffer. For each agonist, a dose-response relation was determined in cells from at least two separate tracheas. Each concentration of agonist was applied to a separate coverslip to avoid receptor desensitization. Once the concentration giving maximal peak response had been determined, measurements were made at that concentration for a total of at least five different HTE samples. These cells responded to bradykinin, ATP, histamine and isoproterenol, but there were substantial differences among the agonists in peak [Ca$^{2+}$]$_i$ achieved (Table 1). No response was observed in response to methoxamine, phenylephrine, carbachol or substance P, with or without phosphoramidon (each was tested in cells from at least three tracheas, data not shown). Values obtained for non-CF HTE cells are compared with measurements in the two CF tracheal epithelial cell cultures and the five CF nasal polyp cultures that became available during the study period. The order of responsiveness to the agonists was similar for normal, and for CF nasal and tracheal samples (Table 1).

### Response to ATP

The largest increases in [Ca$^{2+}$]$_i$ were observed in response to ATP. All cultures responded. At ATP concentrations >5 μM in cells studied in the spectrofluorimeter, peak [Ca$^{2+}$]$_i$ responses were comparable to the ionomycin peak (Fig. 1), and were considered to represent [Ca$^{2+}$]$_i$ >1500 nM. Some response was detected at ATP concentrations as low as 0.05 μM. Following the initial peak, [Ca$^{2+}$]$_i$ remained elevated in both low calcium and high calcium buffers (Fig. 2). Others have reported that activation of a P2u receptor by ATP results in increased [Ca$^{2+}$]$_i$ in human airway epithelial cells (Mason et al., 1991).

ATP stimulated cAMP synthesis in HTE cells, but the dose-

### Table 1. Maximum [Ca$^{2+}$]$_i$ responses of airway epithelial cells

| | [Ca$^{2+}$]$_i$ (nM) (*n*) | | |
| | Non-CF | CF | |
| Agonist | Tracheal | Tracheal | Nasal |
|---|---|---|---|
| Basal level | 138±13 (25) | 91 (2) | 113±23 (5) |
| Bradykinin (10$^{-7}$ M) | 835±195 (17)* | 684 (2) | 466±60 (5)* |
| Isoproterenol (10$^{-5}$ M) | 210±29 (9/14)*,† | 277 (2/2)* | 190±56 (3/5)*,† |
| Histamine (10$^{-5}$ M) | 352±51 (5)* | 382 (1) | 367 (2/3) |
| ATP (5×10$^{-5}$ M) | >1500 (6)* | N.T.‡ | >1500 (2) |

*Significantly different from basal values.
†Fractions indicate proportion of cultures that responded, if not all samples responded.
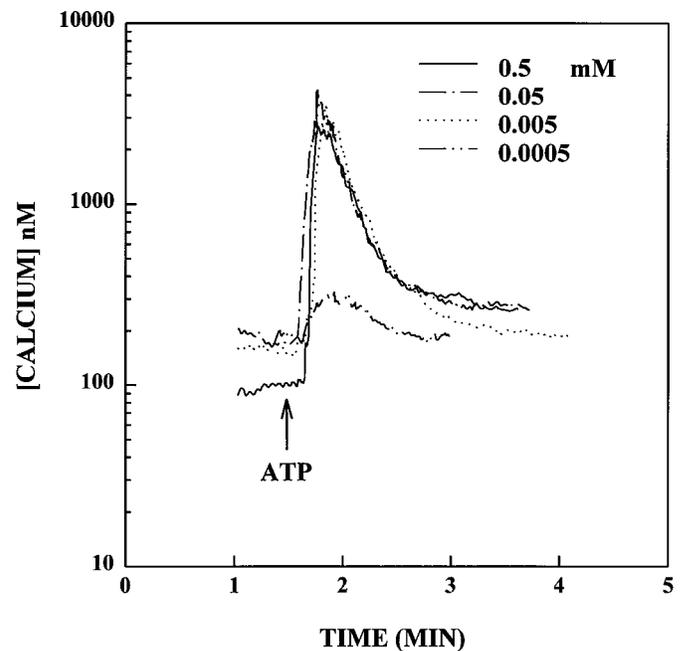‡N.T., not tested.



**Fig. 1.** Response of [Ca$^{2+}$]$_i$ in HTE cells exposed to ATP at various concentrations as shown. Tracings from different Aclar squares from the same trachea in one experiment are superposed for illustrative purposes.

response relationship was different from the dose-response relationship for achieving peak [Ca$^{2+}$]$_i$ values (Table 2). At a concentration at which there was little cAMP stimulation, maximal peak [Ca$^{2+}$]$_i$ values were achieved. Prior treatment of cells with isoproterenol, which markedly increases cAMP (Mason et al., 1991), did not alter the subsequent response to ATP (Fig. 2). Plateau values for [Ca$^{2+}$]$_i$ increased with increasing ATP concentrations, like the cAMP response (Fig. 1, Table 2). However, as noted below, HTE cells treated with isoproterenol, which achieved much higher levels of cAMP (Table 3), showed no sustained (plateau) [Ca$^{2+}$]$_i$ increase, so cAMP cannot by itself account for the plateau. The mechanism by which ATP stimulates cAMP production is not clear, but it is unlikely that it is by way of breakdown products such as adenosine, since adenosine produces a maximal cAMP response that is considerably lower than that stimulated by ATP itself (Table 3).
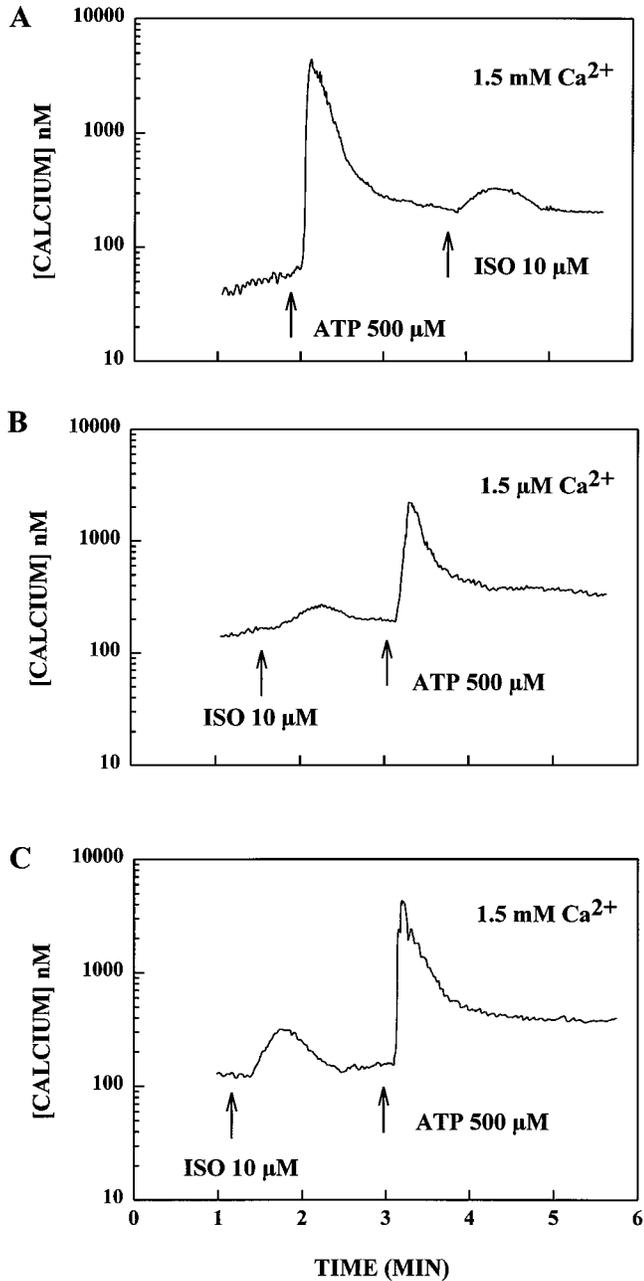
**A**



**B**



**C**



**Fig. 2.** Response of $[Ca^{2+}]_i$ in HTE cells to sequential addition of: (A) ATP (500 μM) followed by isoproterenol (10 μM) in buffer containing 1.5 mM $Ca^{2+}$; (B) isoproterenol (10 μM) followed by ATP (500 μM) in buffer containing 1.5 μM $Ca^{2+}$; (C) isoproterenol (10 μM) followed by ATP (500 μM) in buffer containing 1.5 mM $Ca^{2+}$. Incremental response to isoproterenol (ISO) was 132 nM in A, 40 nM in B, and 195 nM in C.

## Bradykinin responses

Bradykinin stimulated increases in $[Ca^{2+}]_i$, with the maximum response achieved at $10^{-7}$ M (Fig. 3). A plateau phase was evident in cells studied in the high calcium buffer, but not in low calcium buffer or when EGTA was added to the buffer (Fig. 4). All cultures tested responded. The $[Ca^{2+}]_i$ response to bradykinin was the same whether cells were pretreated with isoproterenol (which produces a rise in cAMP) or with iso-proterenol in the presence of propranolol (which inhibits the

**Table 2. Dose-response relation for ATP stimulation of cAMP synthesis**

| [ATP] (μM) | cAMP (pmol/mg protein) (n=4) |
|---|---|
| 0 | 59±30 |
| 0.1 | 48±32 |
| 1 | 71±31 |
| 10 | 150±64 |
| 100 | 322±181 |
| 1000 | 1071±543 |

**Table 3. Intracellular signalling responses of HTE cells**

| Agonist | Intracellular cAMP content* (pmol/mg (n)) | Maximum $[Ca^{2+}]_i$ (nM) (n) |
|---|---|---|
| Buffer only | 80±31 (39) | 119±27 (9)† |
| Isoproterenol ($10^{-5}$ M) | 2180±810 (21)‡ | 210±29 (9)†,‡ |
| Isoproterenol, wash, isoproterenol ($10^{-5}$ M) | 1610±147 (3)*,‡,§ | 124±10 (3)† |
| $PGE_2$ ($10^{-5}$ M) | 244±113 (16)‡ | 127±38 (3)† |
| Forskolin ($10^{-4}$ M) | 1290±79 (9)‡ | 133±34 (4)† |
| Adenosine ($10^{-5}$ M) | 210±22 (7)‡ | 130±25 (4)† |
| ATP ($10^{-3}$ M) | 1071±543 (5)‡ | >1500 (6)‡ |

*cAMP content measured 5 minutes after addition of indicated agonist and 25 minutes after addition of 0.25 mM isobutylmethylxanthine.
†Only data from isoproterenol-responsive cultures are included.
‡Significantly different from buffer only.
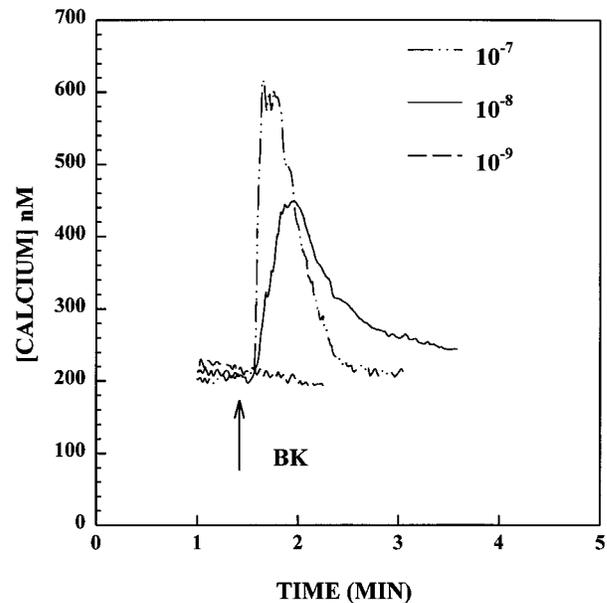§IBMX added only at second exposure to isoproterenol.



**Fig. 3.** Response of $[Ca^{2+}]_i$ in HTE cells exposed to bradykinin at various concentrations as shown. Tracings from different Aclar squares from the same trachea in one experiment are superposed for illustrative purposes.

cAMP increase). Preincubation of the cells with a cyclooxy-genase inhibitor did not inhibit the rise in $[Ca^{2+}]_i$ in response to bradykinin (Table 4).

## Histamine response

The dose-response relation for histamine reached maximal

**Table 4. Effect of cyclooxygenase inhibition on $[Ca^{2+}]_i$ response to bradykinin**

| | $[Ca^{2+}]_i$ (nM) ($n=5$) | |
|---|---|---|
| | Control | Cyclooxygenase-inhibited |
| Buffer | 162±50 | 185±21 |
| Bradykinin | 348±50 | 320±39 |

levels at $10^{-5}$ M. All cultures tested except one CF nasal polyp culture responded to histamine (Table 1).

## Isoproterenol response

Nine of 14 cell preparations displayed a transient increase in $[Ca^{2+}]_i$ in response to isoproterenol or epinephrine. The least detectable increase occurred at 0.5 μM isoproterenol. This increase was prevented by propranolol (Fig. 4), a β-adrenergic antagonist, but not by phentolamine, an α-adrenergic antagonist. There was a small response to epinephrine (a potent α and β-adrenergic agonist), but none to phenylephrine ($10^{-5}$ M), norepinephrine ($10^{-5}$ M) or methoxamine ($10^{-5}$ M), all α-adrenergic agonists (tested in cultures that responded to isoproterenol). Thus the pharmacology of this response was consistent with a β-adrenergic effect.

In cultures that responded, the duration of elevation of $[Ca^{2+}]_i$ was always less than 2 minutes (Figs 2, 4-6) in either low or high calcium buffers. Peak $[Ca^{2+}]_i$ was the same in high calcium or low calcium buffers (Fig. 2) and external $LaCl_3$ or EGTA did not prevent or alter the $Ca^{2+}$ transient (Fig. 4), suggesting that the response does not depend upon external calcium, but rather on release of intracellular stores. When resting $[Ca^{2+}]_i$ was increased by administration of ATP, isoproterenol still produced an increase in $[Ca^{2+}]_i$ comparable to that seen in untreated cells (Fig. 2). Prior administration of dibutyryl-cAMP or CPT-cAMP did not prevent the $[Ca^{2+}]_i$ response to isoproterenol (Figs 5 and 6). Thus, the $[Ca^{2+}]_i$ response to isoproterenol was not affected by either $[Ca^{2+}]_i$ or cAMP levels within the cell.

Both the time-course and desensitization pattern differed between the cAMP and the $[Ca^{2+}]_i$ response to isoproterenol. The $[Ca^{2+}]_i$ response was brief (Figs 2, 4-6), but HTE cells continued to accumulate cAMP for over 30 minutes after stimulation with isoproterenol (Davis et al., 1990). Exposure to isoproterenol followed by washing and re-exposure to isoproterenol eliminated the second $[Ca^{2+}]_i$ response to isoproterenol. However, the cells were still capable of response to bradykinin, histamine or ATP, suggesting that intracellular calcium stores were not depleted. It is, however, possible that a specific calcium pool accessed by the β-adrenergic receptor signal could be depleted, and this, rather than receptor desensitization, results in the failure of the second calcium response. In contrast, the cAMP response to isoproterenol was unaltered by brief prior exposure to the agonist (Table 3). Mechanisms of rapid desensitization of the β-adrenergic receptor include internalization and phosphorylation either by a β-adrenergic receptor kinase or by protein kinase A (Lohse et al., 1990).

In order to test further whether increased intracellular cAMP mediates the isoproterenol effect, we tested receptor-mediated stimulators of cAMP synthesis, prostaglandin $E_2$ (PGE$_2$, $10^{-5}$ M) and adenosine ($2.5\times10^{-5}$ M), a non-receptor-mediated
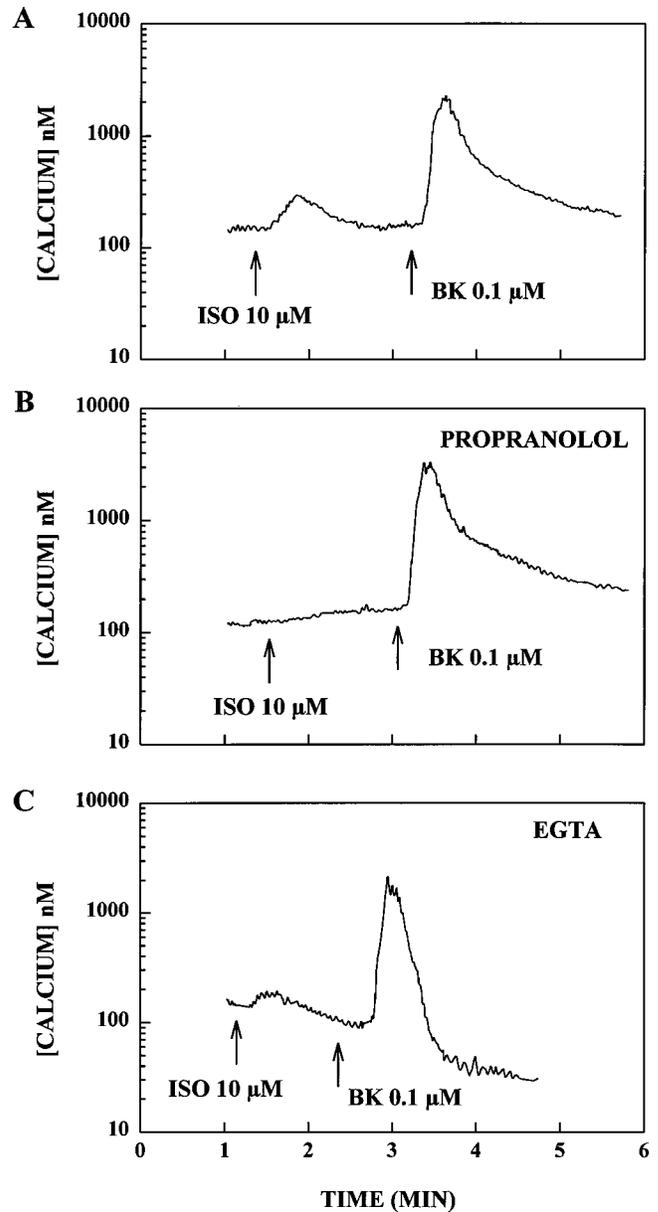


**Fig. 4.** Response of $[Ca^{2+}]_i$ in HTE cells to sequential addition of isoproterenol (10 μM, ISO) and bradykinin (0.1 μM, BK). (A) Buffer contained 1.5 mM $Ca^{2+}$. (B) Buffer contained 1.5 mM $Ca^{2+}$ plus 20 μM propranolol. (C) Buffer contained EGTA sufficient to reduce $Ca^{2+}$ to <1 μM.

cAMP stimulant (forskolin, $10^{-4}$ M), and permeant analogs of cAMP, dibutyryl-cAMP (db-cAMP; 10 and 100 μM) and 8-(4-chlorophenylthio)-cAMP (CPT-cAMP; 10, 100 and 200 μM). Each was tested in at least three tracheal samples that responded to isoproterenol. None of these produced a calcium transient (Table 4, Figs 5 and 6). This order of potency of agonists is similar to that reported (in part) previously (Davis et al., 1990).

## Cell-by-cell responses

In order to asses the heterogeneity of the cell population, we studied the response of individual HTE cells in a video-enhanced fluorescence microscope. A dose-response curve for ATP was
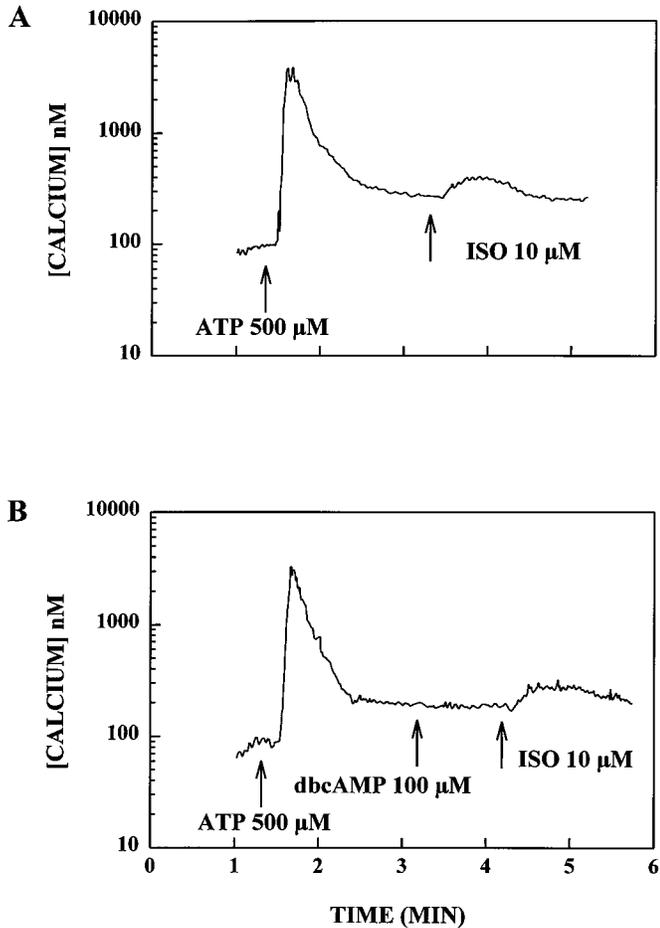
**A**



**B**



**Fig. 5.** Response of $[Ca^{2+}]_i$ in HTE cells to sequential addition of: (A) ATP (500 μM), then isoproterenol (10 μM); or (B) ATP (500 μM), then db-cAMP (100 μM), followed by isoproterenol (10 μM).

determined using cells from four separate tracheas plated on 16 separate coverslips, considering both the proportion of cells that responded and the maximum height of the peak. In the flow-through chamber, using ratio imaging to determine $[Ca^{2+}]_i$, we found a somewhat different ATP dose-response relationship compared to epithelial sheets. Even though all of the cultures tested responded to ATP with an increase in $[Ca^{2+}]_i$, not every cell in the culture responded. The maximal response was not achieved at 5 μM ATP, as it was in the epithelial sheets. At this concentration, fewer than half the cells had responded (ultimately, at higher concentrations, nearly every cell responded) and the peak $[Ca^{2+}]_i$ response was well below the maximum achieved at higher concentrations of ATP. The percentage of cells responding increased at each concentration tested, but the peak $[Ca^{2+}]_i$ response appeared to be biphasic, with one peak at 5-10 μM ATP and a second, higher peak at 50 μM ATP, which did not further increase even at 5 mM ATP (Fig. 7).

The response to isoproterenol was tested in 12 coverslips from two separate tracheas in a total of 295 cells. Only 20 cells on six coverslips responded to isoproterenol (10 μM). Fig. 8 shows the results from one coverslip in which six of 21 cells responded with an increase in $[Ca^{2+}]_i$. Neither the height of the peak nor the proportion of cells responding increased when the isoproterenol concentration was increased to 100 μM. These
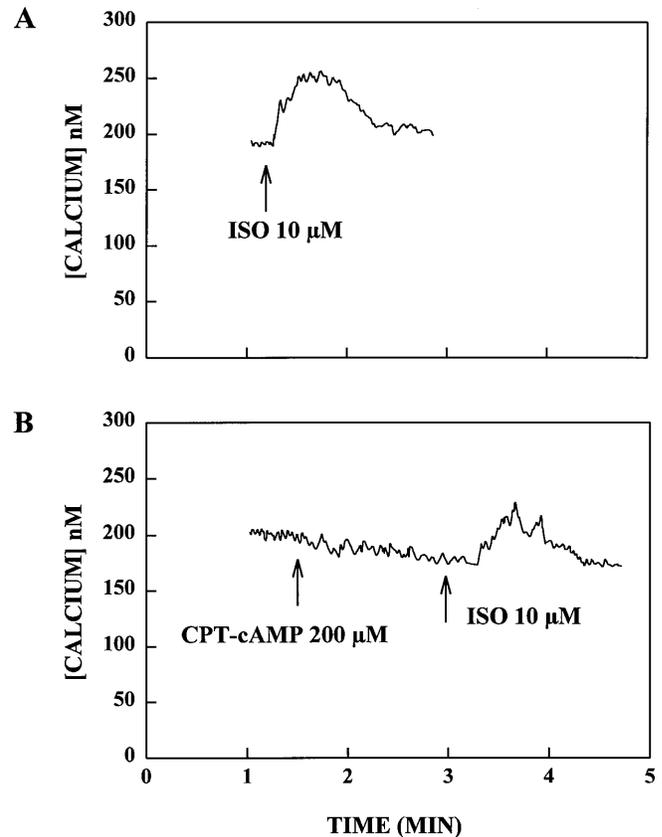
**A**



**B**



**Fig. 6.** HTE cell response to agonists. (A) Response to isoproterenol (10 μM). (B) Response of cells from same trachea to CPT-cAMP (200 μM), followed by isoproterenol (10 μM).

data indicate that HTE cells in primary culture are heterogeneous with respect to their $[Ca^{2+}]_i$ responses to β-adrenergic receptor stimulation.

## cAMP responses

There was no significant increase in cAMP in response to histamine (up to 100 μM) or bradykinin (up to 10 μM) in HTE cell cultures. Comparison of the $[Ca^{2+}]_i$ and cAMP responses to various other agonists are shown in Table 3. Isoproterenol, $PGE_2$, forskolin, ATP and adenosine all increased intracellular cAMP content, but only isoproterenol and ATP produced increases in $[Ca^{2+}]_i$ as well. Dose-response curves for isoproterenol have been reported previously (Davis et al., 1990). The dose-response relation for ATP is shown in Table 2.

## DISCUSSION

Human airway epithelial cells in primary culture respond to bradykinin, histamine, ATP and isoproterenol with an increase in $[Ca^{2+}]_i$. The largest increases were observed in response to ATP, followed by bradykinin and histamine, and the response to isoproterenol was small and occurred in only nine of 14 cultures. This response had the pharmacological characteristics of a β-adrenergic response, which is usually mediated by cAMP. However, there was no increase in $[Ca^{2+}]_i$ in response to other receptor agonists that increase cAMP, such as $PGE_2$
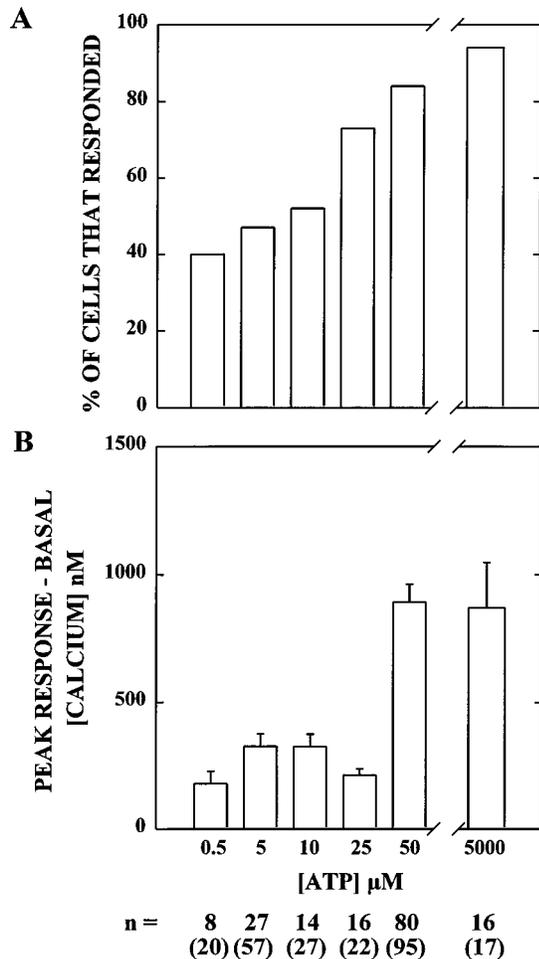
**Fig. 7.** HTE cell response to ATP exposure visualized in a video-enhanced fluorescence microscope. Each bar represents the results from one coverslip at a given ATP concentration; the number of cells that responded in each coverslip is given beneath the bar, with the total number of cells analyzed in parentheses. (A) Percentage of cells that responded; and (B) the average ± s.e.m. of the peak [Ca$^{2+}$]$_i$ response minus basal [Ca$^{2+}$]$_i$ at the indicated ATP concentration for those cells that responded. Buffer contained 1.5 mM Ca$^{2+}$.



**Fig. 8.** HTE cell response to isoproterenol visualized in a video-enhanced fluorescence microscope. Buffer contained 1.5 mM Ca$^{2+}$. A total of 21 cells were analyzed in this particular coverslip, of which only the 6 depicted here showed an increase in calcium concentration.

or adenosine. There was no increase in [Ca$^{2+}$]$_i$ in response to forskolin, which directly stimulates adenylate cyclase, or to db-cAMP or CPT-cAMP (up to 200 μM), permeant analogs of cAMP. Moreover, the dose-response relationship, time-course of response, and pattern of desensitization of cAMP and [Ca$^{2+}$]$_i$ responses to isoproterenol, are quite different. Thus it is unlikely that cAMP mediates the β-adrenergic [Ca$^{2+}$]$_i$ response. Homologous desensitization of the β-adrenergic receptor and inhibition of Mg$^{2+}$ transport are not mediated by cAMP. Also, β-adrenergically mediated increases in [Ca$^{2+}$]$_i$ do not depend on cAMP in tracheal smooth muscle or in the heart (Schubert et al., 1989; Erdos et al., 1981; Barber et al., 1989; Yatani et al., 1988). There may be some non-β$_2$-adrenergic receptors in airway epithelial cells, which might couple to alternative non-cAMP signal transduction systems. Our previous studies indicate that over 90% of the β-adrenergic receptors on these cells are of the β$_2$-adrenergic class and a two-site model fits the data no better than a one-site model (Davis et al., 1990), so the proportion of non-β$_2$-adrenergic
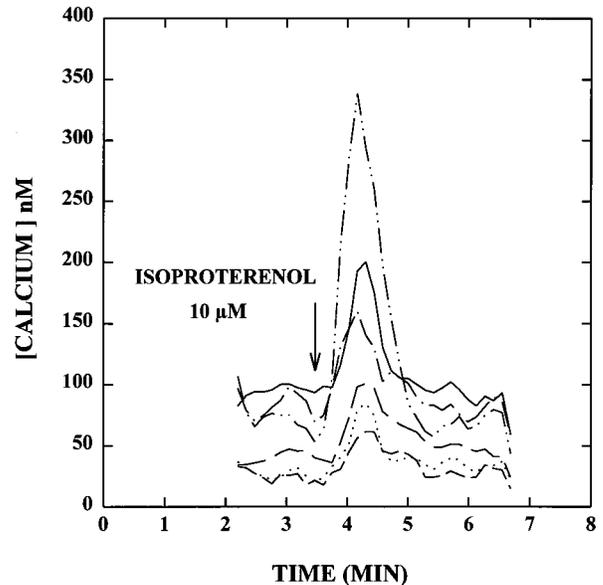
receptors must be very small. On the other hand, the [Ca$^{2+}$]$_i$ response to β-adrenergic agonists is small and inconsistent. In turkey erythrocytes, some β-adrenergic receptors couple to phospholipase C and signal through inositol phosphate turnover (Rooney et al., 1991). However, in tracheal epithelial cells, inositol phosphate turnover does not increase with isoproterenol stimulation, so activation of this pathway, if it occurs, is below the limits of detection of conventional assays (Liedtke, 1992).

In addition, cellular cAMP levels do not affect the response of [Ca$^{2+}$]$_i$ to other agonists in human airway epithelial cells. Pretreatment of cells with agents that increase cAMP does not affect the subsequent [Ca$^{2+}$]$_i$ response to other agonists. For a series of agonists, the cAMP response to that agonist does not correlate with the peak [Ca$^{2+}$]$_i$ response.

In canine airway epithelium, bradykinin not only promotes turnover of inositol phosphates, but also activates phospholipase A$_2$, causing production of arachidonate metabolites. The cyclooxygenase products in turn stimulate adenylate cyclase, raising cAMP levels, and cAMP stimulates release of intracellular Ca$^{2+}$ stores (McCann et al., 1989). In order to determine whether cyclooxygenase products participated in the [Ca$^{2+}$]$_i$ response to bradykinin in human tracheal cells, we preincubated some cultures with cyclooxygenase inhibitors before testing with bradykinin. Cultures in which cyclooxygenase was inhibited responded to bradykinin with a rise in [Ca$^{2+}$]$_i$ to levels comparable to untreated cultures. In addition, we detected no cAMP response to bradykinin in direct assays, and [Ca$^{2+}$]$_i$ did not increase with direct application of PGE$_2$ (10 μM), the major cyclooxygenase product of these cells (Salari and Chan-Yeung, 1989). Thus, human tracheal epithelial cells behave differently from their canine counterparts. However, these experiments were performed in human tracheal epithelial cells in primary culture on plastic, and it is possible that

cells in the native human epithelium, or even cells grown on permeant supports, might behave differently.

It is unlikely that the different magnitudes of response of peak $[Ca^{2+}]_i$ to bradykinin, histamine, ATP and isoproterenol are explained by cAMP modulation of $[Ca^{2+}]_i$. An alternative explanation is that the second messengers for $Ca^{2+}$ mobilization by these agonists may differ, and these second messengers might differ in their ability to mobilize $Ca^{2+}$. Inositol phosphates, cyclic ADP-ribose, sphingosine derivatives or calcium itself have been proposed to trigger release of intracellular $Ca^{2+}$ stores (Ghosh et al., 1990; Galione, 1993), but our experiments do not address the individual signaling systems coupled to each receptor. A second possibility is that different receptors may access different intracellular storage pools of calcium, which might be of different sizes. A third possibility is that the cells are heterogeneous and express different complements of receptors or receptors with different coupling to $Ca^{2+}$ mobilization. Some or all of these explanations may be correct. Our results for the cell-by-cell responses to ATP and isoproterenol indicate that the human tracheal epithelial cell population in primary culture is heterogeneous with respect to receptor responses. Since these cultures were not synchronized, our experiments do not distinguish between whether the cells themselves are heterogeneous or whether receptor responsiveness varies with the stage of the cell cycle. However, because the cultures were derived from a heterogeneous starting population of cells, it seems likely that heterogeneity will persist in culture and contribute to the observed responses.

Our values for basal $[Ca^{2+}]_i$ of human tracheal epithelial cells are in general agreement with the findings of others who studied human airway epithelial cells in suspension, in epithelial sheets or in cell-by-cell in culture. In addition, our results for the response to agonists such as isoproterenol, bradykinin, histamine, muscarinic agonists and cAMP analogs are similar to those reported by Yamaya et al. (1993) for cultured human tracheal surface epithelial cells studied in sheets, and the results of others for single-cell measurements on nasal epithelial cells cultured on coverslips (Boucher et al., 1989; Reinlib et al., 1992). However, Murphy et al. (1988) found no $[Ca^{2+}]_i$ response to isoproterenol in nasal epithelial cells studied in suspension, and Reinlib et al. (1992) found that $PGE_2$ and carbachol stimulate increases in $[Ca^{2+}]_i$ in cell-by-cell studies of human nasal epithelial cells in culture. Reinlib et al. (1992) also found reduced response to histamine in CF cultures. Differences in cell source (nasal vs tracheal), methods of cell separation and culture, and growth conditions between our studies and those of others, may account for the discrepancies between our results and theirs. For example, our cell-by-cell data indicate that the isoproterenol response is produced by only a few cells in our population, so preparative or culture conditions that select against this subpopulation could eliminate any apparent response. Neither we nor Yamaya et al. (1993) observed a significant response to muscarinic agonists in our tracheal cultures, but nasal epithelial cells studied by Reinlib et al. (1992) might be quite different. Our results, however, in contrast to those of Reinlib et al. (1992), do not indicate any substantive difference between CF and normal airway epithelial cell $[Ca^{2+}]_i$ responses.

The data reported here show quantitative differences in $[Ca^{2+}]_i$ measured cell-by-cell by ratio imaging using fluorescence microscopy and measured for whole monolayers by measuring the emission spectrum of fura-2 at 510 nm. However, the qualitative results - order of agonist potency, and comparison of normal and CF samples - are the same in the two methods. The small quantitative differences are probably accounted for by the different methods of measurement and by the fact that agonists in the one case were added to a closed cuvette and in the other case to a flow-through system.

In summary, our results indicate that in HTE cells, unlike canine tracheal epithelial cells, cAMP does not regulate $[Ca^{2+}]_i$. Moreover, some β-adrenergic responses in human airway epithelial cells, including those that produce changes in $[Ca^{2+}]_i$, may be mediated by means other than cAMP. Heterogeneity of HTE cells in primary cultures accounts in part for the variation in the response to agonists that provoke an increase in $[Ca^{2+}]_i$. The order of responsiveness to agonists that stimulate increases in $[Ca^{2+}]_i$ is similar in normal and CF cells.

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