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Regulation of Intracellular Ca^{2+} by CFTR in Chinese Hamster Ovary Cells

V. Urbach, B.J. Harvey

Wellcome Trust Cellular Physiology Research Unit, Department of Physiology, University College, Cork, Ireland

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Abstract. In cystic fibrosis, the mutation of the CFTR protein causes reduced transepithelial Cl^- secretion. As recently proposed, beside its role of Cl^- channel, CFTR may regulate the activity of other channels such as a Ca^{2+}-activated Cl^- channel. Using a calcium imaging system, we show, in adenovirus-CFTR infected Chinese Hamster Ovary (CHO) cell monolayers, that CFTR can act as a regulator of intracellular $[Ca^{2+}]_i$ ($[Ca^{2+}]_i$), involving purino-receptors. Apical exposure to ATP or UTP produced an increase in ($[Ca^{2+}]_i$ in noninfected CHO cell monolayers (CHO-WT), in CHO monolayers infected with an adenovirus-CFTR (CHO-CFTR) or infected with an adenovirus-LacZ (CHO-LacZ). The transient $[Ca^{2+}]_i$ increase produced by ATP or UTP could be mimicked by activation of CFTR with forskolin (20 μM) in CHO-CFTR confluent monolayers. However, forskolin had no significant effect on $[Ca^{2+}]_i$ in noninfected CHO-WT or in CHO-LacZ cells. Pretreatment with purino-receptor antagonists such as suramin (100 μM) or reactive blue-2. (100 μM), and with hexokinase (0.28 U/mg) inhibited the $[Ca^{2+}]_i$ response to forskolin in CHO-CFTR infected cells. Taken together, our experiments provide evidence for purino-receptor activation by ATP released from the cell and regulation of $[Ca^{2+}]_i$ by CFTR in CHO epithelial cell membranes.

Key words: CFTR — Intracellular Ca^{2+} — CHO cells — Forskolin — Purinoreceptors

Introduction

In cystic fibrosis, mutation of the Cystic Fibrosis Transmembrane conductance Regulator (CFTR) produces a reduced cAMP-dependent Cl^- secretion associated with Na^+ hyperabsorption that results in a dehydration of the epithelial lumen. Since identification and cloning of the CFTR protein (Quinton 1983, Riordan et al., 1989, Cliff et al., 1992), it has been suggested that CFTR is involved in epithelial ion transport not only through its Cl^- channel activity but also via interactions with other ion channels such as an outwardly rectifying Cl^- channel (ORCC) (Egan et al., 1992, Gabriel et al., 1993; Schwiebert et al., 1995, 1998*a*) and the epithelial Na^+ channel (Grubb et al., 1994; Chinet et al., 1994; Johnson et al., 1995; Hyde et al., 1993; Ismailov et al., 1996; Stutts et al., 1997; Kunzelman et al., 1997). The mechanism by which CFTR may regulate other ion channels is not well known. It has been proposed that CFTR may act through an autocrine mechanism involving ATP to regulate ORCC channels (Schwiebert et al., 1995). Recently, electrophysiological evidence showed the existence of a CFTR Cl^- pore and a CFTR-associated ATP pore regulated by common gates (Sugita, Yue & Foskett, 1998). Experiments of truncation of CFTR suggested the presence of two distinct domains in the structure of the CFTR protein: a Cl^- channel domain and a ORCC regulator domain (Schwiebert et al., 1998).

In this study, using fura-2 fluorescence spectrometry we have explored the possible role of CFTR in the regulation of intracellular calcium concentration ($[Ca^{2+}]_i$) subsequent to an ATP-release from the cell and activation of purino-receptors. We used the Chinese Hamster Ovary (CHO) cell line as an expression system for CFTR. Monolayers of wild type CHO cells (CHO-WT) were infected with an adenoviral vector containing human CFTR cDNA (H5.110CBCFTR). CHO cells express spontaneously a P_2U purino-receptor (Iredale et al., 1993), and external ATP has been shown to regulate the $[Ca^{2+}]_i$ and Cl^- secretion (Thiele et al., 1998) of these cells. Forskolin (20 μM) was used to stimulate CFTR activity and the effect of forskolin on $[Ca^{2+}]_i$ was compared between CHO cells infected with the adenovirus-CFTR (CHO-CFTR), CHO-WT cells and CHO cells in-

Correspondence to: V. Urbach

fected with an adenovirus LacZ (CHO-LacZ). These results provide direct evidence for a control of $[Ca^{2+}]_i$ by CFTR via stimulation of purino-receptors.

Materials and Methods

Cell Culture and Adenoviral Infection

The Chinese Hamster Ovary cells were grown on plastic culture flasks in Ham's F12 medium (ICN Biomedicals, Aurora, Ohio) supplemented with 10% FCS, 1% glutamin, and antibiotics at 37°C in 5% CO_2. Two days before the experiment, cells were plated at low density on glass coverslip. Confluent CHO monolayers (CHO-WT) were then either directly used for intracellular Ca^{2+} ($[Ca^{2+}]_i$) measurement or for adenoviral infection.

The adenoviral constructs (H5.110CBCFTR and H5.110CBLacZ) were made in the Laboratoire de Therapie Genique (Nantes, France). They are second generation of human serotype 5 adenovirus, containing a deletion of the E1 (Wilson, 1993) and E2 sub360 genes (Logan & Shenk, 1984), and a temperature-sensitive mutation (ts125) of the E2a gene (Ensinger & Ginsburg, 1972). Two different genes were inserted CBCFTR or LacZ. The viruses were grown in 293 cells (Graham et al., 1977) with a CMV/βactin promoter. The properties of these viruses have been initially studied using an epithelial cell line derived from pancreas of CF patient (CFPAC). CFPAC cells exposed to H5.110CBCFTR virus demonstrated high levels of membrane localized CFTR protein which corrected the defect in Cl^- conductance (Yang et al., 1994). Other studies also show successful expression of human CFTR in CHO cells (Mogayzel et al., 1997). In our study, for each experimental condition, monolayers of CHO cells were infected with an adenovirus containing CFTR or with a control adenovirus containing a LacZ insert. Confluent monolayers were exposed to the adenovirus in solution in 2% FCS, for 2 hr, at 37°C and then rinsed with culture medium. Different quantities of viruses were tested: 3×10^8 pfu CFTR with a multiplicity of infection (MOI) approximately equal to 10, 3×10^9 pfu CFTR (MOI = 100) and 3×10^9 pfu LacZ.

Whole-Cell Current Measurements

The whole-cell patch-clamp technique was applied to CHO cells isolated by trypsin (0.25% Trypsin EDTA, Gibco). Patch-pipettes (tip resistance = 5 MΩ) were prepared from borosilicate glass capillaries (Vitrex, Modulohm, Herlev, Denmark), pulled and polished on a programmable puller (DMZ, Zeitz Instrumente, Augsburg, Germany). Patch-pipettes were filled with an intracellular-like K^+-rich solution (KCl 140 mM, NaCl 10 mM, HEPES 10 mM, EGTA 5 mM, $MgCl_2$ 1.22 mM, $CaCl_2$ 2.96 mM (pCa 8), pH 7.2 and 290 mOsM). The cells were bathed in Krebs solution (NaCl 140 mM, KCl 5 mM, $CaCl_2$ 2 mM, HEPES 10 mM, $MgCl_2$ 2 mM, pH 7.4 and 290 mOsM). The whole-cell configuration was obtained from cell-attached mode after breaking the patch membrane by applying a brief negative pressure in the pipette (−20 mbar). Whole-cell currents were amplified (Axopatch 200A, Axon Instrument, CA), and digitized using a 16-bit A/D converter (CED 1401, Cambridge Electronic Design, Cambridge, UK) after a low pass filtering at 5 kHz (Kemo VBF8, Kemo LTD Beckenham Kent, UK) and sampled in real-time on hard disk. Current-voltage relations were analyzed using the CED VClamp software. The membrane conductance, G_m, was calculated at the zero current intercept of the whole-cell *I-V* relations.

Intracellular Ca^{2+} Measurements

Monolayers of wild type CHO cells, CHO cells infected with the H5.110CBCFTR virus or with the H5.110CBLacZ virus were loaded for 45 min, in the dark, at room temperature, with 3 μM of the Ca^{2+}-sensitive fluorescent probe fura-2 acetoxy-methyl ester (fura 2-AM, Molecular Probes, Netherlands). Cells were then rinsed twice with Krebs solution. CHO monolayers were placed in temperature-controlled Peltier perfusion chambers (Medical System Corporation, model TC 202, Hertfordshire, UK) on an inverted microscope equipped for epifluorescence (Diaphot, Nikon, Japan). The light from a Xenon lamp (OSRAM, Germany) was filtered through alternating 340 nm and 380 nm filters mounted in a chopper motorised under computer control (STARWISE FLUO system, IMSTAR Paris, France). The emission fluorescence produced after fura-2 excitation was selected with a filter at 510 nm. The transmitted light image was detected using a video camera (Darkstar, Photonics Sciences, UK) coupled to the microscope. The fluorescence obtained at each excitation wavelength (F_{340} and F_{380}) depends upon the level of Ca^{2+} binding to fura-2, according to an in vivo calibration of the dye performed using a range of EGTA-buffered Ca^{2+} solutions of the fura-2 free acid. Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was calculated automatically by the computer, using the Grynkiewicz equation:

$$[Ca^{2+}]_i = K' \frac{R - R_{min}}{R_{max} - R}$$

Where K' is the dissociation constant of Ca^{2+} binding with fura-2, and R_{max} and R_{min} are the fluorescence ratio (F_{340}/F_{380}) values under saturating and Ca^{2+}-free conditions respectively. The mean Ca^{2+} variations (ΔCa^{2+}) were calculated by subtraction of the maximum $[Ca^{2+}]_i$ value (at the peak) to the initial basal $[Ca^{2+}]_i$.

Suramin was obtained from Calbiochem (Nottingham, UK), reactive blue-2 from Research Biochemicals International (Natick, MA) and the other compounds from SIGMA (Dorset, UK). Results are expressed as mean values ± SEM ($n = a, b$ cells) where a equals to the number of experiments and b the total number of cells analyzed. Statistical analysis was performed using Students *t*-test.

Results

Whole-cell Currents in CFTR-Infected and Noninfected CHO Cells

To test the functional expression of CFTR in CHO cells infected with the adenovirus H5.110CBCFTR, we tested the effect of forskolin (20 μM) a known activator of CFTR, on the whole-cell conductance of CHO cells. The whole-cell current-voltage relationships were studied in solutions containing equal concentrations of Cl^- on both sides of the cell membrane (140 mM NaCl Krebs solution in the bath and 140 mM KCl solution in the patch pipette). The reversal potential of whole-cell currents recorded under these conditions was 0 mV, indicating that Cl^- was the major ion contributing to the whole-cell current. The whole-cell membrane conductance was 0.379 ± 0.086 nS, ($n = 6$) in noninfected wild-type CHO cells (CHO-WT). Infection of CHO cells with adenovirus carrying 3.10^9 pfu of either CFTR

(CHO-CFTR cells) or LacZ (CHO-LacZ cells), did not significantly change the membrane conductance nor the whole cell *I-V* relationship (Fig. 1*A–C*). For CHO-CFTR cells, the $G_m = 0.471 \pm 0.08$ nS ($n = 6$, $P = 0.1$), and for CHO-LacZ cells, the $G_m = 0.326 \pm 0.083$ nS, ($n = 6$, $P = 0.1$). The 2,2′ iminobenzoic acid (DPC 10 μM), used as an inhibitor of CFTR channel activity, did not affect the membrane conductance of any of the three types of CHO cells. This result indicates that CFTR is not spontaneously active in the infected cells.

The CFTR activator, forskolin, produced a threefold increase in the whole-cell conductance of CHO-CFTR cells ($G_m = 1.425 \pm 0.145$ nS, $n = 6$) (Fig. 1*B*). The forskolin-stimulated conductance of CHO-CFTR cells was inhibited by subsequent exposure to DPC (10 μM) ($G_m = 0.597 \pm 0.140$ nS, $n = 6$) (Fig. 1*B*). These data provide strong evidence for successful functional expression of human CFTR Cl^- channels in CHO cells following infection with adenovirus H5.110CBCFTR. As a control, we exposed CHO-WT cells and CHO-LacZ cells to forskolin which did not significantly affect the whole-cell conductance (forskolin on CHO-WT: $G_m = 0.358 \pm 0.087$ nS, $n = 6$; forskolin on CHO-LacZ: $G_m = 0.362 \pm 0.091$ nS, $n = 6$) (Fig. 1*A*).

Basal Intracellular $[Ca^{2+}]$ in Infected and Noninfected CHO Cells

Prior to any stimulation of a $[Ca^{2+}]_i$ response in CHO cells, the basal $[Ca^{2+}]_i$ was compared between noninfected and infected CHO monolayers preparations. Basal $[Ca^{2+}]_i$ was 74 ± 4 nM ($n = 27$, 183 cells) in noninfected CHO cells monolayers (CHO-WT). The basal $[Ca^{2+}]_i$ was slightly but significantly increased in CHO monolayers infected with both types of adenoviral preparations. In CHO monolayers infected with 3.10^9 pfu CFTR (CHO-CFTR), basal $[Ca^{2+}]_i = 123 \pm 5$ nM ($n = 31$, 171 cells, $P < 0.0001$). In CHO monolayers infected with 3.10^9 pfu LacZ (CHO-LacZ) $[Ca^{2+}]_i = 137 \pm 5$ ($n = 7$, 51 cells, $P < 0.0001$). No significant difference was found between the $[Ca^{2+}]_i$ measured in CHO-CFTR and CHO-LacZ ($P > 0.01$), which indicates that the increase in basal $[Ca^{2+}]_i$ measured in CHO-CFTR cells was not due to the CFTR protein expression but most likely a non specific effect of the adenoviral infection.

External Nucleotide Effects on Intracellular $[Ca^{2+}]$

The Ca^{2+} sensitivity to external nucleotides was explored in the three types of CHO monolayers (CHO-WT, CHO-CFTR, CHO-LacZ). External nucleotides produced a rapid and transient increase in $[Ca^{2+}]_i$ in all three types of cell monolayers. Mechanical stimulation or shear stress of cells has been shown to cause release of ATP and calcium changes (Grygorczyk & Hanarahan, 1997*a*) and this may be an associated artefact of solution changes. Pipetting or slow perfusion (1 μl/sec) of normal Krebs solution did not affect basal Ca^{2+} levels. Apical addition of ATP on CHO-WT cell monolayers by direct pipetting of small volumes of drug (1 μl) to the bath (1 ml) or perfusion of drug at slow rates (1 μl/sec) produced similar increases in $[Ca^{2+}]_i$. The changes in $[Ca^{2+}]_i$ were found to occur homogeneously within the cell without evidence for intracellular calcium gradients or waves. Typical responses to ATP in CHO monolayers infected with 3.10^9 pfu CFTR (CHO-CFTR), in noninfected CHO cells (CHO-WT) and in CHO monolayers infected with 3.10^9 pfu LacZ (CHO-LacZ) are shown in Fig. 2. In CHO-CFTR cells, ATP 100 μM produced a transient $[Ca^{2+}]_i$ increase of 185 ± 15 nM ($n = 4$, 25 cells). In noninfected cells and in CHO-LacZ the same ATP concentration stimulated a $[Ca^{2+}]_i$ increase of 209 ± 10 nM ($n = 5$, 30 cells) and 190 ± 10 nM ($n = 4$, 25 cells) respectively. Apical exposure of CHO-WT cells to UTP 100 μM also produced a $[Ca^{2+}]_i$ increase of 220 ± 6 nM ($n = 4$, 24 cells). Lower concentration of these nucleotides such as 10 nM of ATP or 10 nM of UTP also produced a $[Ca^{2+}]_i$ response of 45 ± 21 nM ($n = 3$, 16 cells, $P < 0.001$) and 53 ± 1 nM ($n = 3$, 16 cells, $P < 0.001$), respectively, in CHO-WT cells. ATP or UTP added at 1 nM concentration, did not produce a $[Ca^{2+}]_i$ change in any of the three CHO cell preparations.

Forskolin Effect on $[Ca^{2+}]_i$

The possible role of CFTR in regulating intracellular $[Ca^{2+}]_i$ was investigated by testing the effect of forskolin (20 μM) on non CFTR-infected and CFTR-infected CHO cell monolayers. Two different quantities of adenovirus were tested. In 90% of the CHO cells infected with 3.10^9 pfu CFTR, forskolin produced a large and transient increase in $[Ca^{2+}]_i$ from 127 ± 4 nM to a peak value of 332 ± 7 nM followed by a recovery to a basal $[Ca^{2+}]_i$ ($n = 11$, 73 cells, $P < 0.0005$) (Fig. 3). In CHO cells infected with 3.10^8 pfu CFTR, forskolin produced a $[Ca^{2+}]_i$ response of similar amplitude ($[Ca^{2+}]_i$ increase of 198 ± 9 nM ($n = 4$, 21 responsive cells)) but only in 10% of the cells. External ATP (100 μM) added after the $[Ca^{2+}]_i$ response to forskolin did not produce any further $[Ca^{2+}]_i$ change in CHO-CFTR cells (Fig. 3).

In CHO-WT monolayers, forskolin did not produce the large increase in $[Ca^{2+}]_i$ as observed in CHO-CFTR. The $[Ca^{2+}]_i$ variation of 30 ± 21 nM ($n = 9$, 52 cells) measured in CHO-WT, upon forskolin exposure, was largely reduced compared to the $[Ca^{2+}]_i$ increase of 205 ± 10 nM ($n = 11$, 73 cells, $P < 0.0001$) obtained in CHO-CFTR cells (Fig. 3). The concentration depen-

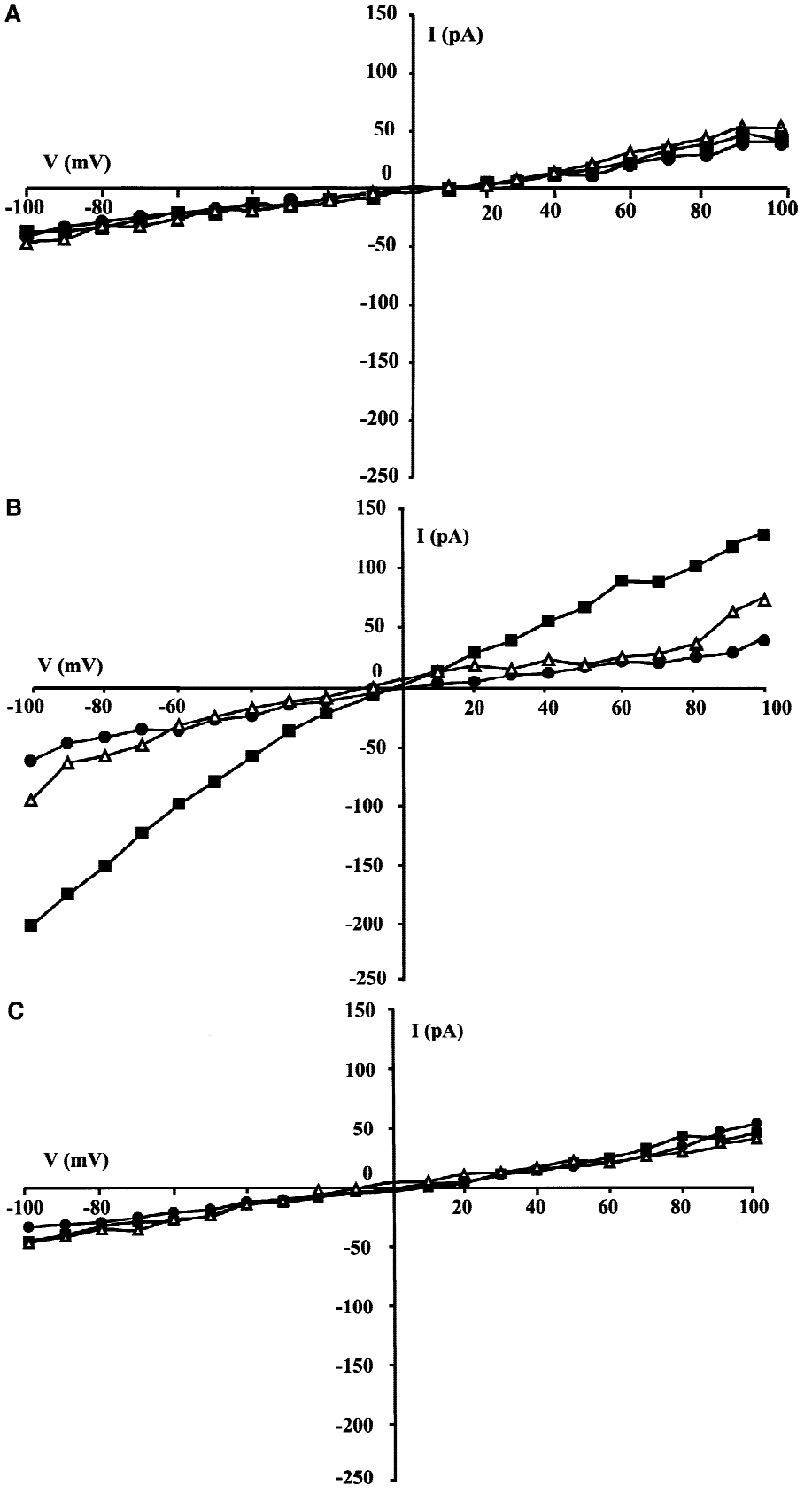

Fig. 1. Typical whole-cell current-voltage relationships obtained in non-infected CHO cells (*A*), in CFTR-infected CHO cells (*B*) and in LacZ-infected CHO cells (*C*). In the three types of cell preparations, the current-voltage relationships are shown under control conditions (●), and following exposure of the cells to forskolin (■), and after a subsequent addition of DPC in the bathing solution (▲).

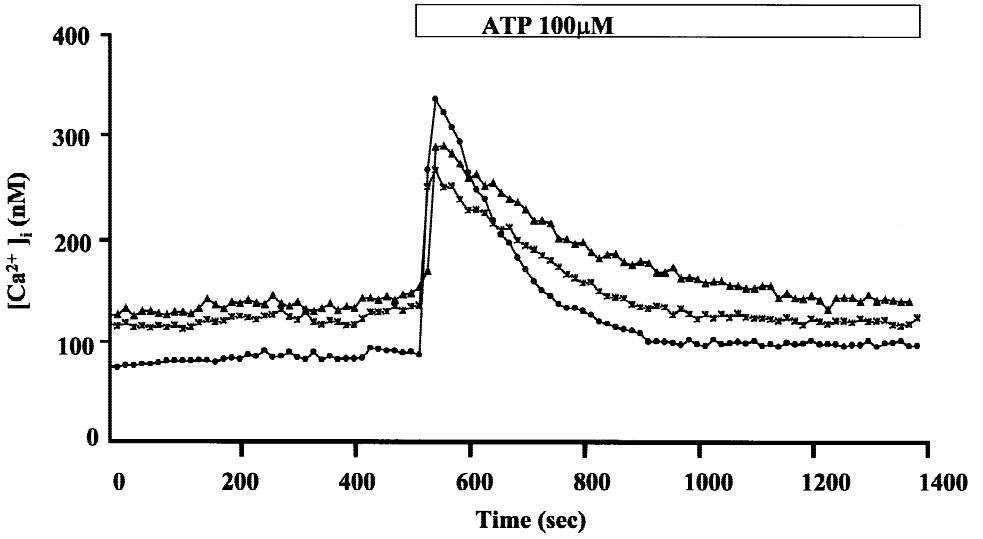

Fig. 2. Typical $[Ca^{2+}]_i$ responses to apical exposure to ATP (100 μM) in CHO-WT monolayers (●), in CHO-CFTR (3.10^9 pfu CFTR) monolayers (*), and in CHO-LacZ monolayers (▲).

dence of the adenovirus-CFTR infection on the $[Ca^{2+}]_i$ response to forskolin and, in particular, the lack of $[Ca^{2+}]_i$ response to forskolin in noninfected CHO monolayers suggests a role of CFTR in regulating the level of $[Ca^{2+}]_i$. In support of this conclusion, pretreatment of the apical membrane of the CHO-CFTR cell monolayers with glibenclamide, used as a CFTR channel inhibitor, significantly inhibited the forskolin induced $[Ca^{2+}]_i$ response. In these experiments, glibenclamide exposure did not affect the basal level in $[Ca^{2+}]_i$ (before glibenclamide 115 ± 20 nM, after glibenclamide 110 ± 32 nM, $n = 5$, 24 cells). In glibenclamide treated CHO-CFTR cells, forskolin addition produced only a reduced $[Ca^{2+}]_i$ increase to 180 ± 36 nM ($n = 5$, 24 cells) ($\Delta[Ca^{2+}]_i = 30 \pm 21$ nM upon forskolin addition to glibenclamide treated CHO-CFTR cells).

To verify that the CFTR protein and not the adenoviral infection, was responsible for the $[Ca^{2+}]_i$ response to forskolin in CHO-CFTR monolayers, we tested the effect of forskolin on CHO cells infected with an adenovirus-LacZ, used as a control adenovirus, non-coding for CFTR. In CHO-LacZ cells, forskolin did not produce any significant $[Ca^{2+}]_i$ increase (7 ± 6 nM ($n = 7$, 37 cells)). Thus CHO-WT and CHO-LacZ cells did not significantly differ in their lack of response to forskolin.

In CHO-WT cells and CHO-LacZ cells, after forskolin exposure, exogenous ATP (100 μM) still produced a significant and large $[Ca^{2+}]_i$ increase of 210 ± 25 nM ($n = 9$, 52 cells) and 175 ± 24 nM ($n = 7$, 37 cells) respectively (Fig. 3). These data show that the adenoviral infection does not interfere with the nucleotide-induced $[Ca^{2+}]_i$ response of these cells.

Hexokinase Effect on the Forskolin-Induced $[Ca^{2+}]_i$ Increase

To elucidate the mechanism of the $[Ca^{2+}]_i$ increase obtained after forskolin in CHO-CFTR cells, we have tested the effect of pretreatment of the CHO-CFTR cells with the enzyme hexokinase (which consumes extracellular ATP by phosphorylation of D-glucose present in the bathing solution) on the $[Ca^{2+}]_i$ response to forskolin. The rationale behind this type of experiment was to test the eventual involvement of endogenous release of ATP in the Ca^{2+} response to forskolin. Treatment of the apical side of polarised CHO-CFTR cell monolayers with hexokinase (0.28 U/mg in the presence of glucose in the bathing solution), for 5 min did not significantly affect the basal $[Ca^{2+}]_i$ level, but significantly inhibited the $[Ca^{2+}]_i$ increase induced by forskolin in CHO-CFTR cells. In CHO-CFTR monolayers, the $[Ca^{2+}]_i$ was initially 108 ± 14 nM and remained at 107 ± 11 nM after 20 min of hexokinase treatment ($n = 4$, 30 cells). Pretreatment of CHO-CFTR monolayers with hexokinase significantly inhibited the calcium response to forskolin (Fig. 4). Under these conditions, the rise in $[Ca^{2+}]_i$ induced by forskolin was only 90 ± 23 nM ($n = 4$, 30 cells), which is significantly reduced compared to the increase of 205 ± 10 nM ($n = 11$, 73 cells, $P < 0.0001$) obtained in CHO-CFTR cells without hexokinase treatment (Fig. 4).

Control experiments were performed showing that apical hexokinase pretreatment inhibited the $[Ca^{2+}]_i$ change usually observed after addition of external apical ATP. After pretreatment of CHO-WT cells with hexokinase, the $[Ca^{2+}]_i$ response to ATP 100 μM exposure

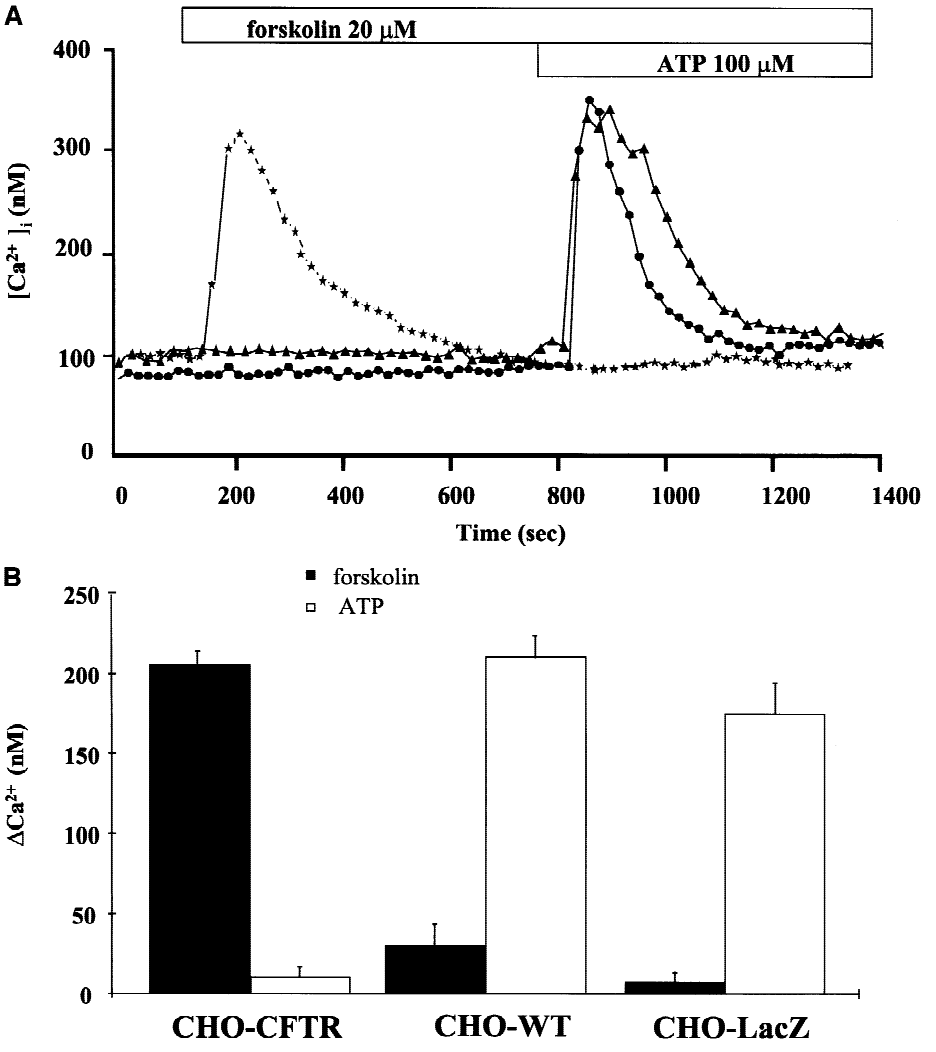

Fig. 3. (*A*) Forskolin (20 μM), followed by ATP (100 μM) effects on $[Ca^{2+}]_i$ in CHO-WT monolayers (●), in CHO-CFTR (3×10^9 pfu CFTR) monolayers (★) and in CHO-LacZ monolayers (▲). (*B*) Mean intracellular $[Ca^{2+}]$ variations (ΔCa^{2+}) in CHO-CFTR (3×10^9 pfu CFTR) monolayers, in CHO-WT monolayers and in CHO-LacZ monolayers after forskolin (20 μM) and ATP (100 μM) added subsequently.

was reduced to 89 ± 40 nM, compared to the control response of 209 ± 10 nM ($n = 5$, 30 cells, $P < 0.001$) obtained with the same ATP concentration. However hexokinase treatment did not affect the $[Ca^{2+}]_i$ response to apical exposure to UTP (100 μM) (Fig. 4*B*). These experiments show that hexokinase blocks the forskolin effect at a step prior to the activation of purino-receptors and provide evidence that endogenous release of ATP across the apical membranes of the CHO-CFTR cells mediates the $[Ca^{2+}]_i$ response to forskolin.

Suramin and Reactive Blue-2 Inhibition of Forskolin-Induced $[Ca^{2+}]_i$ Increase

To investigate the role of purino-receptors in the $[Ca^{2+}]_i$ response to forskolin, the effect of pretreatment of CHO-CFTR monolayers with suramin (100 μM) and the reactive blue-2 (100 μM), two purino-receptor antagonists was tested on the response to forskolin. Both suramin and reactive blue-2, did not cause any change in the basal $[Ca^{2+}]_i$ level, but inhibited the forskolin-induced $[Ca^{2+}]_i$ increase in CHO-CFTR monolayers (Fig. 4). The $[Ca^{2+}]_i$ levels measured in CHO-CFTR monolayers before and after suramin treatment were 132 ± 54 nM and 140 ± 45 nM ($n = 5$, 29 cells), respectively. The $[Ca^{2+}]_i$ levels before and after reactive blue-2 treatment were 102 ± 20 nM and 88 ± 15 nM ($n = 5$, 20 cells), respectively. The $[Ca^{2+}]_i$ levels measured following forskolin were 145 ± 39 nM ($n = 5$, 29 cells) and 116 ± 43 nM ($n = 5$, 20 cells), in suramin and reactive blue-2 pretreated cells, respectively. Interestingly, suramin and reactive blue-2 abolished the $[Ca^{2+}]_i$ response to ATP but suramin only partially reduced the response to UTP

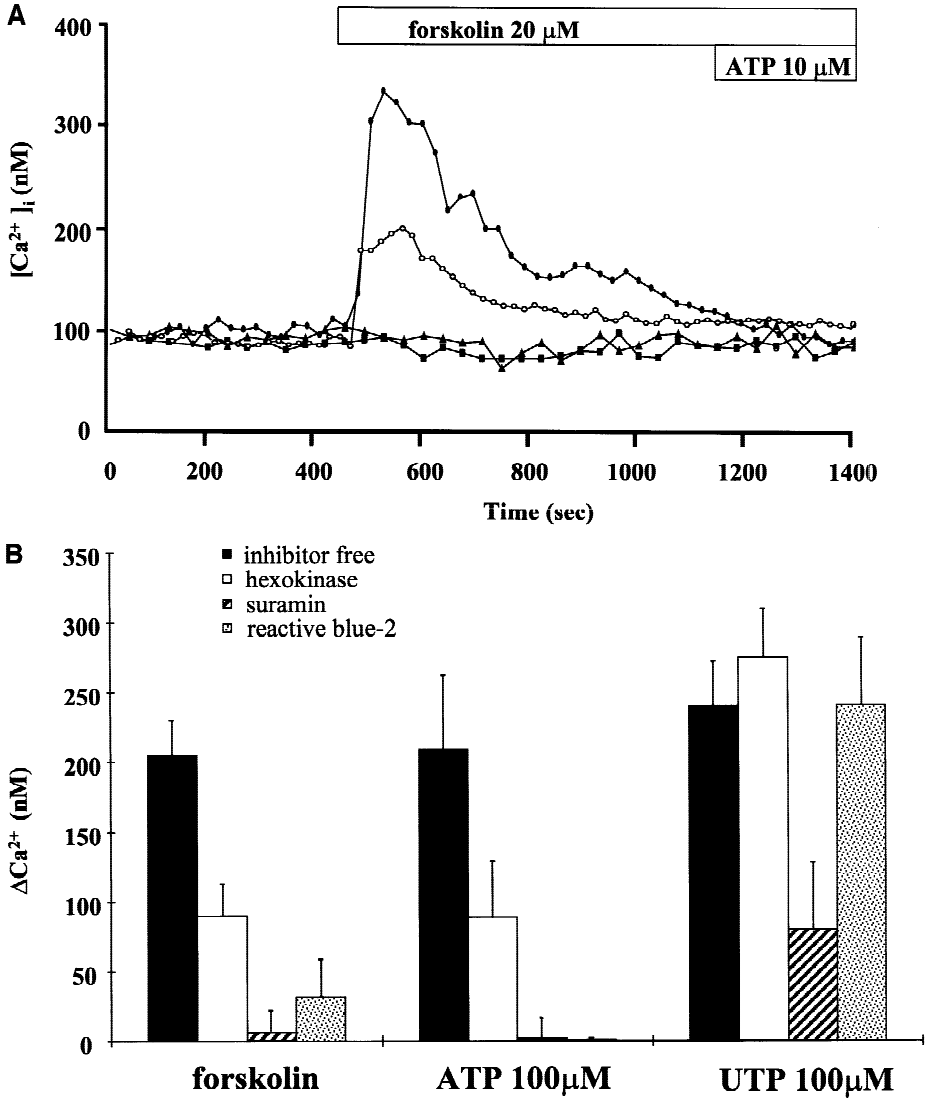

Fig. 4. Inhibition of the $[Ca^{2+}]_i$ response induced by forskolin. (*A*) Typical $[Ca^{2+}]_i$ responses to forskolin in CHO-CFTR without inhibitor present (●), in CHO-CFTR cells pretreated with hexokinase (○) or with suramin (■) or with reactive blue-2 (▲). (*B*) Mean intracellular $[Ca^{2+}]$ variations (ΔCa^{2+}) measured upon exposure to forskolin in CHO-CFTR cells, or upon exposure to ATP or UTP in CHO-WT cells with or without inhibitors as indicated.

and reactive blue-2 did not affect the response to UTP (Fig. 4).

Taken together these results show that CFTR can act as a regulator of $[Ca^{2+}]_i$ in CHO cells via a mechanism involving purino-receptor activation possibly by endogenous release of ATP from the cell.

Discussion

In this study, the hypothesis of a control of $[Ca^{2+}]_i$ by CFTR via stimulation of purino-receptors was tested in CHO cells used as an epithelial system of human CFTR expression. We verified, initially, that exposure to ATP or UTP on the apical side of confluent CHO cell monolayers produced an increase in $[Ca^{2+}]_i$. This result is in agreement with the identification of purino-receptors (P_2 type) already described, in the plasma-membrane of Chinese Hamster Ovary (CHO) cells (Iredale et al., 1993).

Previous reports have shown successful expression of human CFTR in CHO cells (Mogayzel et al., 1997). In this study, the functional expression of human CFTR in the plasma membrane of CHO cells infected with the H5.110CBCFTR adenovirus has been verified by the forskolin stimulation of a DPC-sensitive whole-cell conductance. The conductance is generated by chloride ions, given the equivalence of the reversal potential and the equilibrium potential for Cl^- across the membrane. The forskolin-stimulated conductance was observed only in CHO-CFTR cells. Forskolin had no significant effect in

noninfected cells or in CHO cells infected with the H5.110CBLacZ adenoviral construct, used as control for a non-CFTR adenoviral infection of CHO cells.

We showed that the basal $[Ca^{2+}]_i$ was slightly increased by both adenoviral infections (H5.110CBLacZ and H5.110CBCFTR) indicating that the elevated basal $[Ca^{2+}]_i$ was due to the adenoviral infection itself and not to the presence of CFTR. In the three types of CHO cell preparations (CHO-WT, CHO-CFTR, CHO-LacZ) a similar $[Ca^{2+}]_i$ increase was obtained after apical ATP exposure. This result shows that the adenoviral infection did not affect the $[Ca^{2+}]_i$-sensitivity of these cells to external ATP.

In this study, using forskolin as an activator of CFTR in CHO-CFTR infected cells, we provided the first direct evidence for regulation of $[Ca^{2+}]_i$ by CFTR. The effect of forskolin on $[Ca^{2+}]_i$ was compared between noninfected and adenovirus-CFTR infected monolayers coming from the same cell culture batch (same clone, same passage numbers of CHO cells). Forskolin failed to produce any significant $[Ca^{2+}]_i$ increase in wild type CHO cells, which is consistent with previous report of $[Ca^{2+}]_i$ insensitivity of these cells to forskolin (Van Sande et al., 1990). In contrast, forskolin (20 μM) produced a $[Ca^{2+}]_i$ increase in monolayers of CHO cells infected with CFTR. Since the only difference between the two cell preparations was the adenoviral-CFTR infection, this result suggests a role of the CFTR protein in the regulation of $[Ca^{2+}]_i$. In addition, the absence of $[Ca^{2+}]_i$ response to forskolin in CHO-LacZ cells excludes the possibility that the forskolin induced $[Ca^{2+}]_i$ increase observed in CHO-CFTR cells was due to the adenoviral infection and not the CFTR protein. The activation of CFTR (by the cAMP agonist, forskolin) was necessary to produce a $[Ca^{2+}]_i$ increase, since no difference of the basal level in $[Ca^{2+}]_i$ was detected between CHO-LacZ and CHO-CFTR cells. Our results, therefore, provide evidence for the control of $[Ca^{2+}]_i$ by CFTR activation in CHO-CFTR infected monolayers.

The evidence for a role of CFTR in the regulation of $[Ca^{2+}]_i$ via stimulation of purino-receptors is supported by the inhibitory effect of two purino-receptor antagonists, suramin and reactive blue-2, on the $[Ca^{2+}]_i$ response induced by forskolin in CHO-CFTR cells. The hypothesis that released endogenous nucleotides are involved in this response, was verified by the inhibition of the $[Ca^{2+}]_i$ rise induced by forskolin in CHO-CFTR cells after pretreatment with hexokinase (which consumes extracellular ATP by phosphorylation of D-glucose). The specificity of the inhibitory effect of suramin or reactive blue-2 on purino-receptors during the forskolin induced $[Ca^{2+}]_i$ increase was tested by showing that these known purino-receptors antagonists inhibited the $[Ca^{2+}]_i$ response induced by external ATP. Similarly, the role of hexokinase on external ATP, in the $[Ca^{2+}]_i$ response to forskolin, is supported by the inhibitory effect of hexokinase on the $[Ca^{2+}]_i$ response to both forskolin and external ATP.

Previous studies in intact tracheal and intestinal epithelia have shown an increase in $[Ca^{2+}]_i$ in response to forskolin (Grubb et al., 1994, MacVinish et al., 1998). Forskolin was observed to $[Ca^{2+}]_i$ in both normal and CF tracheal cells, suggesting this as a mechanism to induce Cl^- secretion. However, we show that forskolin has little effect on $[Ca^{2+}]_i$ in CHO cells which lack CFTR. Thus forskolin may have nonspecific effects on calcium homeostasis independent of its action on CFTR. Forskolin may affect $[Ca^{2+}]_i$ signaling via phopholipase A_2 metabolism or a cAMP sensitivity of the IP_3 receptors (Taylor & Traynor, 1995; Zhong et al., 1998). However, the restriction of the forskolin effect on $[Ca^{2+}]_i$ to CFTR-infected CHO cells and the abolition of this response by inhibitors acting at steps proximal to the intracellular Ca^{2+} signaling cascade, argue in favor of an autocrine role for CFTR protein in regulating $[Ca^{2+}]_i$ via the release of nucleotides.

Hexokinase is known to have a greater affinity for ATP than for UTP (Simofuruaya & Suzuki, 1991) and our control experiments are in agreement with this report. Hexokinase inhibits the $[Ca^{2+}]_i$ response to ATP and does not affect the $[Ca^{2+}]_i$ response to UTP. In addition, we found reactive blue-2 to be a more potent inhibitor of the $[Ca^{2+}]_i$ response to ATP, compared to UTP. The reduced or absent sensitivity to reactive blue-2 of the response to UTP has also been reported in nonepithelial and epithelial tissues (Ikeda et al., 1995; Matsumoto, Nakane & Chiba, 1997; Zhang et al., 1997; Matsumoto et al., 1997; Matsuo et al., 1997; Huber-Lang et al., 1997; Zhong et al., 1998; Srinivas et al., 1998). Taken together, the selective inhibition by hexokinase and reactive blue-2 of the $[Ca^{2+}]_i$ response to ATP compared to UTP and the large inhibition of the forskolin induced $[Ca^{2+}]_i$ increase in CHO-CFTR cells, suggests that the nucleotide involved in the $[Ca^{2+}]_i$ response to forskolin is most likely ATP rather than UTP.

The role of CFTR channel as a regulatory protein in epithelia has been widely reported in the literature, but the mechanisms involved are complex and not yet well described. There is evidence for the regulation of Na^+ channels in the apical membrane of respiratory epithelia by CFTR via direct protein-protein interaction (Stutts et al., 1995; Mall et al., 1996). A stimulatory role of CFTR on water permeability has also been described in *Xenopus* oocytes expressing CFTR (Hasegawa et al., 1992; Schreiber et al., 1997). In airway epithelia, the outwardly rectifying Cl^- channel (ORCC) has been shown to be stimulated by intracellular cAMP which activates CFTR (Schwiebert et al., 1995). In the latter experiments, hexokinase inhibited the current due to ORCC activation by cAMP but not the current due to CFTR

channel activation. These data suggested a role of CFTR in the indirect regulation of ORCC involving an ATP release from the cell (Schwiebert et al., 1995).

Our results provide evidence for an autocrine regulatory role of CFTR on intracellular $[Ca^{2+}]_i$ signalling via purino-receptor activation by external ATP released from the cell. Different hypotheses have been put forward to explain the role of CFTR in the stimulation of purino-receptors in the apical membrane of epithelia: ATP could be transported through the CFTR protein itself or through an associated protein controlled and by CFTR. Contradictory results have been reported concerning the ATP conductance of the CFTR channel. Electrophysiological experiments showed an ATP conductance of the CFTR channel in CFTR-transfected mammary cells (Reisen et al., 1994), in the endoplasmic reticulum and the plasma-membrane of CFTR-transfected CHO cells (Pasyk & Foskett, 1997) and in airway epithelia (Schwiebert et al., 1995). However, other patch-clamp studies reported the failure of CFTR to conduct ATP in human sweat duct and in the Calu-3 submucosal gland serous cell line (Reddy et al., 1996), in CFTR-transfected Chinese hamster ovary (CHO) cells (Grygorczyk, Tabcharini & Hanrahan, 1996; Li, Ramjeesingh & Bear, 1996; Reddy et al., 1996) and in planar lipid bilayers (Reddy et al., 1996). Studies using luciferin/luciferase (Prat et al., 1996; Taylor et al., 1998; Jiang et al., 1998) or [g-^{32}P] ATP release assay in airway epithelia (Schwiebert et al., 1995) show that CFTR expression is associated with ATP release from the cells. Others failed to observe any association of CFTR with ATP release (Grygorczyk & Hanrahan, 1997*a*,*b*). This divergence of results suggests that CFTR-associated ATP release/transport may be dependent on the type of epithelium and/or cofactors regulated by the external environment of the cells (reviewed by Schwiebert et al., 1998*b*). More recently, electrophysiological evidence, in MDCK epithelial cells, points to the existence of a CFTR Cl^- pore and a CFTR-associated ATP pore regulated by common gates (Sugita et al., 1998). Experiments with truncated CFTR proteins expressed in *Xenopus* oocytes also suggests the presence of different functional domains in the CFTR protein: a Cl^- channel domain and an ORCC regulator domain (Schwiebert et al., 1998*a*), and that CFTR-associated ATP release is controlled by a Cl^- sensor domain of the CFTR protein (Jiang et al., 1998). In our study, the calcium response to CFTR activation is inhibited by glibenclamide, a blocker of CFTR. This would favor the hypothesis that ATP is trafficked via CFTR (or chloride conductance through CFTR is necessary for ATP release).

The involvement of CFTR in the regulation of $[Ca^{2+}]_i$ could explain the pleiotropic role of CFTR in the regulation of other transporters. The $[Ca^{2+}]_i$ increase controlled by CFTR could directly regulate Ca^{2+}-sensitive ion channels or could trigger other Ca^{2+}-dependent cellular mechanisms, such as vesicle trafficking or PKC stimulation, involved in regulation of ion channel activity. An autocrine regulation of $[Ca^{2+}]_i$ by CFTR activation could stimulate Ca^{2+}-activated Cl^- current (Mason, Paradiso & Boucher, 1991; Urbach et al., 1994; Schwiebert et al., 1995). In addition, amiloride-sensitive sodium absorption through epithelia has been shown to be indirectly inhibited by experimental manoeuvres designed to increase $[Ca^{2+}]_i$ (Graham et al., 1992; Ismailov, Berdiev & Benos, 1995; Harvey, 1995; Maguire et al., 1999). Therefore, the CFTR protein activation could control the balance between secretion and absorption by simultaneous stimulation of chloride secretion through other Cl^- channels while downregulating sodium absorption in normal epithelia.

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