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Optical imaging of Cl^- permeabilities in normal and CFTR-expressing mouse L cells

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Single cell optical imaging techniques were used to compare Cl^- conductances in cystic fibrosis transmembrane conductance regulator (CFTR)-expressing and control mouse L cell fibroblasts. Elevation of intracellular cAMP levels in control cells was without effect on plasma membrane Cl^- permeability, whereas cells engineered to stably express CFTR displayed a 20-fold enhancement of plasma membrane Cl^- permeability in response to cAMP. Control L cells displayed Ca^{2+}-, as well as swelling-activated Cl^- permeabilities, which were small compared with cAMP-stimulated permeability in CFTR-expressing cells. CFTR-expressing cells also displayed a similar swelling-activated Cl^- permeability. Whereas 50% of the CFTR-expressing cells possessed a small Ca^{2+}-activated Cl^- permeability similar to control cells, the other cells displayed an enhanced response which was never observed in control cells. Intracellular cAMP determinations suggested that this latter result might be explained by a Ca^{2+}-induced rise of cAMP. The cAMP-activated and Ca^{2+}-activated Cl^- conductances had different anion selectivities, as measured by light scattering of suspended cells. Activation of protein kinase C was without effect on Cl^- permeability in CFTR-expressing cells, nor did it modify cAMP-activation of Cl^- permeability. Thus, expression of human CFTR in L cells does not confer cAMP-sensitivity to pre-existing, endogenous Ca^{2+}- or swelling-activated Cl^- channels, but rather confers a novel Cl^- conductance which is regulated by cAMP. Osmotic cell swelling and PKC activation are without specific effect in CFTR-expressing L cells. However, elevated $[Ca^{2+}]_i$ may play a role in activating a Cl^- conductance specifically associated with CFTR.

Introduction

Cystic fibrosis (CF) is the most common lethal autosomal recessive disease among Caucasians. Although the spectrum of clinical manifestations of CF is quite wide, it is generally believed that the underlying basis of the disease is abnormal regulation of epithelial ion and fluid transport, in particular Cl^- secretion [1,2]. Cloning of the CF gene and subsequent studies of the protein which it encodes, the cystic fibrosis transmembrane conductance regulator (CFTR), have established that CFTR is a cAMP-regulated, low conductance (5–10 pS), linear Cl^- channel. Thus, transfection of CFTR into cells caused the appearance of a cAMP-regulated Cl^- channel not normally present in these cells [3–9]; mutation of charged residues in the transmembrane domains of CFTR changed the anion selectivity of the Cl^- channel associated with its expression [10]; and purification and reconstitution of CFTR into lipid bilayers caused the appearance of a protein kinase A-stimulated Cl^- channel with properties similar to the cAMP-activated Cl^- channels expressed endogenously in epithelial cells [11]. In addition to its regulation by cAMP-mediated phosphorylation, CFTR Cl^- channel activity might also be regulated by protein kinase C [6,12] and by adenine nucleotides [13,14].

In addition to CFTR, epithelial cells also express other plasma membrane Cl^- channels, including Ca^{2+}-activated Cl^- channels and Cl^- channels which are activated by cell swelling. Data from several studies suggest that the CFTR Cl^- channel is different from the Ca^{2+}- and volume-activated channels. First, in cells from CF patients, cAMP-activated Cl^- currrents are absent, whereas Ca^{2+}-or volume-activated currents are still present [15–19]. Second, when examined in the same cell, the three conductances have different biophysical characteristics, including time-dependences of their responses to voltage, anion selectivities, current/voltage relations, and sensitivities to inhibitors [16–18,20,21]. Nevertheless, other data suggest that in addi-

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tion to conferring the small linear cAMP-activated Cl^- channel, CFTR expression may also affect other Cl^- conductances. For example, expression of CFTR in CF cells restores regulation by cAMP of an outwardly rectifying Cl^- channel [22,23] which is different from CFTR [24]. Both cAMP-, as well as Ca^{2+}-activated Cl^- conductances were absent in G_1-phase lymphocytes obtained from CF patients [25]. In a colonic epithelial cell line (HT_{29}) which expresses CFTR, cAMP, Ca^{2+} and hypotonicity appeared to activate the same low-conductance Cl^- channel [26]. In respiratory epithelial cells, an antibody directed against CFTR inhibited both cAMP-activated, as well as swelling-activated Cl^- currents [16]. CFTR expression in trachea of transgenic mice with a disrupted *CFTR* gene, by infusion of liposomes carrying a CFTR expression plasmid, conferred cAMP regulation of transepithelial Cl^- secretion, but also enhanced Ca^{2+}-activated Cl^- secretion, as well as Na^+ absorption [7]. Although CFTR is a cAMP-regulated Cl^- channel, these studies taken together raise the possibility that expression of CFTR might confer novel regulation of or affect other plasma membrane ion conductances, including other Cl^- channels.

We have initiated experiments to determine the applicability of optical imaging techniques for assaying plasma membrane Cl^- permeabilities in transient expression systems which employ mammalian cells. One problem in such systems is that transfection efficiencies are generally significantly less than 100%. Nevertheless, optical imaging affords the opportunity to examine many cells simultaneously, thus allowing the identification by functional responses of those cells which successfully express the gene of interest. We intend to utilize such an approach to initially screen mutant CFTRs transiently transfected into mouse L-cell fibroblasts, to identify functional mutants for subsequent development of stable cell lines. We previously showed, using optical imaging techniques, that stable expression of CFTR in mouse L-cell fibroblasts conferred a Cl^- conductance which was highly regulated by cAMP [4]. In light of the studies cited above which suggested that in some cell types CFTR expression may also influence other plasma membrane Cl^- conductances, it became necessary before proceeding with transient expression studies, to determine the endogenous Cl^- conductances in L cells and the possible effects of CFTR expression on them. Therefore, in the present study we have used optical imaging techniques to compare the Cl^- conductances in CFTR-expressing and control L cells, in order to define the magnitudes and types of Cl^- conductances present in these cells, to identify specific Cl^- channel regulation conferred by CFTR, and to determine whether CFTR-associated Cl^- conductance might be regulated by putative modulators of epithelial Cl^- secretion other than cAMP.

Materials and Methods

Cells

Stably-transfected mouse L-cell lines described by Rommens et al. [4] were employed. The CFTR-expressing cells used in the present study were from clones 4a-3I, 4a-3K and 10–4C. The control cells were from clones 6b-I and 2–2B which were transfected with CFTR cDNA in which a single nucleotide was deleted 35 bp downstream from the initiation codon. A severely truncated, non-functional protein would be predicted from this frameshift construct [4]. Cells were maintained in α-MEM medium supplemented with 7% fetal bovine serum (Sigma) and HAT (Gibco; 750 μl of 10 × concentrate per 100 ml medium).

SPQ fluorescence assay of intracellular Cl^- concentration

L cells, grown on glass coverslips for 1–2 days, were loaded with the Cl^--indicator dye 6-methoxy-1-(3-sulfonatopropyl)quinolinium (SPQ, Molecular Probes) by incubation in Ca^{2+}-free (no EGTA), hypotonic (50% dilution) medium containing 20 mM SPQ at room temperature for 4 min. The coverslip was subsequently placed in isotonic medium containing 20 mM SPQ and 200 μM Ca^{2+} for 30 min and then washed in SPQ-free medium containing 1 mM Ca^{2+} for 10 min. The coverslip was mounted in a chamber and perfused continuously (bath volume 50 μl; flow rate approx. 2 ml/min) with a medium containing 138 mM NaCl, 2.4 mM K_2HPO_4, 0.8 mM KH_2PO_4, 10 mM Hepes, 1 mM $CaCl_2$, 10 mM glucose, 10 μM bumetanide (pH 7.4) on the stage of an inverted microscope. Experiments were conducted at 37°C. NO_3^- medium was identical, except that NO_3^- replaced all but 10 mM Cl^-. To minimize Cl^- fluxes through non-conductive pathways, the experiments were performed in the absence of HCO_3^- and in the presence of bumetanide, to inhibit the anion exchanger and Cl^-/cation cotransporters, respectively. Fluorescence and differential interference contrast (DIC) imaging were performed simultaneously, as previously described [27]. SPQ fluorescence intensities (F) were normalized to the total SPQ fluorescence F_0, determined as F measured in the absence of intracellular Cl^-, since autofluorescence was negligible. For normalization in cells for which F_0 was not determined, the resting F in these cells, together with an average value of F_0/F which was previously determined for this cell type [4], was used to calculate F_0. Cl^- fluxes (J_{Cl}) were calculated according to the following:

$$J_{Cl}(\text{mM/s}) = dF/dt \cdot K_{Cl} \cdot F_0/F^2 \quad (1)$$

where dF/dt is the initial rate of change of SPQ fluorescence (arbitrary units) upon replacement of the

Cl^- medium by the NO_3^- medium (normally the rate measured between 5–35 s following replacement), K_{Cl} is the effective quenching constant for Cl^- quenching of intracellular SPQ in L cells (14.9 M^{-1} [4]) and F is the SPQ fluorescence at time t [28]. Because the Cl^- flux as determined in our experiments was essentially a measurement of unidirectional flux, with the assumption of a similar resting $[Cl^-]_i$ in all cells, then J_{Cl} is equivalent to a permeability.

$[Ca^{2+}]_i$ imaging

L cells grown on glass coverslips for 1–2 days were loaded with the Ca^{2+}-indicator dye fura-2 by incubation with 5 μM fura-2 acetoxymethylester (1 : 1 mixture of fura-2 AM in DMSO and 25% pluronic acid) dissolved in growth medium, for 90 min at room temperature under continuous gassing with 5% CO_2/95% O_2. The coverslip was mounted in a chamber and perfused continuously as described above. Low-light level fura-2 imaging, construction of ratio images and quantitation were as previously described [27]. $[Ca^{2+}]_i$ was estimated from ratio images by averaging pixel intensities throughout the cell.

Fura-2 calibration

Fluorescence ratios were converted to $[Ca^{2+}]_i$ by calibration of fura-2 free acid (50 μM) in 50-μm-thick glass capillaries. $[Ca^{2+}]_i = B \cdot K_d((R - R_{min})/(R_{max} - R))$, where R is the measured ratio, R_{max} is the measured ratio in the presence of 1 mM (saturating) Ca^{2+}, R_{min} is the ratio in the absence (< 10 nM) of Ca^{2+}, K_d (the dissociation constant for the dye) was assumed to be 225 nM and B is the ratio of fluorescence at 380 nm excitation for fura-2 (unbound) to fura-2 (bound) [29]. Calibrations were performed using each neutral density filter employed in experiments to control for their unique spectral characteristics. In vitro calibration may not accurately describe the behavior of the dye inside cells, which would result in errors in the absolute values of $[Ca^{2+}]_i$. Nevertheless, for the present study in which the fura-2 signal was used to compare responses among groups of cells, this in vitro calibration procedure was considered to be sufficient.

Light scattering

Right-angle light scattering was used to detect changes in plasma membrane Cl^- conductance, as described previously [30]. Briefly, L cells, loosely grown on bacteriological petri dishes, were resuspended at a concentration of 10^6 cells/ml in Cl^--containing medium (136 mM NaCl, 3 mM KCl, 20 mM Hepes, 10 mM glucose, 0.5 mM $CaCl_2$) plus 10 μM gramicidin. Following 5–9 min equilibration, 10 μM forskolin or 1 μM ionomycin was added and the rate of increase in light scattering (340 nm excitation and emission), which is a measure of the rate of increase of cell size, was determined. In anion replacement experiments, 130 mM Cl^- was replaced by an equal concentration of the indicated anion.

cAMP determinations

CFTR-expressing L cells were grown to approx. 90% confluence on 60-mm petri dishes. Following 30 min preincubation in Cl^--containing medium at 37°C, fresh medium was added and equilibrated at 37°C for 5 min. Stimulation was facilitated by the addition of forskolin (10 μM) or ionomycin (1 μM). The phosphodiesterase inhibitor IBMX (100 μM) was added to all samples, including the control, to maximize the changes in cAMP (in the absence of the inhibitor the stimulated increases in cAMP were very small). After 5 min, the medium was rapidly aspirated and cAMP was extracted using 1.5 ml 0.1 M HCl. The amount of cAMP was determined using a cAMP[^{125}I] scintillation proximity assay kit (Amersham).

Statistics

Unless otherwise indicated, results are expressed as the mean ± S.E., and significance determined using the unpaired Student's t-test.

Results

One of the primary objectives of this work was to characterize CFTR-specific Cl^- permeabilities by measuring and comparing Cl^- permeabilities present in mouse L cells stably expressing either full-length CFTR or a severely truncated, non-functional form of the protein [4]. A second objective was to evaluate the applicability of optical imaging techniques for determining the types and magnitudes of the plasma membrane Cl^- conductances expressed by mouse L cells, as discussed in the Introduction.

cAMP-activated Cl^- permeability

The basic protocol for determining Cl^- permeability using optical imaging of the Cl^--indicator dye SPQ, which we previously described for evaluating cAMP activation of Cl^- conductance in these same L cells [4], is demonstrated in Fig. 1. Replacement of most of the extracellular Cl^- by NO_3^- causes Cl^- to leave the cell in exchange for NO_3^-. Because Cl^- quenches SPQ fluorescence but NO_3^- does not, SPQ fluorescence intensity increases at a rate which is a measure of either Cl^- or NO_3^- permeability, whichever is rate-limiting. Since NO_3^- is generally at least as permeable as Cl^- through Cl^- channels (see selectivity results below), it is likely that the rate of change of SPQ fluorescence intensity measures Cl^-, rather than NO_3^-, permeability. In unstimulated cells, replacement of extracellular Cl^- with NO_3^- resulted in small, slow changes in SPQ fluorescence (Fig. 1), indicating that

the basal Cl^- permeability was low. Cl^- flux under unstimulated conditions ranged from not measurable to 0.2 mM/s (mean ± S.E. = 0.09 ± 0.01 mM/s for 217 cells on 23 coverslips). Exposure for 2 min to a Cl^--containing medium containing the membrane-permeable cAMP analog 8-(4-chlorophenylthio)-cAMP (cpt-cAMP; 500 μM) and 100 μM IBMX (to raise intracellular cAMP levels) was without effect on SPQ fluorescence (Fig. 1) or cell volume of cells expressing CFTR (not shown). Nevertheless, probing the Cl^- permeability again by removal of extracellular Cl^- demonstrated that a substantial permeability had been activated. The cAMP-activated Cl^- flux ranged from 0.59 to 2.33 mM/s, with a mean of 1.67 ± 0.07 mM/s ($n = 45$ cells on 6 coverslips from three passages), which represents a nearly 20-fold increase compared to the Cl^- flux in the absence of raised intracellular levels of cAMP. A similar exposure to cpt-cAMP plus IBMX of the cells expressing truncated CFTR was without effect, since the Cl^- flux measured under these conditions was not different from those determined in the same cells under unstimulated conditions (data not shown and Ref. 4).

Calcium-activated Cl^- permeability

To determine whether expression of CFTR modifies Ca^{2+}-activated Cl^- permeabilities in L cells, we compared the Cl^- permeability responses to elevated $[Ca^{2+}]_i$ in CFTR-expressing and control cells. $[Ca^{2+}]_i$ was elevated by exposure of the cells to the Ca^{2+} ionophore ionomycin, thereby by-passing receptor-associated mechanisms. In an initial series of experiments, we determined the effectiveness of ionomycin to raise $[Ca^{2+}]_i$ in these cells, by monitoring $[Ca^{2+}]_i$ inside single cells using ratio imaging of intracellular fura-2. In the presence of 1 mM extracellular Ca^{2+}, perfusion with ionomycin caused a rapid rise of $[Ca^{2+}]_i$ to a peak, which was normally followed by relatively sustained or slowly decreasing $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ oscillations or spiking were never observed. A similar $[Ca^{2+}]_i$ response ($P > 0.05$) to ionomycin was observed in both control (resting $[Ca^{2+}]_i = 41 \pm 3$ nM; peak $[Ca^{2+}]_i = 878 \pm 162$ nM; $n = 21$ cells) and CFTR-expressing (resting $[Ca^{2+}]_i = 47 \pm 2$ nM; peak $[Ca^{2+}]_i = 913 \pm 96$ nM; $n = 55$ cells) cells.

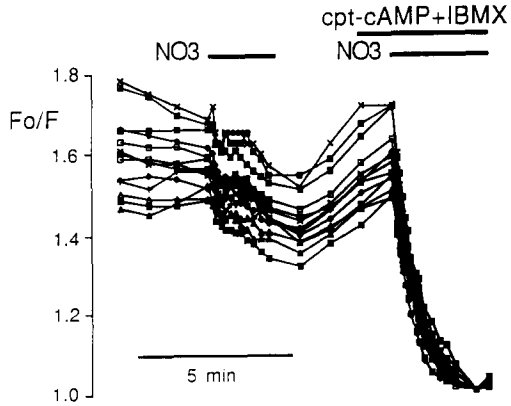

Fig. 1. The effect of cpt-cAMP on CFTR-expressing L cells. SPQ-loaded cells, initially perfused with Cl^--containing media, were perfused with NO_3^--containing media to measure the resting Cl^- permeability. Following a 3-min recovery in Cl^- medium, 500 μM cpt-cAMP plus 100 μM IBMX in Cl^- media was perfused for 2 min and the Cl^- permeability was probed again by replacement of extracellular Cl^- by NO_3^- in the continued presence of cpt-cAMP + IBMX. Each line is the SPQ fluoresence (F) of one cell on the coverslip expressed relative to the maximum fluorescence measured in 0 mM Cl^- (F_0). This coverslip is representative of 11 others from six different passages and three different CFTR-expressing clones.

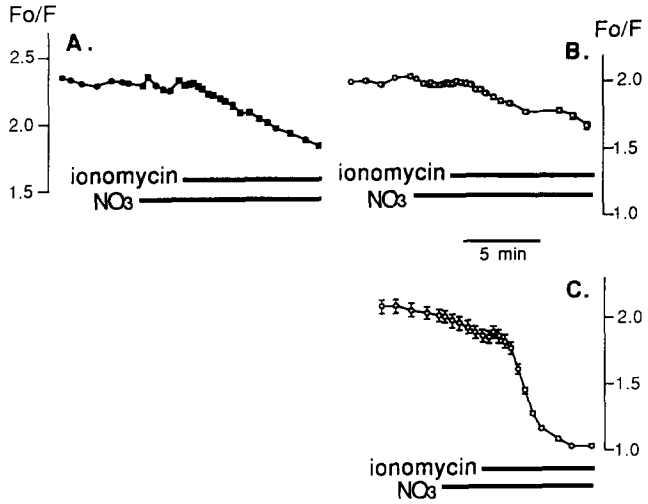

Fig. 2. The effect of elevated intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) on the Cl^- permeabilities of CFTR-expressing and control cells. The Cl^- permeabilities of SPQ-loaded control (A) and CFTR-expressing (B,C) cells were measured by replacement of extracellular Cl^- with NO_3^- as indicated. After perfusing with NO_3^--containing media for 2 min to assess the resting anion permeability, ionomycin (3 μM) was added to the perfusate to raise $[Ca^{2+}]_i$. Each panel shows the mean (±S.E.) response of all the cells on a coverslip (number of cells and coverslips indicated in text). SPQ fluorescence (F) is expressed relative to the maximum fluorescence measured in 0 mM Cl^- (F_0).

To compare the extent of activation of Ca^{2+}-activated Cl^- conductances between CFTR-expressing cells and control cells expressing non-functional truncated CFTR, we performed the following protocol. Basal Cl^- permeability was first assayed by substituting most of the extracellular Cl^- for NO_3^-, as above. After 2 min, the perfusate was exchanged for an identical one which, in addition, contained ionomycin to raise $[Ca^{2+}]_i$. Cl^- fluxes were measured during ionomycin treatment and compared to those measured in the same cell prior to stimulation. In cells which did not express CFTR, ionomycin elicited a small but significant ($P < 0.001$) increase in Cl^- permeability (Fig. 2A): Cl^- flux increased approx. 1.5-fold, from 0.039 ± 0.004 mM/s under resting conditions to 0.059 ± 0.003 mM/s when $[Ca^{2+}]_i$ was elevated ($n = 50$ cells on 5

coverslips from three different passages). In contrast, a rise of $[Ca^{2+}]_i$-activated Cl^- permeability to a greater extent in CFTR-expressing cells: Cl^- permeability increased 2.8-fold, from 0.046 ± 0.004 mM/s to 0.129 ± 0.008 mM/s ($n = 85$ cells on 6 coverslips from five different passages). In the CFTR-expressing cells, the permeability which was induced by elevated $[Ca^{2+}]_i$ was considerably less than that which was activated by cAMP. However, it was significantly greater than the stimulation by elevated $[Ca^{2+}]_i$ in the control cells ($P < 0.001$). In contrast to the control cells, the Ca^{2+}-activated Cl^- permeability responses of CFTR-expressing cells could be separated into two categories: cells either responded similarly to control cells (i.e., $J_{Cl} < 0.1$ mM/s; Fig. 2B) or else they responded with larger permeabilities (≥ 0.1 mM/s; Fig. 2C) which were never observed in the control cells. This enhanced Cl^- permeability response was observed in 50% of the CFTR-expressing cells.

One possible mechanism whereby elevated $[Ca^{2+}]_i$ could enhance CFTR-mediated Cl^- permeability is if it secondarily caused a rise of $[cAMP]_i$. Implicit in this interpretation is that such an effect of elevated $[Ca^{2+}]_i$ must occur in only some of the cells, since all the cells expressed CFTR (as evidenced by the response of 100% of the cells to cAMP). If so, the response of $[cAMP]_i$ as determined in a population of cells would likely be small. Nevertheless, we measured $[cAMP]_i$ in CFTR-expressing cells exposed for 5 min to either 10 μM forskolin or 1 μM ionomycin. Ionomycin stimulated a small increase in intracellular cAMP in each of two experiments, from an unstimulated level of 11.5 ± 0.4 pmol/mg (mean $\pm$ S.D.) to 13.6 ± 0.3 pmol/mg. In comparison, forskolin raised cAMP to 20.0 ± 1.3 pmol/mg. Although it is impossible to determine whether ionomycin raised $[cAMP]_i$ equally in all cells or more effectively in a subset of the cells, the small elevation of $[cAMP]_i$ caused by ionomycin suggests that the enhanced ionomycin-stimulated Cl^- permeability-response observed only in a sub-population of the CFTR-expressing cells might have been caused by a secondary elevation of $[cAMP]_i$ by elevated $[Ca^{2+}]_i$. However, the possibility that the enhanced Cl^- permeability response was mediated by other mechanisms cannot be ruled out (see Discussion).

Comparison of the anion selectivities of the Ca^{2+}-and cAMP-activated Cl^- conductances

The observation that a cAMP-stimulated Cl^- permeability was observed only in the cells which expressed CFTR, whereas a small Ca^{2+}-stimulated Cl^- permeability was detected in CFTR-expressing as well as control cells, suggested that these two permeabilities are conferred by different anion channels. Nevertheless, the possibility that CFTR expression conferred a sensitivity to cAMP of a Ca^{2+}-activated Cl^- permeability could not be ruled out. Therefore, to further characterize these two Cl^- permeabilities, we determined their anion selectivities using right-angle light scattering [30]. Right-angle light scattering measures changes in cell volume of cells in suspension which have been treated with gramicidin to enhance their plasma membrane cation conductance [30]. Under such conditions, the magnitude and kinetics of the activation of anion conductance are rate-limiting for cell swelling due to NaCl influx, accompanied by osmotically-obliged water. Cell swelling was observed as an increase in right-angle light scatter [30]. We used this protocol rather than single cell imaging of cell swelling because it avoids problems of cell heterogeneity due to cell size, shape and other factors inherent in such determinations in non-spherical cells grown on a support. In Cl^--containing medium, forskolin (10 μM) caused a rapid, substantial increase in light scattering which was dependent upon the anion present in the media (Fig. 3). Since the rate of cell swelling, and therefore rate of increase of light scattering, is determined by the mag-

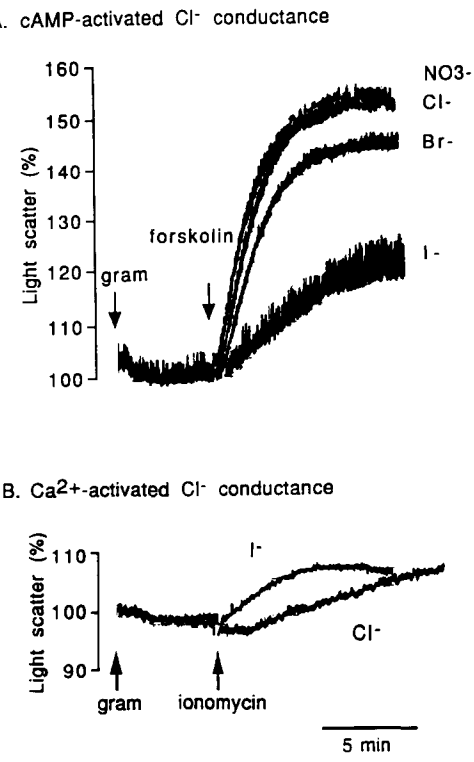

Fig. 3. The effect of extracellular anion substitution on light scattering by forskolin-stimulated L cells. 10^6 CFTR-expressing L cells were resuspended in 1 ml of medium containing either Cl^-, Br^-, NO_3^- or I^- as the dominant anion as indicated and changes in right-angle light scatter were measured. The cell suspension was exposed to 10 μM gramicidin for 5–9 min to enhance plasma membrane cation permeability. Forskolin (10 μM) (A) or ionomycin (1 μM) (B) was then added to activate plasma membrane cAMP- or Ca^{2+}-dependent halide conductances, respectively. Rate of cell swelling reflects magnitude of the anion conductance. Each trace is representative of three independent experiments.

TABLE I

Anion selectivity of the cAMP- and Ca^{2+}-activated Cl^- conductances

Anion permeabilities are expressed relative to Cl^-. Data are mean ± S.E. of four (cAMP) and five (Ca^{2+}) independent experiments.

Anion	cAMP	Ca^{2+}
Cl^-	1.0	1.0
NO_3^-	1.1 ± 0.2	-
Br^-	1.1 ± 0.2	-
I^-	0.3 ± 0.2 *	1.7 ± 0.1 *

* Significant difference compared to rate in Cl^- media (unpaired *t*-test; $P < 0.001$).

nitude of the halide conductance, analysis of the data shown in Fig. 3 allowed the anion selectivity of the cAMP-activated Cl^- channel to be determined as $NO_3^- \geq Cl^- \geq Br^- \gg I^-$. This sequence is similar to that described for the cAMP-stimulated Cl^- channel in a number of cell types, including epithelial cells [15,21,31] and non-epithelial cells expressing recombinant CFTR [10,30,32]. In contrast, the Ca^{2+}-activated anion conductance measured in I^--containing medium was nearly twice that observed in Cl^--containing media (Fig. 3B, Table I). This selectivity is similar to that observed for Ca^{2+}-activated Cl^- currents found in other cell types including airway epithelium [15], T84 cells [21] and CHO cells [30]. Although different anion selectivity is not definitive evidence for different Cl^- channels, it provides support for this hypothesis and suggests that the specific cAMP-activated Cl^- permeability conferred by expression of CFTR is distinct from the endogenous Ca^{2+}-activated Cl^- permeability. Thus, CFTR expression does not confer cAMP sensitivity to pre-existing Ca^{2+}-activated Cl^- channels in L cells.

Protein kinase C

We performed a series of experiments designed to explore the potential involvement of PKC in regulation of Cl^- conductances in control and CFTR-expressing L cells. We first explored the effects of PKC activation on Cl^- permeability. To activate PKC, SPQ-loaded L cells were incubated in media containing phorbol myristate acetate (PMA) at concentrations ranging from 0.1–1 μM, for times ranging from 2–15 min. The Cl^- permeability was then evaluated by switching from the Cl^- containing medium to the NO_3^- medium, as above. However, no increase in Cl^- permeability was observed: resting Cl^- permeability was 0.13 ± 0.03 mM/s, whereas it was 0.10 ± 0.03 mM/s following incubation with 1 μM PMA ($n = 45$ cells on 6 coverslips from three experiments). Li et al. [23] described dual effects of PKC on Cl^- channel activity in airway epithelial cells: whereas at low (< 10 nM) $[Ca^{2+}]$ PKC activation was stimulatory, higher $[Ca^{2+}]$ were inhibitory. The stimulatory effect, but not the inhibitory effect, was absent in CF tissues. Thus, the failure to observe an effect of PKC activation might be explained if $[Ca^{2+}]_i$ in L cells was too high. Additional experiments were conducted in nominally Ca^{2+}-free medium to lower $[Ca^{2+}]_i$. If the model proposed by Li et al. [23] is relevant, this protocol should have increased the likelihood that PKC would stimulate the Cl^- permeability. Nevertheless, there was still no apparent stimulation with PMA (1 μM) in either CFTR-expressing or control cells (data not shown). Taken together, these results suggest that CFTR-expression does not confer PKC-sensitivity to Cl^- conductance in mouse L cells.

Exogenous bovine brain PKC potentiated cAMP-activation of a Cl^- channel in CFTR-expressing CHO cells [6], whereas phorbol esters inhibited cAMP-stimulated Cl^- permeability in intact epithelia [33,34]. Although PKC activation appeared to be without direct effect on Cl^- permeability in CFTR-transfected L cells, we examined whether it modulated the effects of elevated cAMP, by pretreating cells for 15–30 min with 1 μM PMA followed by stimulation with cAMP. However, PKC activation caused neither a consistent inhibition nor enhancement of the Cl^- flux induced by cpt-cAMP (data not shown).

Taken together, our results indicate that expression of CFTR in L cells does not confer a PMA-sensitive Cl^- permeability, nor does PKC modify cAMP activation of the the CFTR-specific Cl^- conductance. L cells express PKC, and physiological consequences of exposure to low doses of PMA have been demonstrated in this cell type [35]. We point out, however, that L cells might not posess appropriate PKC isoforms necessary for activation of the CFTR-mediated Cl^- conductance.

Swelling-activated Cl^- permeability

Osmotic swelling activates Cl^- conductances in non-epithelial as well as epithelial cells, including those which express CFTR, such as human airway [17,36,37], sweat gland [37] and T84 [38] cells. Outwardly-rectifying Cl^- currents displaying time-dependent effects of voltage are associated with swelling of some cell types [17,37], but it is unknown whether only one type of Cl^- channel is involved in diverse cell types. P-glycoprotein, which is structurally similar to CFTR, not only transports drugs from cells, but also conducts Cl^- ions in response to cell swelling [39]. However, it is unknown whether the CFTR-specific Cl^- conductance can be regulated by cell swelling. We examined the effects of acute osmotic swelling on Cl^- permeability in both control and CFTR-expressing L cells. SPQ-loaded control or CFTR-expressing L cells were perfused with Cl^- containing media, and then basal Cl^- permeability was measured by switching to a NO_3^--containing medium as described previously. After 2 min, cells were perfused with a similar NO_3^--containing medium

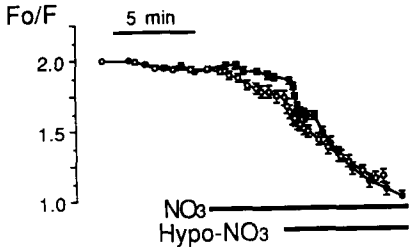

Fig. 4. The effect of osmotic cell swelling on Cl^- permeability in CFTR-expressing and control L cells. SPQ-loaded CFTR-expressing (○) and control cells (●) were perfused with NO_3^--containing media to measure the resting Cl^- permeability. The NO_3^--containing media were subsequently diluted by 30% (Hypo-NO_3). The initial rapid decrease of F_0/F is due to osmotic dilution of $[Cl^-]_i$. Subsequent decrease of F_0/F indicates enhancement of Cl^- permeability by osmotic cell swelling. Each trace is the mean response (±S.E.) of seven CFTR-expressing and nine control cells on two different coverslips examined on the same day and is representative of three other experiments.

diluted 25% with distilled water (the Ca^{2+} concentration was maintained at 1 mM). An initial rapid enhancement of SPQ fluorescence was observed, which corresponded to the rapid cell swelling and consequent dilution of intracellular Cl^- (Fig. 4). Following a subsequent lag period between 30–60 s, Cl^- permeability increased, as indicated by a secondary, rapid increase in SPQ fluorescence (decrease in F_0/F; Fig. 4). This swelling-enhanced loss of intracellular Cl^- was likely through a conductive pathway (channel), in exchange for NO_3^- through the same pathway, since Cl^- loss was not associated with volume reduction (not shown). In both the control and CFTR-expressing cells, the swelling-induced Cl^- permeability was similar, increasing from 0.06 ± 0.02 mM/s to 0.16 ± 0.01 mM/s in control cells and from 0.03 ± 0.01 mM/s to 0.14 ± 0.02 mM/s in CFTR-expressing cells. Thus, expression of CFTR did not impart, or modify the magnitude of, a specific swelling-activated Cl^- permeability.

Discussion

Our results, obtained by fluorescence imaging of a Cl^- indicator dye, demonstrate that mouse L cells which have not been transfected with CFTR possess Ca^{2+}-activated and swelling-activated Cl^- permeabilities, but no detectable cAMP-activated Cl^- conductance. In contrast, L cells expressing CFTR displayed a cAMP-activated Cl^- conductance which was considerably larger than the other Cl^- permeabilities. The swelling-activated Cl^- permeability was unaffected by CFTR expression. However, the Ca^{2+}-activated permeability was increased in a subpopulation of CFTR-expressing cells.

The observation that elevated $[Ca^{2+}]_i$ increased Cl^- permeability to levels greater than the endogenous Ca^{2+}-activated Cl^- permeability in some of the CFTR-expressing cells, may suggest the presence of a protein kinase A-independent mechanism which can activate the CFTR-specific Cl^- conductance. This stimulation is unlikely to be mediated by protein kinase C, since direct activation of PKC with phorbol esters was without effect. It also seems unlikely that Ca^{2+} is a direct mediator in this pathway. $[Ca^{2+}]_i$ was elevated similarly by ionomycin in all the cells, so the heterogeneous Cl^- permeability-response was unlikely to be due to heterogeneous $[Ca^{2+}]_i$ responses. Furthermore, there was often a considerable lag (up to 5 min) between the elevation of $[Ca^{2+}]_i$ and activation of the Cl^- permeability, which is inconsistent with a direct effect of Ca^{2+} on the CFTR-associated Cl^- permeability. The data suggest that a rise of $[Ca^{2+}]_i$ triggered down-stream events which were of sufficient magnitude to activate Cl^- permeability in only some of the cells. Calcium-calmodulin-dependent protein kinase II (CaMKII) regulates Cl^- channels in airway epithelial cells [17] and T84 cells [40], and CaMKI can phosphorylate CFTR [41]. Activation of CFTR by one of these Ca^{2+}-dependent kinases might underlie the enhanced Cl^- permeability elicited by elevated $[Ca^{2+}]_i$ in some of the CFTR-expressing L cells. Alternatively, CFTR might confer Ca^{2+} sensitivity to another, unidentified channel which is endogenous to L cells. This mechanism would be analogous to the apparent CFTR regulation of the outwardly rectifying Cl^- channel [24]. We considered the possibility that the enhanced Ca^{2+}-activated Cl^- permeability observed in a sub-population of the CFTR-expressing L cells was caused by an increase in $[cAMP]_i$ by elevated $[Ca^{2+}]_i$. Since we measured a small increase in $[cAMP]_i$ in L cells exposed to ionomycin, it seems likely that at least part of the observed Cl^- permeability response to elevated $[Ca^{2+}]_i$ was caused by a secondary elevation of $[cAMP]_i$. Indeed, Ca^{2+} ionophore-stimulated increases in $[cAMP]_i$ have been reported in several tissues, including canine trachea [33,34] and colonic epithelium [42]. In spite of the fact that all the cells expressed CFTR, variability among cells in the level of CFTR expression and/or the effectiveness of elevated $[Ca^{2+}]_i$ to raise $[cAMP]_i$ may have accounted for the heterogeneous Cl^- permeability responses.

The CFTR-specific conductance had different characteristics compared with the endogenous Ca^{2+}- or swelling-activated Cl^- channels in L cells. The anion selectivity of the cAMP-activated Cl^- conductance was different compared to that of the Ca^{2+}-activated conductance; and the swelling-activated Cl^- current in CFTR-expressing L cells is outwardly rectifying, compared with the linearity of the cAMP-activated Cl^- current in these same cells (Wang, X.-D., personal communication). These differences, taken together with the finding that the cAMP-activated Cl^- conductance was observed only in cells expressing CFTR, indicate

that CFTR expression does not confer cAMP-regulation of endogeneous Ca^{2+}- or swelling-activated Cl^- channels, and that the major effect of CFTR expression in L cells is the de novo appearance of a unique Cl^- conductance.

In conclusion, human CFTR expression in mouse fibroblast L cells confers a novel Cl^- conductance which is regulated by cAMP. L cells which have not been transfected with CFTR possess Cl^- permeabilities which are activated by cell swelling or an elevation of $[Ca^{2+}]_i$. Nevertheless, by comparison with the cAMP-activated conductance in the CFTR-expressing cells, these conductances are small. Osmotic cell swelling and PKC activation are without specific effect in CFTR-expressing L cells. However, elevated $[Ca^{2+}]_i$ may play a role in activating a Cl^- conductance specifically associated with CFTR expression, in addition to that expressed endogenously by these cells. Finally, these data suggest that CFTR does not confer cAMP-sensitivity to pre-existing, endogenous Ca^{2+}- or swelling-activated Cl^- channels.

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