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Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) Confers Glibenclamide Sensitivity to Outwardly Rectifying Chloride Channel (ORCC) in Hi-5 Insect Cells

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Abstract. Increasing evidence is now accumulating for the involvement of the cystic fibrosis transmembrane conductance regulator (CFTR) in the control of the outwardly rectifying chloride channel (ORCC). We have examined the sensitivity of ORCC to the sulfonylurea drug glibenclamide in Hi-5 (*Trichoplusia ni*) insect cells infected with recombinant baculovirus expressing either wild-type CFTR, ΔF508-CFTR or *E. coli* β galactosidase cDNA and in control cells either infected with virus alone or uninfected. Iodide efflux and single channel patch-clamp experiments confirmed that forskolin and 1-methyl-3-isobutyl xanthine (IBMX) or 7-methyl-1,3 dipropyl xanthine (DPMX) activate CFTR channels (unitary conductance: 9.1 ± 1.6 pS) only in cells expressing CFTR. In contrast, we identified 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid (SITS)-sensitive ORCC in excised membrane patches in any of the cells studied, with similar conductance (22 ± 2.5 pS at −80 mV; 55 ± 4.1 pS at +80 mV) and properties. In the presence of 500 μM SITS, channel open probability (P_o) of ORCC was reversibly reduced to 0.05 ± 0.01 in CFTR-cells, to 0.07 ± 0.02 in non-CFTR expressing cells and to 0.05 ± 0.02 in ΔF508-cells. In Hi-5 cells that did not express CFTR, glibenclamide failed to inhibit ORCC activity even at high concentrations (100 μM), whereas 500 μM SITS reversibly inhibited ORCC. In contrast in cells expressing CFTR or ΔF508, glibenclamide dose dependently (IC_{50} = 17 μM, Hill coefficient 1.2) and reversibly inhibited ORCC. Cytoplasmic application of 100 μM glibenclamide reversibly reduced P_o from 0.88 ± 0.03 to 0.09 ± 0.02 (wash: P_o = 0.85 ± 0.1) in CFTR cells and from 0.89 ± 0.05 to 0.08 ± 0.05 (wash: P_o = 0.87 ± 0.1) in ΔF508 cells. In non-CFTR expressing cells, glibenclamide (100 μM) was without effect on P_o (control: P_o = 0.89 ± 0.09, glib.: P_o = 0.86 ± 0.02; wash: P_o = 0.87 ± 0.05). These data strongly suggest that the expression of CFTR confers glibenclamide sensitivity to the ORCC in Hi-5 cells.

Key words: *Trichoplusia ni* insect cells (Hi-5) — Baculovirus — Cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel — Glibenclamide — Outwardly rectifying chloride channel (ORCC)

Introduction

The cystic fibrosis transmembrane conductance regulator (CFTR) is a cAMP-activated, ATP-dependent chloride channel located in the apical membrane of epithelial cells where it plays a crucial role in cAMP and hormonal-dependent anion secretion (Riordan et al., 1989). The regulation and permeation properties of the CFTR channel have been extensively studied (reviewed in Hanrahan et al., 1995).

CFTR can also function as a regulator of other cellular functions as originally proposed by Riordan et al. (1989). Increasing evidence is now accumulating for the involvement of CFTR in the regulation of amiloride-sensitive Na^+ channels (Stutts, Rossier & Boucher, 1997), epithelial K^+ conductance (Loussouarn et al., 1996) and Cl^- channels (Egan et al., 1992; Gabriel et al., 1993; Jovov et al., 1995; Schwiebert et al., 1995). Other studies have also suggested that CFTR regulates positively ATP transport (Schwiebert et al., 1995) and pH

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regulation (Barasch et al., 1991). In this regard the relation between CFTR and the outwardly rectifying chloride channel (ORCC) is of particular interest (Egan et al., 1992; Schwiebert et al., 1995) since ORCC was originally considered to be the chloride channel defective in cystic fibrosis (Frizzell, Rechkemmer & Shoemaker, 1986; Welsh & Liedtke, 1986; for a review *see* Guggino, 1993) before the CF gene was cloned (Riordan et al., 1989).

The sulfonylurea drugs glibenclamide and tolbutamide are blockers of K_{ATP} channels (Ashcroft & Ashcroft, 1990). Recently, these drugs have been shown to inhibit the activity of CFTR (Sheppard & Welsh, 1992; Schultz et al., 1996; Venglarik et al., 1996; Sheppard & Robinson, 1997). It was then found that glibenclamide also inhibits the activity of ORCC in HT29 and T84 cells (Rabe, Disser & Frömter, 1995) and M-1 mouse cortical collecting duct cells (Volk, Rabe & Korbmacher, 1995). In mammalian cardiac myocytes, glibenclamide inhibits swelling-activated- and Ca^{2+}-activated chloride channels (Sakaguchi, Matsuura & Ehara, 1997; Yamasaki & Hume, 1997).

Baculovirus has been used to express CFTR in the insect cells Hi-5 (Yang et al., 1997) and Sf9 cells (Larsen et al., 1996). The outwardly rectifying chloride channel is endogenously expressed in Hi-5 cells (Yang et al., 1997). Therefore, these cells are well suited to evaluate the interactions between CFTR and ORCC. We have infected these cells with recombinant baculovirus to express wild type CFTR, CFTR bearing the most common CF-associated mutation ΔF508 and βgal to study the glibenclamide sensitivity of the endogenous ORCC. We did not find any evidence of inhibition of ORCC by glibenclamide in uninfected Hi-5 cells, mock-infected cells or βgal expressing cells. By contrast, inhibition of ORCC by glibenclamide was observed when CFTR or ΔF508 proteins were expressed in Hi-5 insect cells. The stilbene derivative SITS, which did not affect the activity of CFTR, reversibly inhibited ORCC irrespective of the cell used. The results show that the inhibition of the ORCC by glibenclamide but not by SITS is dependent on the expression of CFTR. The possible molecular interactions between CFTR and ORCC are discussed.

Materials and Methods

Cell Culture

Spodopterae frugiperda (Sf9) and *Trichoplusia ni* (Hi-5) insect cells were grown at 28°C in TC 100 media supplemented with 5% decomplemented calf serum.

Plasmid Construction

Two baculovirus transfer vectors were used *pGmAc115T* (Royer et al., 1991) and *pGmAc217* (Gaymard et al., 1996). In the *pGmAc115T* transfer vector the initiator ATG codon of polyhedrin was removed by changing G to T and a *Bgl II* cloning site was introduced at position +34 (+1 is the first nucleotide of the polyhedrin initiator ATG codon) and residues +34 to +407 were deleted. In the *pGmAC217* residues −8 to +502 were deleted and a *Bgl II* site was introduced just downstream at position −8. Such a deletion in the promoter region has been shown to decrease expression levels. Two oligonucleotides (GATCTAAGCTAGCTAAGGCCTAAGAGCTCGGTACCAGGATCCACTGCAGAAG and ATTCGATCGATTCCGGATTCTCGAGCCATGGTCCTAG GTGACGTCTTCCTAG) were hybridized and inserted in the *Bgl II* site. The 4.5 kb fragment encoding CFTR or ΔF508 was excised respectively from pTG5960 and pTG5962 (Transgene, Strasbourg, Fr.) and was ligated into *Sac I Pst I* site modified transfer vector. Restriction analysis was used to control for proper orientation of coding nucleotide sequence towards polyhedrin promoter.

Production of Recombinant Baculoviruses

To obtain recombinant viruses, Sf9 cells were cotransfected with *pGmAc115T* or *pGmAc217* containing the coding regions of CFTR or ΔF508 and with purified DNA from wild type *Autographa California nuclear polyhedrosis virus* (AcMNPV) using a liposome (DOTAP, Boehringer Mannheim) mediated technique (Royer et al., 1991; Gaymard et al., 1996). The recombinant viruses were purified by several rounds of plaque purification and named *pGmAc115T-CFTR, pGmAc115T-ΔF508, pGmAc217-CFTR* and *pGmAc217-ΔF508.* They were amplified to 10^8 plaque forming units/ml and used for protein expression. A wile-type virus (mock) and recombinant baculovirus expressing the E. coli β galactosidase gene were used as controls.

Immunoblot

Hi-5 cells were layered at a density of $5 \cdot 10^5$ cells/ml and infected with recombinant baculovirus at a multiplicity of infection of 5 (m.o.i.). Mock-infected and wild-type baculovirus were used as controls. After 2 days of incubation at 28°C, cellular extracts were analyzed on an 8% SDS PAGE. Proteins were electroblotted onto a nitrocellulose membrane at 0.8 V/cm^2 in a solution containing 25 mM Tris, 192 mM glycine, 0.1% SDS and 20% methanol. Membranes were blocked in TS (20 mM Tris-Cl, pH 7.5, 150 mM NaCl) containing 5% nonfat milk for 1hr at room temperature. Primary antibody raised against the C-terminus of CFTR (Genzyme, Cambridge, MA) diluted in TS containing 0.1% Tween 20 (TS-T) was bound overnight at 4°C. After a 15 min wash in TS-T anti mouse IgG, secondary antibody coupled to peroxidase (Sigma, St. Louis, MO) diluted in the same buffer was added. Blots were incubated 2hr at 37°C and washed as described above. Peroxidase activity was detected using a chemiluminescent method (Super Signal Pierce).

Determination of Iodide Efflux

Hi-5 cells were seeded in 24-well plates and infected 3 days later at 3 m.o.i. for 2 days. Experiments were performed as previously described (Becq et al., 1996). The iodide efflux medium contained (in mM): 140 NaCl, 1.0 $MgCl_2$, 1.0 $CaCl_2$, 5.5 glucose and 10 MES, pH 6.5. After loading solution (0.5 μCi/ml of ^{125}IK; 60 min) was removed, cells layers were washed and media containing stimulators were added and removed sequentially at 1-min time intervals over a 6-min period. Experiments were performed in triplicate at room temperature. Efflux curves were constructed by plotting the percent of cellular content accumulated in the medium versus time. The efflux rate constants (*k*, min^{-1}) were determined by fitting efflux curves to monoexponential functions using linear regression of Neperian logarithms of the efflux data (expressed as % of iodide incorporated at time 0). Data are expressed as means ± SD and *F* test was used to determine significance ($P < 0.05$).

Single-Channel Patch-Clamp Recording

Hi-5 cells were plated on coverslips, cultured at 28°C and infected 3 days later at 5 m.o.i. for 2 days before use. Single-channel currents were recorded from cell-attached and excised inside-out patches. To stimulate CFTR channels, cells were exposed to forskolin (10 μM) and 7-methyl-1,3 dipropyl xanthine (DPMX, 250 μM, Chappe et al., 1998). Experiments were performed at room temperature. Results were displayed conventionally with inward currents (outward flow of anions) indicated by downward deflections. In all the figures, dashed lines give the zero current baselines when the channels were in the closed state. Potentials are expressed as the bath potential minus the patch electrode potential. The pipette solution contained 150 mM NaCl, 2 mM $MgCl_2$, and 10 mM TES (pH 7.4); the bath contained 145 mM NaCl, 4 mM KCl, 2 mM $MgCl_2$ and 10 mM TES (pH 6.5). Channel open probability (P_o) or NP_o were calculated. N is the number of channels in the membrane patch and P_o the time averaged open probability of an individual channel. Other experimental details are given in Becq et al. (1993) and Chappe et al. (1998). Data are presented as the mean ± SD of n separate experiments and statistical analyses were performed using the *t* test.

Chemicals

Forskolin was from Calbiochem (San Diego, CA). DPMX was from Research Biochemicals International (RBI, Natik, MA). All inhibitors were prepared freshly before experiments and dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO in the experiments was less than 0.1% and was found to have no significant effect on iodide efflux or membrane currents. Other chemicals were from Sigma Chemical (St. Louis, MO).

Abbreviations

CFTR = Cystic Fibrosis Transmembrane Conductance Regulator; ORCC = Outwardly Rectifying Chloride Channel; SITS = 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid; IBMX = 1-methyl-3-isobutyl xanthine; DPMX = 7-methyl-1,3 dipropyl xanthine.

Results

Expression of CFTR In HI-5 Cells

Hi-5 insect cells were infected with wild-type baculovirus or viruses recombinant for βgal, CFTR (*pGmAc115T-CFTR*, *pGmAc217-CFTR*) or ΔF508 (*pGmAc115T-ΔF508*, *pGmAc217*-ΔF508) cDNA. *pGmAc217-CFTR* was obtained using a transfer vector that contained a deletion in the promotor region designed to decrease the level of expression of foreign sequences. Antibody raised against the C terminus of CFTR detected a characteristic 140-kDa band in CFTR-infected cells (Fig. 1: *pGmAc115T-CFTR*, lane 5; *pGmAc217-CFTR*, lane 1) and ΔF508-infected cells (Fig. 1: *pGmAc115T-ΔF508*, lane 6; *pGmAc217-ΔF508*, lane 2) but not in noninfected (Fig. 1, lane 3) and mock-infected cells (Fig. 1, lane 4). In cells infected with *pGmAc115T-CFTR* recombinant virus, forskolin (10 μM) added together with IBMX (250 μM) increased the rate constant (k, min^{-1}) of iodide efflux from 0.086 ± 0.005 min^{-1} to 0.133 ± 0.010 min^{-1} ($n = 18$, Fig. 2*A*, $P < 0.05$). Similar results were obtained with *pGmAc217-CFTR* recombinant virus (*not shown*). In CFTR-infected Hi-5 cells, the cAMP-stimulated iodide efflux was lower than observed with CHO cells stably transfected with CFTR (Chappe et al., 1998). This difference may be explained by the temperature used for insect cells (25°C instead of 37°C for mammalian cells).

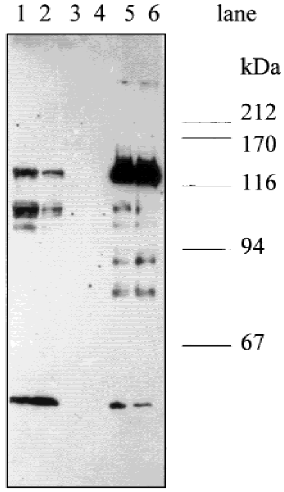

Fig. 1. Expression of wild type and ΔF508CFTR in Hi-5 cells. The blot was probed using an antibody directed against the C-terminus of CFTR. Proteins (280 μg: lanes 1, 2, 3, 4; 7 μg: lanes 5, 6) were extracted from Hi-5 cells infected with (lanes 1, 2, 5, 6) or without (lane 3) baculovirus recombinant for CFTR (lanes 1, 5) or ΔF508 (lanes 2, 6) cDNA and using low (*pGmAc217*: lanes 1, 2) or high (*pGmAc115T*: lanes 5, 6) promotors. Lane 4: mock-infected cells.

The properties of single CFTR channels were than analyzed using the cell-attached patch-clamp configuration. In the absence of cAMP agonists, no spontaneous chloride channel activity was recorded (noted basal in Fig. 2*B*). After the simultaneous addition to the bath of forskolin (10 μM) and DPMX (250 μM) chloride channel activity with characteristics of CFTR was observed in both *pGmAc115T-CFTR*- and *pGmAc217-CFTR*-infected cells (Fig. 2*B*). Since both constructs gave similar results, we pooled the data and these two cells are thereafter noted CFTR-expressing cells. CFTR channels were not observed in uninfected Hi-5, mock-βgal- or ΔF508 infected Hi-5 cells (Fig. 2*B*). Typical CFTR activity in a cell-attached membrane patch from an infected cell exposed to forskolin (10 μM) and DPMX (250 μM) is presented in Fig. 2*C*. The chloride channel activated in cell-attached patches, had a linear current-voltage relation-

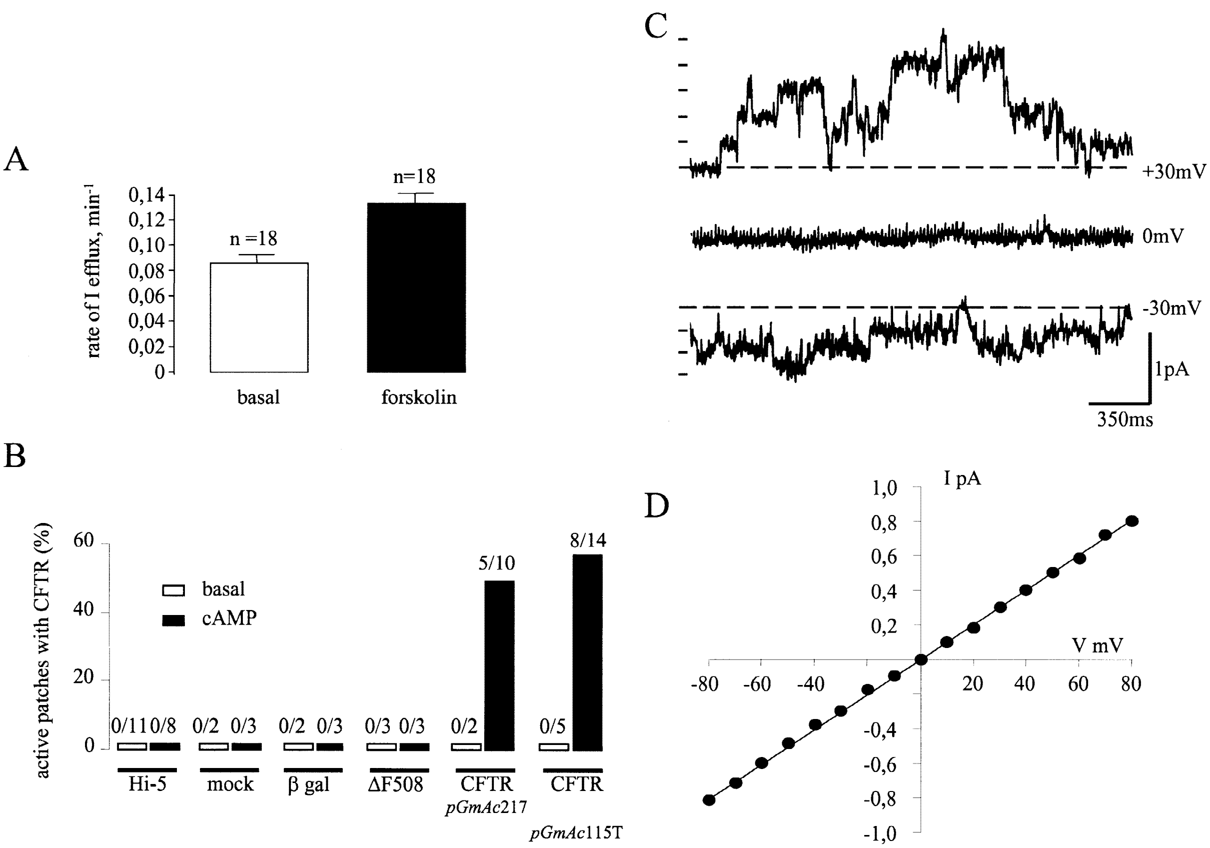

Fig. 2. Activity of cystic fibrosis transmembrane conductance regulator (CFTR) chloride channels in cell-attached patches from Hi-5 cells. (*A*) Histograms showing the rate constant of the iodide efflux in Hi-5 expressing CFTR in the absence (empty bar, noted basal) and presence of 10 μM forskolin and 250 μM IBMX (filled bar, noted forskolin). The number of experiments is given at the top of each bar. (*B*) Summary of experiments performed in cell-attached patch-clamp configuration on uninfected Hi-5 cells (noted Hi-5) or cells infected with baculovirus alone (noted mock) or containing βgal (noted βgal), ΔF508 (noted ΔF508) or CFTR (noted CFTR *pGmAc115T* and CFTR *pGmAc217*) cDNA. In black, the cells were stimulated with a cAMP cocktail (noted cAMP) containing 10 μM forskolin and 250 μM DPMX. The number of experiments is given at the top of each bar. (*C*) Example recordings of CFTR chloride channel activity in a cell-attached experiment performed on a *pGmAc115T-CFTR* infected cell in the presence of the cAMP cocktail described in *B.* Open channel current levels are indicated by lines to the left of the traces. (*D*) Current-voltage relationship of CFTR channel activated in cAMP-treated CFTR-expressing Hi-5 cells.

ship (Fig. 2*D*) and unitary conductance (9.1 ± 1.6 pS, n = 13) consistent with that of CFTR reported in Hi-5 (Yang et al., 1997) and Sf9 cells (Kartner et al., 1991; Egan et al., 1992; Larsen et al., 1996) and also similar to those described for other preparations (Gray et al., 1989; Dalemans et al., 1991; Tabcharani et al., 1991; Becq, Hollande & Gola, 1993; Hanrahan et al., 1995).

Outwardly Rectifying Chloride Channels (ORCC) in HI-5 Cells

Except for CFTR, no chloride channel activity was detected in cell-attached patches either in the presence or absence of cAMP agonists. In inside-out patches however, the activity of large conductance chloride channels was recorded, either spontaneously or following depolarization (Fig. 3*A*), irrespective of the expression of CFTR (Fig. 3*B*). Figure 3*C* shows representative current recordings from an excised inside-out patch held at various potentials in CFTR expressing cells. The chloride channels detected in the five different cells had similar conductances and pronounced outward rectification (unitary conductances, 22 ± 2.5 pS at −80 mV and 55 ± 4.1 pS at +80 mV, n = 34, pooled data in Fig. 3*D*). All these properties are characteristic of the signature of ORCC described in other cells (Egan et al., 1992; Rabe et al., 1995; Schwiebert et al., 1995; Volk et al., 1995).

SITS and Glibenclamide are Inhibitors of ORCC In CFTR-Expressing Cells

We examined the sensitivity of ORCC in Hi-5 cells expressing CFTR to SITS and glibenclamide using excised inside-out membrane patches. Addition of SITS (500

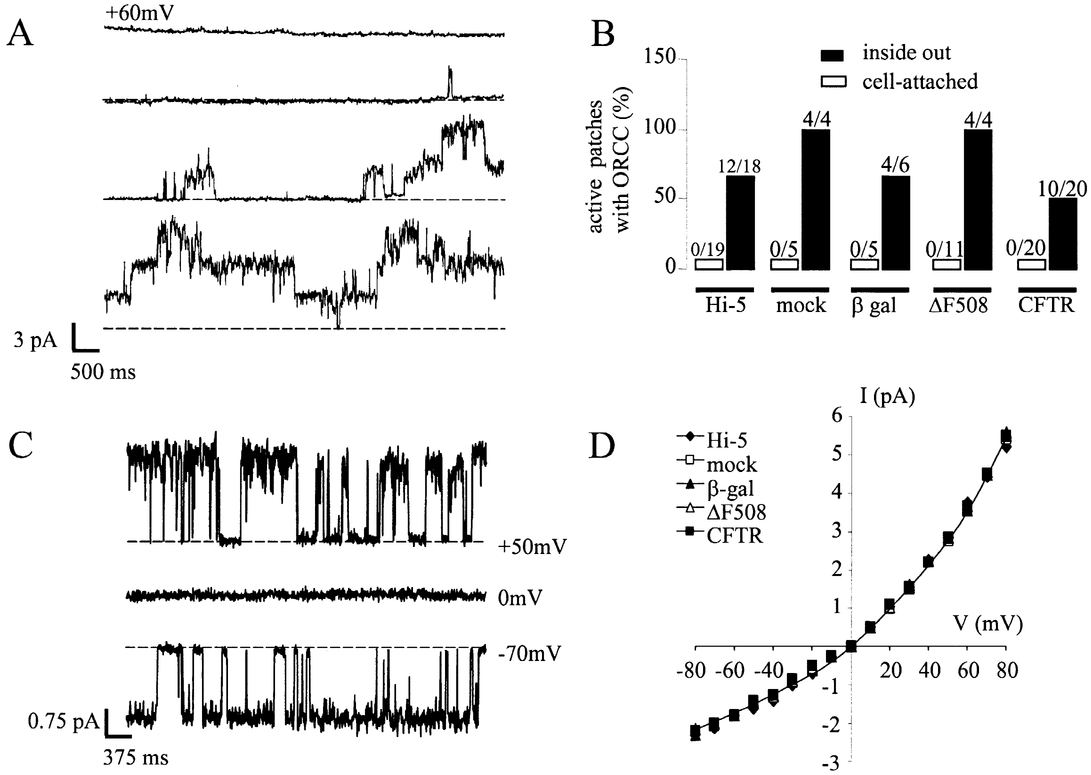

Fig. 3. Activity of outwardly rectifying chloride channels in excised inside-out patches of Hi-5 cells. (*A*) Experiment performed in the excised inside-out configuration showing the activation of large conductance outwardly rectifying chloride channels (ORCC) in uninfected Hi-5 cell. (*B*) Summary of experiments performed in cell-attached and inside-out patch-clamp configuration with uninfected Hi-5 or cells infected with baculovirus alone or containing βgal, ΔF508 or CFTR cDNA. The cells were stimulated with a cAMP cocktail (noted cAMP) containing 10 μM forskolin and 250 μM DPMX. The number of experiments is given at the top of each bar. (*C*) Example recordings of ORCC activity in an inside-out experiment performed on a CFTR-expressing cell. (*D*) Current-voltage relationship of ORCC in inside-out configuration in the five different cells studied as indicated.

μM) to the bath induced a reversible inhibition of ORCC activity in 10 out of 10 experiments (Fig. 4*A*). Previous reports (Rabe et al., 1995; Volk et al., 1995) have shown that glibenclamide blocked ORCC in T84 and M-1 mouse cortical collecting duct cells. Figure 4*B* shows that in Hi-5 cells expressing CFTR, glibenclamide (100 μM) fully and reversibly inhibited ORCC activity in 10 out of 10 experiments and caused a flickery channel block. The type and magnitude of the inhibition was independent of the membrane potential (*data not shown*). Figure 4C shows an experiment in which glibenclamide (100 μM) then SITS (500 μM) were sequentially added to the solution bathing an excised inside-out patch with active ORCCs ($n = 3$). The reverse experiment gave a similar result ($n = 2$, *not shown*). These observations further confirmed that both drugs acted as reversible inhibitors of ORCC. Exposure to glibenclamide caused a reversible reduction of the channel open probability P_o, with an half maximal inhibition of 17 μM (Fig. 4*D*). Best fit to Hill function gave a Hill coefficient $n = 1.2 \pm 0.3$. Cytoplasmic application of 100 μM glibenclamide reversibly reduced channel open probability (P_o) from 0.88 ± 0.03 ($n = 10$) to 0.09 ± 0.02 ($n = 10$; $P < 0.001$; wash: $P_o = 0.85 \pm 0.1$, $n = 10$). Similarly, P_o was reversibly reduced to 0.05 ± 0.01 ($n = 10$; $P < 0.001$) in the presence of 500 μM SITS.

SITS But Not Glibenclamide Inhibit ORCC In Uninfected HI-5 Cells

Since ORCCs were detected in patches excised from both Hi-5 expressing CFTR and uninfected cells, we investigated the effect of glibenclamide upon the activity of ORCC in uninfected Hi-5 cells. However glibenclamide (100 μM) was without effect on the activity of ORCC in these cells in 12 out of 12 experiments (Fig. 5*B–D*). Moreover, the repetitive addition of glibenclamide (100 μM) had no effect on the channel activity (NP_o) whereas the addition of both glibenclamide (100 μM) and SITS (500 μM) reversibly inhibited the ORCC activity in the patch (Fig. 5*C*). As shown in Fig. 5*A*, the activity of ORCC in uninfected Hi-5 cells was inhibited in the presence of SITS in the bath (500 μM, $n = 10$).

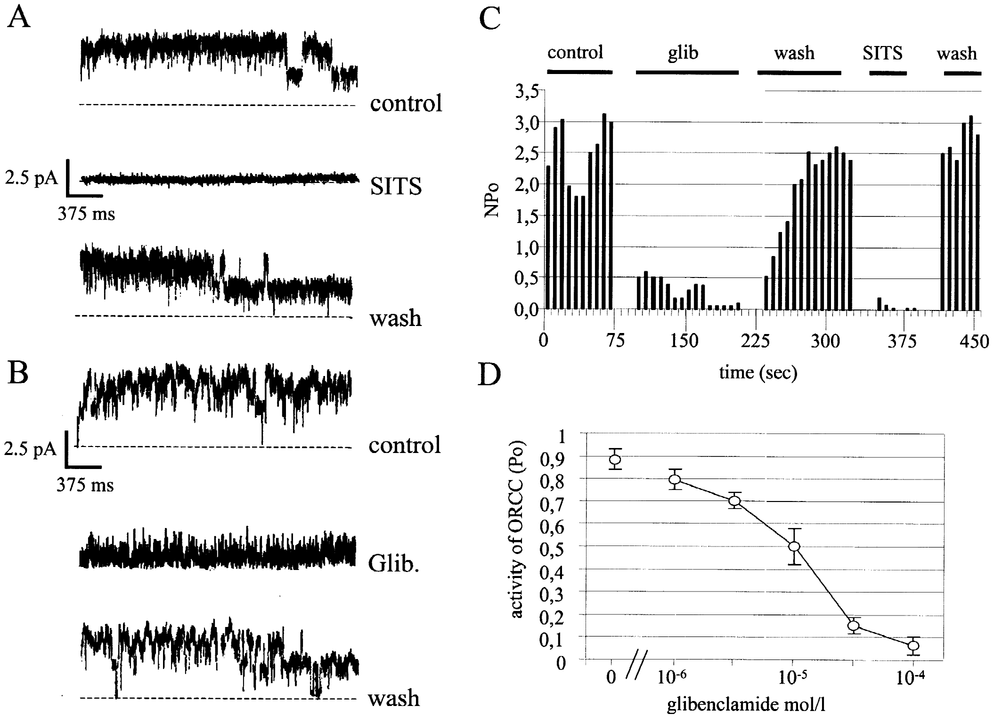

Fig. 4. Effect of SITS and glibenclamide on the activity of ORCC in excised inside out patches from Hi-5 cells expressing CFTR. Reversible inhibition of ORCC by 500 μM SITS (*A*) and 100 μM glibenclamide (*B,* noted Glib.). In *A* and *B,* V = +40 mV; two channels were present in the patch. (*C*) An experiment showing the reversible inhibition by SITS and glibenclamide on ORCC activity evaluated as NP_o (*see* Materials and Methods). NP_o is plotted as function of time to show the effect of the sequential addition of 100 μM glibenclamide and 500 μM SITS. Note that during the washing periods ORCC were reactivated. (*D*) Concentration-dependent inhibition of ORCC activity by glibenclamide, evaluated as P_o. $V = +30$ mV.

Furthermore, glibenclamide used at concentrations ranging from 1 to 100 μM have no effect on ORCC channel open probability (Fig. 5*D, n* = 3). Cytoplasmic application of 100 μM glibenclamide was without effect on P_o (control: $P_o = 0.89 \pm 0.09$, $n = 12$; glib.: $P_o = 0.86 \pm 0.02$, $n = 12$; wash: $P_o = 0.87 \pm 0.05$, $n = 12$). In contrast, P_o was reversibly reduced to 0.07 ± 0.02 ($n = 10$; $P < 0.001$) in the presence of SITS (500 μM).

Glibenclamide Fails to Inhibit ORCC in Mock and βGAL HI-5 Cells

We were surprised to observe that glibenclamide failed to inhibit the activity of ORCC in uninfected Hi-5 cells. Glibenclamide solutions were prepared fresh before experiments and tested on both CFTR and non-CFTR expressing cells during the same set of experiments and we used cell cultures of similar age and similar infection procedures. Since the same aliquots of glibenclamide were found effective on ORCC in CFTR expressing cells but were ineffective in uninfected Hi-5 cells, we eliminated the possibility of solution contamination.

Alternatively, the blocking effect of glibenclamide might have been due to the baculovirus itself. To test this hypothesis, we performed additional experiments with mock and βgal-infected cells. Glibenclamide was again unable to inhibit ORCC in either mock- (4 out of 4 experiments, Fig. 6*B* and *C*) or βgal-infected cells (4 out of 4 experiments, Fig. 7*A–C*). Because the lack of effect of glibenclamide on ORCC might be due to alteration of the protein within the patch membrane, we systematically verified the channel sensitivity to SITS. As shown Figs. 6*A* and *B* and 7*B,* SITS (500 μM) reversibly blocked channel activity in mock and βgal cells ($n = 4$ for each cell). These data indicated that ORCCs, although not affected by glibenclamide, were still sensitive to stilbene derivatives.

Glibenclamide Inhibits ORCC in ΔF508-Expressing HI-5 Cells

The experiments reported above strongly suggested that the expression of CFTR is required for the inhibition of ORCC activity by glibenclamide. The effect of SITS on the contrary appeared to be independent on the expression of CFTR, since SITS inhibited ORCCs in any of the cells tested. We then tested whether the most common CF mutation ΔF508 (Riordan et al., 1989), might have

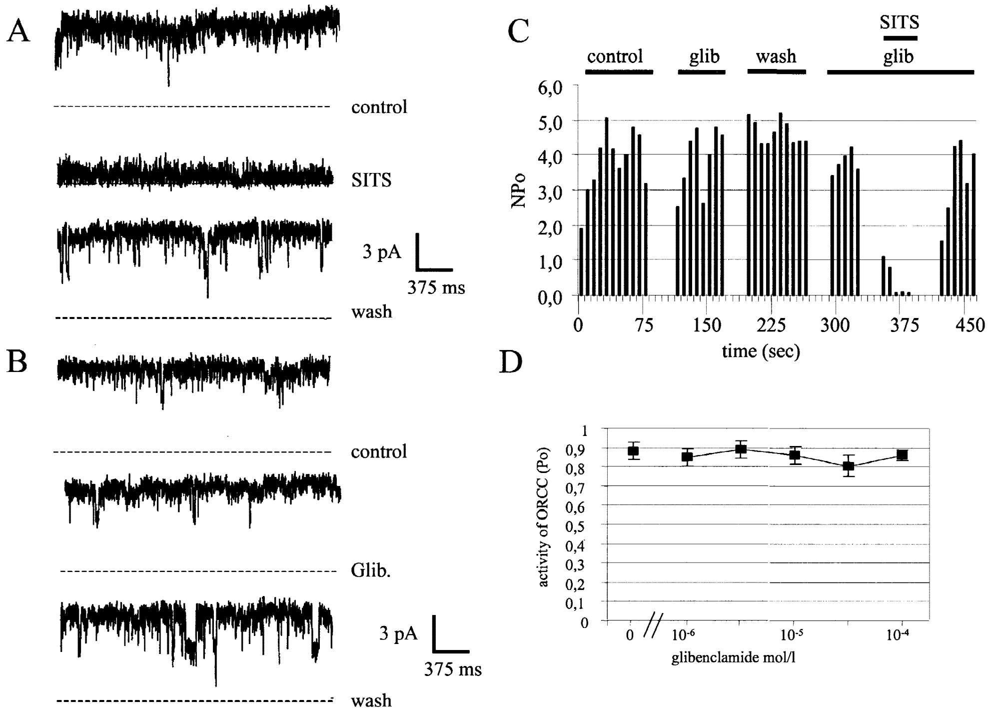

Fig. 5. Effect of SITS and glibenclamide on the activity of ORCC in excised inside-out patches from uninfected Hi-5 cells. (*A*) Reversible inhibition of ORCC by 500 μM SITS. (*B*) No effect of 100 μM glibenclamide (noted Glib.) on the activity of ORCC. (*A*) and (*B*) Experiments performed in inside-out configuration at $V = +50$ mV. Two channels were present in the patch. (*C*) An experiment showing the reversible inhibition by SITS and glibenclamide on ORCC activity evaluated as NP_o (*see*Materials and Methods). NP_o is plotted as function of time to show the effect of the sequential addition of 100 μM glibenclamide and 500 μM SITS+ 100 μM glibenclamide. (*D*) Concentration-dependent relationship of ORCC activity in the presence of increasing doses of glibenclamide evaluated as P_o, $V = +30$ mV. Note that P_o was not affected.

altered the glibenclamide- and/or SITS-sensitivity of ORCC. We first observed that ORCC in ΔF508-infected cells was reversibly inhibited by SITS (500 μM, 4 out of 4 experiments, Fig. 8*B*). Figure 8 shows that glibenclamide (100 μM) reversibly inhibited ORCC activity in ΔF508-infected cells (3 out of 3 experiments). Block was fast and voltage-independent (*not shown*). Inhibition resulted from a flickery type block (Fig. 8*A*) similar to that observed in CFTR-expressing cells (*see* Fig. 4*B*). In the presence of 100 μM glibenclamide, P_o was 0.08 ± 0.05 (control: $P_o = 0.89 \pm 0.05$; wash: $P_o = 0.87 \pm 0.1$, Fig. 8*B*, $P < 0.01$).

Discussion

In this study, we demonstrate for the first time that glibenclamide inhibits the activity of outwardly rectifying chloride channels in excised patches only when CFTR is expressed. The blocking action of glibenclamide is reversible, concentration dependent and not voltage-dependent. The glibenclamide sensitivity of ORCC is preserved in cells expressing ΔF508-CFTR. In contrast, the stilbene disulfonate derivative SITS inhibits ORCC activity even in the absence of CFTR suggesting that only the glibenclamide sensitivity of ORCC is under the control of CFTR.

Outwardly Rectifying Chloride Channels: Inhibition by Stilbene Derivatives

In the first part of our study, we confirmed the existence of ORCC and the absence of CFTR in the parental insect cell line Hi-5 (Yang et al., 1997). Stilbene derivatives such as DIDS or SITS did not affect the activity of CFTR when added to the extracellular side of the membrane (Gray et al., 1989; Tabcharani et al., 1990; Kartner et al., 1991; Fuller & Benos, 1992; Becq et al., 1993). On the other hand, these compounds reversibly inhibited ORCC as previously demonstrated in a variety of cells (Frizzell et al., 1986; Li et al., 1988; Tabcharani et al., 1990; Singh et al., 1991; Becq et al., 1992; *see also* Fuller & Benos, 1992). However, a recent study by Linsdell & Hanrahan (1996) showed that DNDS and DIDS, two stilbene derivatives, are effective voltage-dependent blockers of CFTR channels stably expressed in baby hamster kidney cells when applied to the cytoplasmic face of the membrane. These compounds are nevertheless accepted to be relatively ineffective at blocking

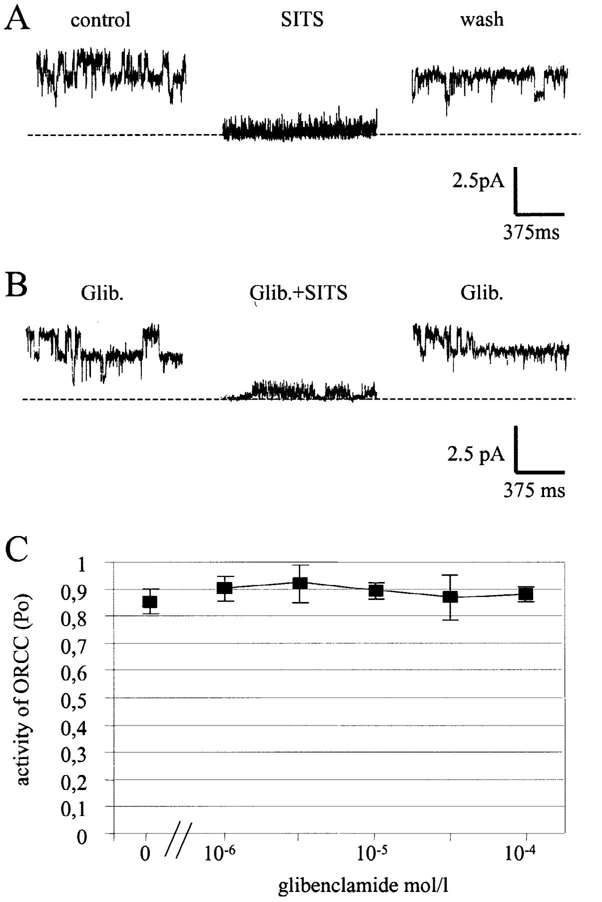

Fig. 6. Effect of glibenclamide and SITS on the activity of ORCC in excised inside out patches from Hi-5 cells infected with the baculovirus alone (mock-cells). (*A*) Reversible inhibition of ORCC by 500 μM SITS. (*B*) No effect of 100 μM glibenclamide (noted Glib.) on the activity of ORCC. (*A*) and (*B*) Experiments performed in inside-out configuration at $V = +30$ mV; three channels were present in the patch. (*C*) Concentration-dependent relation of ORCC activity in the presence of increasing doses of glibenclamide evaluated as P_o. Note that P_o was not affected.

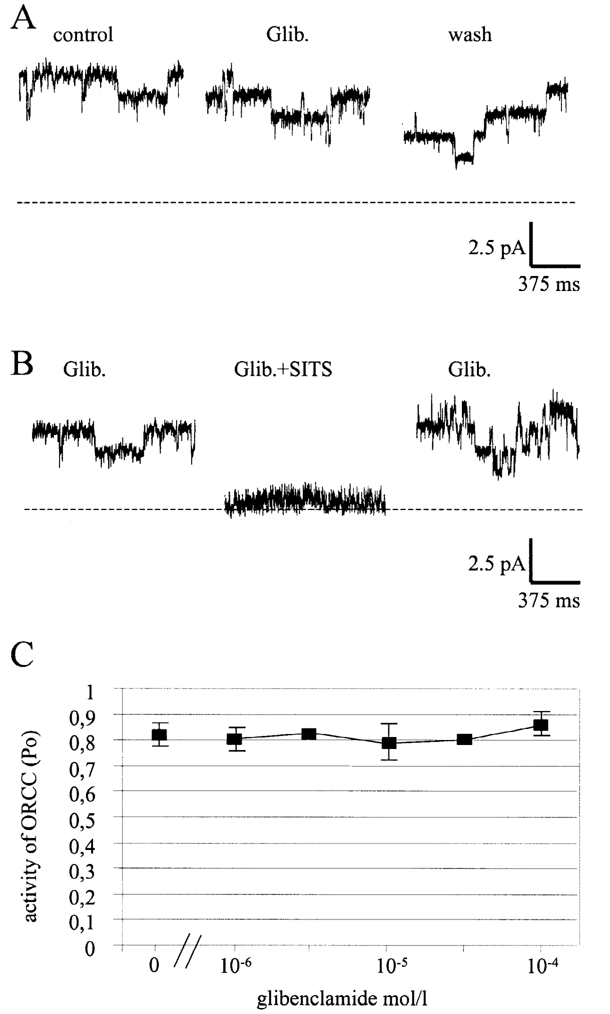

Fig. 7. Effect of glibenclamide and SITS on the activity of ORCC in excised inside-out patches from Hi-5 cells expressing βgal. (*A*) No effect of 100 μM glibenclamide (noted Glib.) on the activity of ORCC. (*B*) Reversible inhibition of ORCC by 500 μM SITS. Note that glibenclamide (100 μM) was present before, during and after the perfusion of SITS. Only the presence of 500 μM SITS induced the inhibition of ORCC, which was again reversible. (*A*) and (*B*) Experiments performed in inside-out configuration at $V = +30$ mV, 4 to 5 channels were present in the patch. (*C*) Concentration-dependent relation of ORCC activity in the presence of increasing dose of glibenclamide evaluated as P_o; $V = +30$ mV. Note that P_o was not affected.

CFTR from the extracellular side of the membrane (for a review *see* Fuller & Benos, 1992).

In Hi-5 cells, we observed a high density of ORCC in all the cells tested, irrespective of the absence or presence of high (*pGmAc115T*-) or low (*pGmAc217*-) expression of CFTR (*see* Fig. 3*B*). The activity of ORCC was reversibly inhibited by SITS in all cells tested. The inhibition of chloride channels by stilbene derivatives has been extensively studied and a direct binding of these drugs to the ORCC protein has been suggested (Singh, Venglarik & Bridges, 1995). This hypothesis is consistent with our results since SITS inhibited ORCC irrespective of the cell studied and of the expression of CFTR.

Similarly, in cells coexpressing Kir6.1 and CFTR and in those expressing only Kir6.1, the K^+ channel Kir6.1 was inhibited by Ba^{2+} (Ishida-Takahashi et al., 1998) showing that the Ba^{2+} block is CFTR-independent. Thus, some intrinsic properties of ORCC (i.e., its sensitivity to SITS) and Kir6.1 (i.e., its sensitivity to Ba^{2+}) are preserved regardless of the presence of CFTR.

Outwardly Rectifying Chloride Channels: Inhibition by Glibenclamide

Glibenclamide inhibits ORCC in M-1 mouse cortical collecting duct cells (Korbmacher et al., 1993; Volk et al., 1995). Cytoplasmic application of 100 μM glibenclamide reversibly reduced channel open probability (P_o) from 0.92 to 0.24 in M-1 cells (Volk et al., 1995). Similarly, in Hi-5 cells expressing CFTR the P_o was reduced from 0.88 to 0.09 in the presence of 100 μM glibenclamide (this study). The IC_{50} for the inhibition of ORCC by glibenclamide was 17 μM in outside-out configuration and 34 μM in inside-out configuration in HT29 cells (Rabe et al., 1995), 36 μM in M-1 mouse cortical collecting duct cells (Volk et al., 1995), and 17 μM in Hi-5 cells expressing CFTR (this study).

We were surprised to observe that glibenclamide failed to inhibit ORCC both in noninfected Hi-5 cells, and in mock- and βgal-infected cells. This result was unexpected since other investigators report the inhibition of such channels by glibenclamide (Rabe et al., 1995; Volk et al., 1995). However, it is important to keep in mind that the HT29 cell line used in the study of Rabe et al. (1995) expresses a high level of CFTR (Riordan et al., 1989).

The fact that glibenclamide failed to inhibit ORCC activity in cells infected or not with wild-type baculovirus or by virus recombinant for βgal cDNA exclude the possibility that virus infection alone could cause the inhibition.

The glibenclamide inhibition of ORCC is not absolutely linked to CFTR channel activity since (i) inhibition occurred in excised patches devoid of detectable CFTR activity, (ii) it was observed in Hi-5 cells infected with viruses recombinant for ΔF508-CFTR in which we were unable to record channel activity. Our results using cells expressing the mutant ΔF508 do not exclude protein-protein interactions at the membrane level because this mutation is temperature-sensitive (Denning et al., 1992). At 37°C, ΔF508 is mislocalized, retained in the ER and degraded (Cheng et al., 1990) but when cells expressing ΔF508 are cultured at reduced temperatures, some ΔF508 protein is delivered to the plasma membrane (Denning et al., 1992). The blocking action of glibenclamide on ORCC occurs in the absence of ATP or PKA in the bath solution, which suggests that activation of CFTR and/or phosphorylation of CFTR is not necessary. These observations are also reminiscent of the recent observations of McNicholas et al. (1996) who showed that the channel activity of CFTR was also not necessary to observe the inhibition of ROMK2 channels by glibenclamide.

ABC Transporters and Glibenclamide

Since the discovery by Sheppard and Welsh (1992) that glibenclamide and other K_{ATP} channel modulators affect the activity of CFTR, several investigators have first confirmed these observations using various cell preparations (Shultz et al., 1996; Sheppard & Robinson, 1997; Chappe et al., 1998) and then extended them to other ABC proteins. Glibenclamide has been shown to be a potent inhibitor of ABC1 (Becq et al., 1997) in *Xenopus*

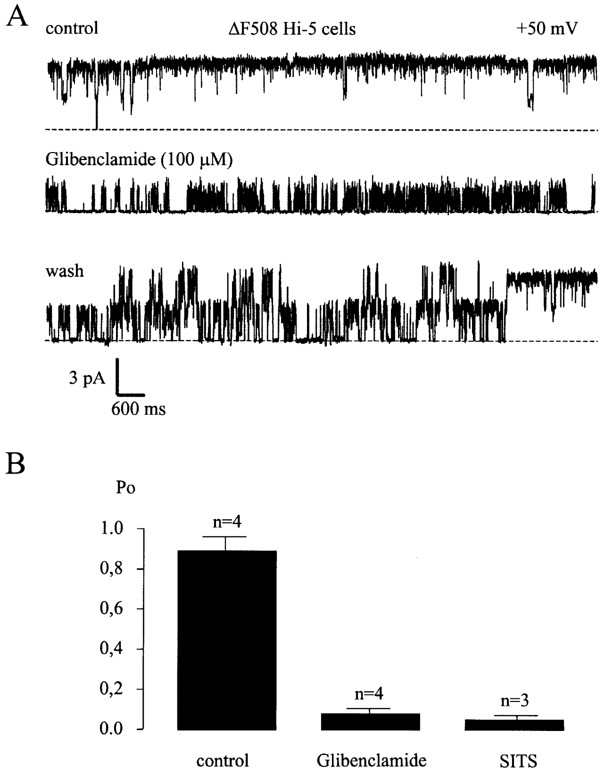

Fig. 8. Effect of glibenclamide and SITS on the activity of ORCC in excised inside-out patches from Hi-5 cells expressing ΔF508-CFTR. (*A*) Reversible inhibition of ORCC by 100 μM glibenclamide. Experiments performed in inside-out configuration at $V = +50$ mV. (*B*) Histograms showing the open probability of ORCC in the presence of 500 μM SITS or 100 μM glibenclamide. Both drugs blocked ORCC ($P < 0.01$ for inhibitor *v.* control).

oocytes or macrophages (Becq et al., 1997; Hamon et al., 1997). ABC proteins in plants are also sensitive to glibenclamide (Leonhardt et al., 1997). Finally, glibenclamide is well known to interact with the K_{ATP} channel through the binding to the ABC protein SUR (Inagaki et al., 1995; Shyng, Ferrigni & Nichols, 1995). Therefore, the glibenclamide-sensitivity of ABC proteins appears to be widely distributed in humans, animals and plants suggesting the conservation of a binding site within ABC proteins. Such a mode of interaction may also explain recent results from our laboratory which show that the anion transport mediated by the ABC transporter ABC1 and the interleukin IL1β transport capability of macrophages expressing ABC1 are both dose-dependently inhibited by glibenclamide (Becq et al., 1997; Hamon et al., 1997).

ABC Transporters Confer Glibenclamide-Sensitivity to a Variety of Ionic Channels. A Potential Molecular Association Between ORCC and CFTR

We provide evidence in this study that CFTR mediates sulfonylurea inhibition of the ORCC in baculovirus infected Hi-5 cells. Our results, taken together with those obtained by others on the relationship between the sulfonylurea receptor SUR and K_{ATP} channel (Inagaki et al., 1995; Shyng et al., 1997), between CFTR and the epithelial K^+ channel ROMK2 (McNicholas et al., 1996, 1997) and between CFTR and the K_{ATP} channel Kir6.1 (Ishida-Takahashi et al., 1998), suggest that CFTR by itself confers glibenclamide sensitivity to several channels such as ROMK2, K_{ATP} and ORCC through a similar mechanism. A striking observation was made by Furukawa et al. (1993) who compared the glibenclamide and stilbene derivatives sensitivity of K_{ATP} channel in guinea pig ventricular myocytes. These authors showed that stilbene derivatives, including SITS block the K_{ATP} channel by binding to their target site with one-to-one stoichiometry. Thus, two different channels ORCC and K_{ATP} channel are both sensitive to SITS and glibenclamide, the latter property being dependent on CFTR expression (this study and Ishida-Takahashi et al., 1998).

An interesting hypothesis would be that glibenclamide, through the binding to an ABC protein (perhaps on specific binding sites) affects the activity of other transporters located in the vicinity of the ABC protein. Based on a kinetic study, Sheppard and Robinson (1997) suggested that glibenclamide and chloride ions may compete for a common binding site located within a large intracellular vestibule that is part of the CFTR pore. The binding of sulfonylureas to CFTR has also been proposed by Venglarik et al. (1996) and Schultz et al. (1996). These authors concluded that glibenclamide blocks CFTR by a closed-open-blocked mechanism.

Based on the functional analysis of mutant forms of SUR, Shyng et al. (1997) proposed that SUR sensitizes the K_{ATP} channel to ATP inhibition, and nucleotide hydrolysis at the nucleotide binding folds blocks this effect. When coupled to the channel subunit, SUR exerts a hypersensitizing effect on channel activity, which is abolished when the channels are treated with trypsin (Shyng et al., 1997).

In conclusion, considering the diversity of proteins that have been reported to be influenced by CFTR (EnaC, K_{ATP}, ROMK2, ORCC . . .), it seems more likely that those channels interact with some intermediate protein rather than directly binding to CFTR.

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