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Comparative Pharmacology of the Activity of Wild-type and G551D Mutated CFTR Chloride Channel: Effect of the Benzimidazolone Derivative NS004

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Abstract. The pharmacological activation of the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel mutated at glycine 551 (G551D-CFTR) was studied in the presence of the benzimidazolone derivative NS004 and compared to that of wild-type (wt) CFTR. Using iodide (^{125}I) efflux and whole-cell patch-clamp techniques we found dose-dependent stimulation of phosphorylated wt-CFTR channels by NS004 with an $EC_{50} \approx 11$ μM. With non-phosphorylated CFTR, the effect of NS004 was apparent only at concentration >100 μM. In G551D-CFTR-expressing CHO cells, neither forskolin (from 0.1 to 10 μM) nor NS004 (from 0.1 to 200 μM) added separately were able to stimulate channel activity. However, in the presence of 10 μM forskolin, NS004 stimulated G551D-CFTR activity in a dose-dependent manner with an $EC_{50} \approx 1.5$ μM. We also determined the half-maximal effective concentration of forskolin ($EC_{50} \approx 3.2$ μM) required to stimulate G551D channel activity in presence of 1.5 μM NS004. No inhibitory effect was observed at high concentration of NS004 with both wt- and G551D-CFTR. Whole-cell recordings of CFTR chloride currents from cells expressing wild-type or G551D-CFTR in the presence of NS004 were linear, time- and voltage-independent. The inhibitory profile of G551D-CFTR channel activity was similar to that of wild type, i.e., inhibition by glibenclamide (100 μM) and DPC (250 μM) but not by DIDS (200 μM) nor calixarene (100 nM). These results show that NS004 activates wt-CFTR channel and restores G551D-CFTR channel activity, the potency of which depends on both the concentration of NS004 and the phosphorylation status of CFTR.

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Introduction

Cystic fibrosis (CF), the most common lethal autosomal recessive genetic disease among Caucasians, results from mutations of the gene encoding the Cystic Fibrosis Transmembrane conductance Regulator (CFTR), a chloride channel that normally mediates Cl^- transepithelial transport in epithelia (Riordan et al., 1989; Tabcharani et al., 1991; Quinton, 1999). CFTR activity is controlled by protein kinase (PKA)-dependent phosphorylation and is gated by ATP at two distinct nucleotide binding domains, NBD 1 and 2 (Riordan et al., 1989; Anderson et al., 1991; Gadsby & Nairn, 1994). Mutations of the CF gene (http://www.genet.sickkids.on.ca/cftr) can be assigned to five classes of mutations (Welsh & Smith, 1993), leading to a protein having an altered chloride channel activity (classes III and IV) or being absent from the apical plasma membrane (classes I, II and V).

The class III mutation glycine-to-aspartic acid at codon 551 (G551D) is located within the NBD1 domain (Cutting et al., 1990). Glycine 551 lies within a sequence in NBD1, D-X-[G/A]-G-Q, which shows remarkable conservation in ABC transporters. With a frequency of 2–5%, depending on the population of origin, G551D is one of the five most frequent CF mutations and is always associated with a severe CF phenotype (Cutting et al., 1990), pulmonary dysfunction and pancreatic insufficiency. G551D-mutated protein is fully glycosylated, correctly located at the apical membrane (i.e., normal biosynthesis, trafficking and processing) (Smit et al., 1993; Welsh & Smith, 1993) and normally phosphorylated at the R

domain by cAMP-dependent protein kinase (Chang et al., 1993). However, the mutant can not be activated by cAMP-elevating agents (Cutting et al., 1990; Welsh & Smith, 1993; Becq et al., 1994). G551D mutation confers a decreased nucleotide binding (Logan et al., 1994) and a reduced ATPase activity at NBD1 (Li et al., 1996; Howell, Borchardt & Cohn, 2000).

The pharmacological modulation of G551D-CFTR chloride-channel activity has not been clearly and systematically characterized and compared to that of wild-type CFTR channel. The ability of high doses of IBMX to activate mutated CFTR, including G551D, has, however, been reported (Drumm et al., 1991; Becq et al., 1994) and the phenylimidazothiazoles bromotetramisole and levamisole (Becq et al., 1994; 1996) as well as genistein (Illek et al., 1999) have been shown to activate the G551D-CFTR channel. Recently, we demonstrated that benzoquinolizinium derivatives activate wt-, G551D-CFTR chloride channels and modulate the trafficking of delF508-CFTR (Becq et al., 1999, Dérand et al., 2001, Dormer et al., 2001).

In this report, we have investigated and compared the effect of the benzimidazolone NS004 on the activation of both wild-type (wt) and G551D-CFTR chloride channels stably expressed in CHO cells, using iodide (^{125}I) efflux and whole-cell patch-clamp techniques.

Materials and Methods

Cell Culture

Chinese Hamster Ovary (CHO) cells stably transfected with pNUT vector alone (pNUT CHO) or containing wild-type CFTR (CFTR(+) CHO) or G551D (G551D CHO) mutation were provided by J.R. Riordan and X.-B. Chang, Scottsdale, AZ, USA (Tabcharani et al., 1991; Chang et al., 1993). Cells cultured at 37°C in 5% CO_2 were maintained in αMEM containing 7% fetal bovine serum, 0.5% antibiotics (50 IU/ml penicillin and 50 μg/ml streptomycin) and 100 μM or 20 μM methotrexate for CFTR(+), G551D and pNUT CHO cells, respectively. For detailed procedures *see* elsewhere (Bulteau et al., 2000; Dérand et al., 2001).

Iodide Efflux Experiments

CFTR chloride-channel activity was assayed by measuring the rate of iodide (^{125}I) efflux from transfected CHO cells, as previously described (Bulteau et al., 2000; Dérand et al., 2001). All experiments were performed at 37°C. Cells were cultured in 24-well plates in order to perform parallel experiments and comparison analysis. At the beginning of each experiment, cells were washed with efflux buffer containing (in mM): 137 NaCl, 5.36 KCl, 0.8 $MgCl_2$, 5.5 glucose and 10 HEPES, pH 7.4. Cells were then incubated in efflux buffer containing 1 μM KI (1 μCi $Na^{125}I$ /ml, NEN, Boston, MA) for 30 min at 37°C to permit the ^{125}I to reach equilibrium. Cells were then washed with efflux medium to remove extracellular ^{125}I. The loss of intracellular ^{125}I was determined by removing the medium with efflux buffer every 1 min for up to 11 min. The first four aliquots were used to establish a stable baseline in efflux buffer alone. A medium containing the appropriate drug was used for the remaining aliquots. Residual radioactivity was extracted with 0.1 N NaOH, and determined using a gamma counter (Cobra II, PackardBell). The fraction of initial intracellular ^{125}I lost during each time point was determined and time-dependent rates of ^{125}I efflux were calculated from: $\ln(^{125}I_{t1}/^{125}I_{t2})/(t_1-t_2)$, where $^{125}I_t$ is the intracellular ^{125}I at time t, and t_1 and t_2 are successive time points (Venglarik, Bridges & Frizzell, 1990). Curves were constructed by plotting rate of ^{125}I efflux versus time. Relative rates (R) were determined and correspond to: R_{peak}/R_{basal}. All comparisons are based on maximal values for the time-dependent rates, excluding the points used to establish the baseline (Bulteau et al., 2000; Dérand et al., 2001). In experiments using the chloride transport inhibitors (DIDS, DPC, glibenclamide and TS-TM calix[4]arene), these agents were present in the loading solution and in the efflux buffer, as indicated in the Results section.

Patch-clamp Experiments

Whole-cell recordings were performed on CHO cells at room temperature. Currents were recorded with a List EPC-7 patch-clamp amplifier. *I-V* relationships were built by clamping the membrane potential to −40 mV and by pulses from −100 mV to +100 mV in 20-mV increments. Pipettes with resistance of 3–5 MΩ were pulled from borosilicate glass capillary tubing (GCL150-T10, Clark Electromedical, Reading, UK), using a two-step vertical puller (Narishige, Japan). They were connected to the head stage of the amplifier through an Ag-AgCl pellet. Seal resistances ranging from 5 to 15 GΩ were obtained. Media generating a chloride gradient of external concentration 151 mM and internal concentration 28 mM were used (E_{Cl} = −42 mV). The pipette solution contained (mM): 113 L-aspartic acid, 113 CsOH, 27 CsCl, 1 NaCl, 1 $MgCl_2$, 1 EGTA, 10 TES, 285 mOsm. (pH 7.2). MgATP (3 mM) was added just before patch-clamp experiments. The external solution consists of (mM) 145 NaCl, 4 CsCl, 1 $CaCl_2$, 5 glucose, 10 TES, 340 mOsm. (pH 7.4). Results were analyzed with the pCLAMP6 package software (pCLAMP, Axon Instruments). Cells were stimulated with Forskolin or appropriate compound at the concentration indicated in the text (dissolved in dimethyl sulfoxide DMSO; final DMSO concentration <0.1%). In control experiments, the currents were not altered by DMSO. For other details see Dérand et al., 2001.

Chemicals

Forskolin, glibenclamide, DPC (diphenylamine-2-carboxylic acid), DIDS (4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid) were from Sigma Chemicals (St Louis, MO). NS004 (5-trifluoromethyl-(5-chloro-2-hydroxyphenyl)-1,3-dihydro-2H-benzimidazol-2-one) and TS-TM calix[4]arene (5,11,17,23-tetrasulfonato-25,26,27,28-tetramethoxy-calix[4]arene) were generous gifts of Dr. Barbry (IPMC, Sofia Antipolis, France) and Drs. Singh and Bridges (University of Pittsburgh, Pittsburgh, USA), respectively. All other products were from Sigma (St Louis, MO), except αMEM was from Fisher PAA.

Statistics

Results were expressed as means ± SEM of n observations. To compare sets of data, we used either an analysis of variance (ANOVA) or Student's t-test. Differences were considered statistically significant when $P < 0.05$. ns: non-significant difference,

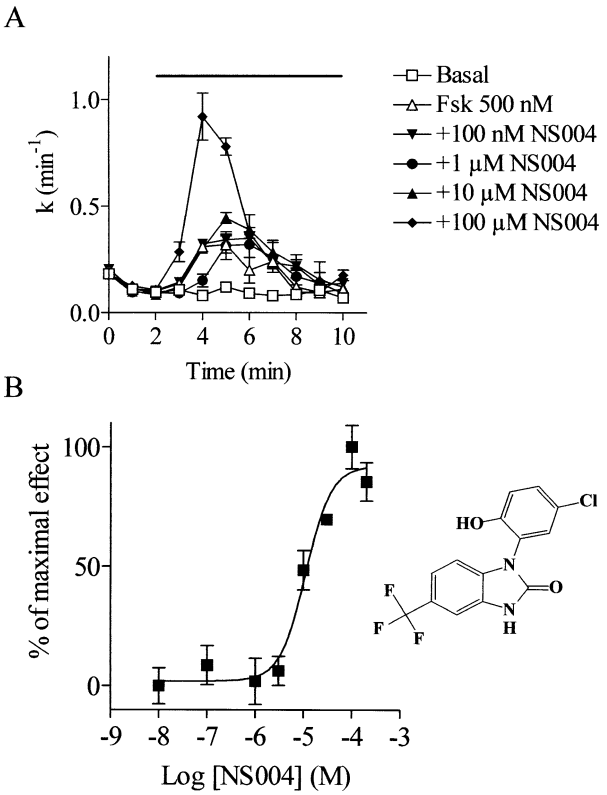

Fig. 1. Effect of NS004 on forskolin-stimulated CFTR activity. (*A*) ^{125}I efflux in the presence of 500 nM Fsk. Basal: cells were not stimulated. *Empty triangle:* forskolin alone. *Black symbols:* cells were stimulated with 500 nM forskolin and NS004 at the indicated concentration. In this and subsequent figures the presence of agonist is indicated by the horizontal bar. (*B*) Dose-response relationship of the effect of NS004 on CFTR in the presence of 500 nM Fsk. The percentage of maximal effect is plotted as function of the concentration of NS004. The half-maximal effective concentration EC_{50} was 11 ± 1.3 μM. The structure of the benzimidazolone NS004 drug is shown. Error bars are SEM for $n = 6$ in *A* and *B*. Some error bars are smaller than the symbol.

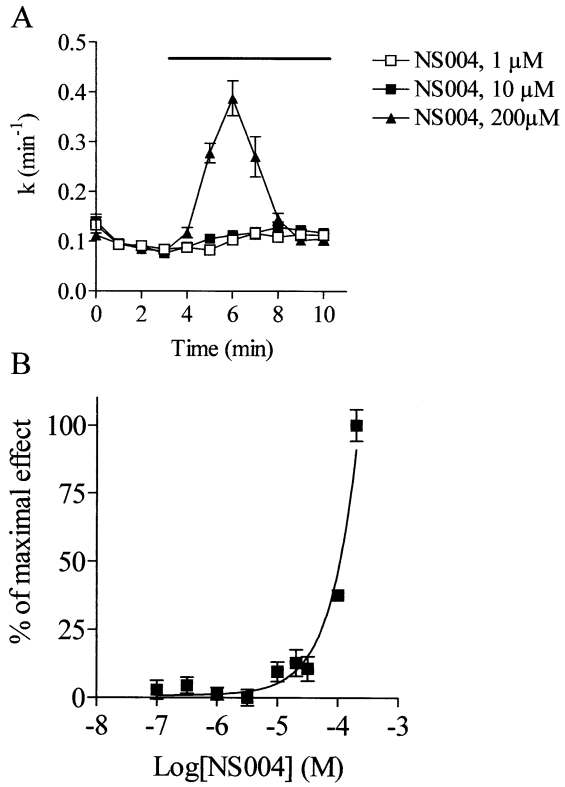

Fig. 2. Effect of NS004 on CFTR activity in the absence of forskolin. (*A*) Examples of ^{125}I efflux in the presence of three concentrations of NS004 as indicated. No forskolin present. (*B*) Dose-response relationship of the effect of NS004 on CFTR in the absence of forskolin. The percentage of maximal effect is plotted as function of the concentration of NS004. The half-maximal effective concentration EC_{50} is estimated to lie above 100 μM. Note that no plateau was obtained. Error bars are SEM for $n = 6$ in *A* and *B*. Some error bars are smaller than the symbol.

$^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. All statistical tests were performed using GraphPad Prism version 3.0 for Windows (Graphpad Software, San Diego, California, USA).

Results

Activation of wt-CFTR by NS004

We first performed iodide efflux experiments with CHO cells stably expressing wt-CFTR and incubated with the benzimidazolone derivative NS004 (*see* Fig. 1 for chemical structure). Because CFTR is mainly regulated by a phosphorylation process, we thought it important to study the effect of NS004 on partially phosphorylated and non-phosphorylated wild-type CFTR. In the first part of our study, wt-CFTR cells were simultaneously exposed to 500 nM forskolin (Fsk) and to increasing concentrations of NS004. We found that NS004 potentiates Fsk-activated ^{125}I efflux in a dose-dependent manner (Fig. 1*A*). The half-maximal effective concentration EC_{50} = 11 ± 1.3 μM was determined from 6 separate experiments, in which we performed complete dose-reponse tests (Fig. 1*B*). In contrast, with non-phosphorylated CFTR (in the absence of Fsk), NS004 had only a moderate effect on wt-CFTR activity (Fig. 2), although pronounced stimulation of CFTR activity was observed at high concentrations, i.e., 100 and 200 μM of NS004, as shown Fig. 2*A* and *B*. For example, the application of 200 μM NS004 stimulated the ^{125}I efflux 4.99 ± 0.21-fold ($n = 4$) when compared to control. Because we could not use NS004 at higher concentration (>200 μM), only a partial dose-response relationship was constructed for this protocol, as shown in Fig. 2*B* (note the absence of a plateau). The half-maximal effective concentration (EC_{50}) may be estimated to be above 100 μM, indicating a low efficacy of NS004 on non-phosphorylated wt-CFTR channel activity.

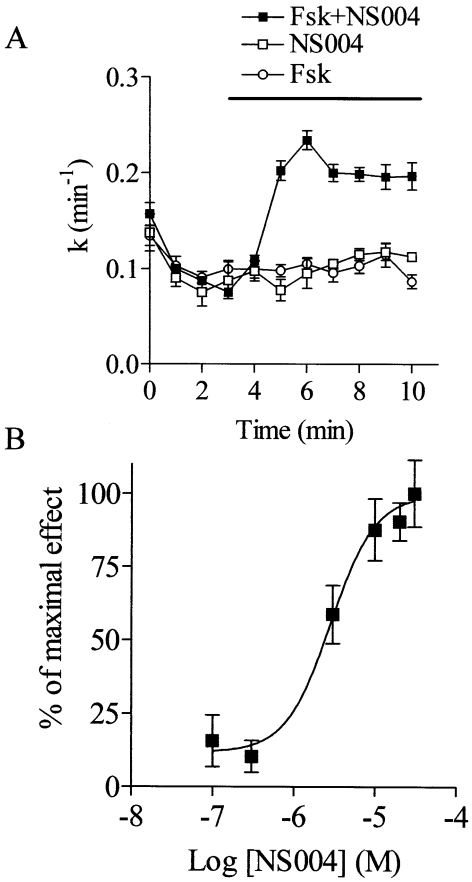

Fig. 3. Iodide efflux experiments in G551D-CFTR CHO cells. (*A*) Comparative curves of ^{125}I efflux obtained in the presence of 10 μM Fsk (○, $n = 14$), 20 μM NS004 (□, $n = 6$) or 20 μM NS004 + 10 μM Fsk (■, $n = 14$). Some error bars are smaller than the symbol. (*B*) Dose-response relationship of the effect of NS004 on G551D-CFTR in the presence of 10 μM forskolin. The dose-response curve was constructed from 4–6 separate experiments. EC_{50} was 1.47 ± 0.07 μM. All results are means ± SEM.

Stimulation of G551D-CFTR-mediated ^{125}I Efflux by NS004

The pharmacological properties of CFTR chloride channel having the glycine-to-aspartate mutation at codon 551 (G551D) were then studied. In the presence of 5 μM Fsk, CFTR-dependent iodide efflux was stimulated ≈5-fold from 0.12 ± 0.01 min^{-1} to 0.63 ± 0.05 min^{-1} ($n = 10$) in wt-CFTR cells (*not shown*). In G551D-CFTR cells, in the absence of Fsk, the peak rate of ^{125}I efflux was 0.09 ± 0.01 min^{-1} ($n = 14$, *not shown*) and did not differ significantly from experiments in which 10 μM Fsk was added (0.10 ± 0.01 min^{-1}, $n = 14$, Fig. 3*A*). We then studied the effect of NS004 on G551D-CFTR activity in the presence of 10 μM Fsk. When Fsk and NS004 were simultaneously added, a dramatic increase of the efflux was observed (Fig. 3*A*). In G551D-CFTR cells, ^{125}I efflux rates were 1.35 ± 0.17 ($n = 12$), 1.13 ± 0.04 ($n = 8$), and 3.41 ± 0.15 ($n = 28$) with 10 μM Fsk, 20 μM NS004 and 10 μM Fsk + 20 μM NS004, respectively. Figure 3*A* also shows that the addition of 20 μM NS004, without Fsk, failed to activate any significant ^{125}I efflux in G551D-CFTR cells. Similar lack of activation was noted with concentrations of NS004 ranging from 0.1 to 200 μM (*not shown*). In contrast, in the presence of 10 μM Fsk, the stimulation of G551D-CFTR activity by NS004 increased dose-dependently with an $EC_{50} = 1.47 \pm 0.07$ μM (Fig. 3*B*). We also searched for the minimal concentration of forskolin required to stimulate G551D in the presence of a low concentration of NS004. For this protocol, G551D-CFTR cells were simultaneously exposed to 1.5 μM NS004 and to increasing concentrations of Fsk. Stimulation of the iodide efflux could be obtained above 1 μM Fsk (Fig. 4). The half-maximal effective concentration of forskolin needed to stimulate G551D-CFTR with 1.5 μM NS004 was $EC_{50} = 3.15 \pm 0.2$ μM ($n = 4$, Fig. 4*B*). These data indicate a synergistic effect of NS004 and forskolin, with the level of stimulation of G551D-CFTR activity being dependent on both NS004 and channel phosphorylation.

Activation of G551D-CFTR Chloride Current by NS004

Whole-cell patch-clamp experiments were next performed, using G551D-CFTR cells. Several control experiments were first performed. As expected from the iodide efflux experiments, G551D-CFTR cells are not responsive to up to 10 μM Fsk in whole-cell patch-clamp experiments, as previously reported (Dérand et al., 2001; Illek et al., 1999). When measured at +40 mV, the current density was only 1.67 ± 0.29 pA/pF ($n = 7$), a value that did not differ from control condition (Figs. 5*A*, 5*B* and 6). For comparison, wild-type CFTR current activated by 10 μM Fsk has a current density of 21.06 ± 2.82 pA/pF when measured at +40 mV ($n = 9$, *not shown*). This lack of responsiveness of G551D-CFTR to cAMP agonists constitutes a hallmark of the mutant and a major difference with wild-type CFTR.

To determine the effect of NS004 on G551D-CFTR current, cells were first exposed to 10 μM Fsk and then to 20 μM NS004. Using this protocol, a large time- and voltage-independent current was stimulated only in cells treated with Fsk + NS004 (Figs. 5*C* and 6). This current reversed near −40 mV (Fig. 6), which corresponds to the reversal potential measured for current activated by Fsk in wt-CFTR-expressing CHO cells (Bulteau et al., 2000; Dérand et al., 2001). This value, being close to the theorical Cl^- equilibrium potential imposed in our experimental conditions ($E_{Cl} = -42$ mV), confirms the chloride nature of the current. The NS004-activated conductance had a current density of 29.97 ± 4.89 pA/pF ($n = 7$) and

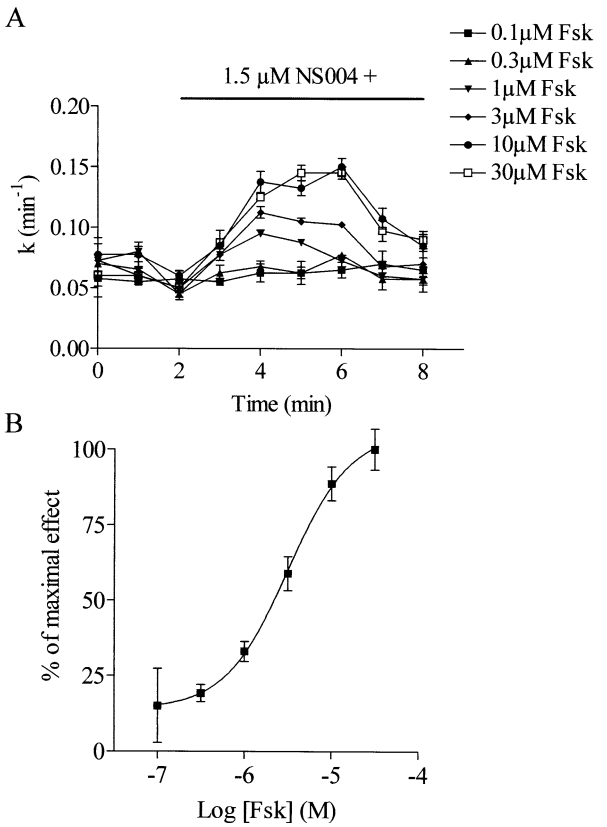

Fig. 4. Minimum concentration of forskolin to stimulate G551D-CFTR activity. (*A*) Comparative curves of ^{125}I efflux obtained in the presence of increasing concentrations of forskolin and in presence of 1.5 μM NS004 ($n = 4$ for each concentration). Some error bars are smaller than the symbol. (*B*) Dose-response relationship of forskolin on G551D-CFTR in the presence of 1.5 μM NS004. The dose-response curve was constructed from 4 separate experiments. EC_{50} was 3.15 ± 0.02 μM. All results are means ± SEM.

was significantly different from control condition (1.24 ± 0.45 pA/pF, $n = 7$, $P < 0.001$) when measured at +40 mV (Fig. 6). Additional experiments were conducted using pNUT CHO cells, but neither Fsk, NS004 nor Fsk + NS004 stimulated a significant ^{125}I efflux or a whole-cell current (*not shown*). These observations are consistent with the hypothesis that no endogenous chloride transporters in CHO cells are stimulated by this compound.

Inhibitory Profile of G551D-CFTR Chloride Channel Activity Stimulated by NS004

Several inhibitors were then used to further characterize G551D-CFTR channel activated by NS004. Figure 5*F* shows the effect of glibenclamide. G551D-CFTR currents were first activated by Fsk + NS004 (Fig. 5*E*) and then inhibited in the presence of 100 μM glibenclamide (Figs. 5*F* and 6). Figure 7 displays an example time course of current mesured at +40 mV after stimulation by Fsk and NS004 and challenged with 100 nM TS-TM calix[4]arene, a specific inhibitor of outwardly rectifying chloride channel (Singh, Venglarik & Bridges, 1995) and then with 100 μM glibenclamide. As expected, TS-TM calix[4]arene did not affect the current, whereas glibenclamide fully reversed it. Similarly, iodide efflux stimulated by Fsk + NS004 was inhibited by 70% and 45% with, respectively, 100 μM glibenclamide and 250 μM DPC, but only by 6% and 1% in presence of 200 μM DIDS or 100 nM TS-TM calix[4]arene (Fig. 8). From these observations, we concluded that the inhibitory profile of G551D-CFTR activated by NS004 did not differ from that of wild-type CFTR, and that G551D mutation did not affect these pharmacological properties of CFTR.

Discussion

The present work reports the characteristics of activation of G551D-CFTR channels by the benzimidazolone derivative NS004 and offers a comparison with wt-CFTR channel activity. Several notable results were obtained. First, NS004 acts on phosphorylated wt and G551D-CFTR. Second, in sharp contrast with the isoflavone genistein, no inhibitory effect at high concentration of NS004 could be detected on wt-CFTR. Third, the inhibitory profile of G551D-CFTR channel activity appears similar to that of wt-CFTR.

It is well established that the cAMP-PKA-signaling pathway is the major mechanism for regulation of CFTR chloride channel activity (Riordan et al., 1989; Anderson et al., 1991; Tabcharani et al., 1991). It involves phosphorylation at multiple serine residues within the regulatory *R* domain of CFTR and/or interaction with one or both nucleotide binding domains (NBD1 and NBD2). Consistent with this hypothesis, we observed a potentiation of forskolin (Fsk)-induced efflux with NS004 in wt-CFTR CHO cells. The EC_{50} was dramatically shifted (≈10-fold) towards lower concentrations of NS004 in the presence of Fsk (500 nM). These observations clearly suggest that NS004 has a better affinity for phosphorylated wild-type CFTR. These data are in good agreement with previous work from our lab (Dérand, Bulteau-Pignoux & Becq, 2002) and others (Illek et al., 1999), suggesting that another drug, genistein, preferentially acts on phosphorylated CFTR. Phosphorylation of CFTR may facilitate the accessibility of the NS004 binding site(s) through domain interactions or conformation changes. Indeed, one major difference concerning the phosphorylation process is that higher concentrations of forskolin are required to achieve stimulation of the G551D-CFTR channel activity by NS004. Based on these observations, we could then speculate that NS004 activates G551D-

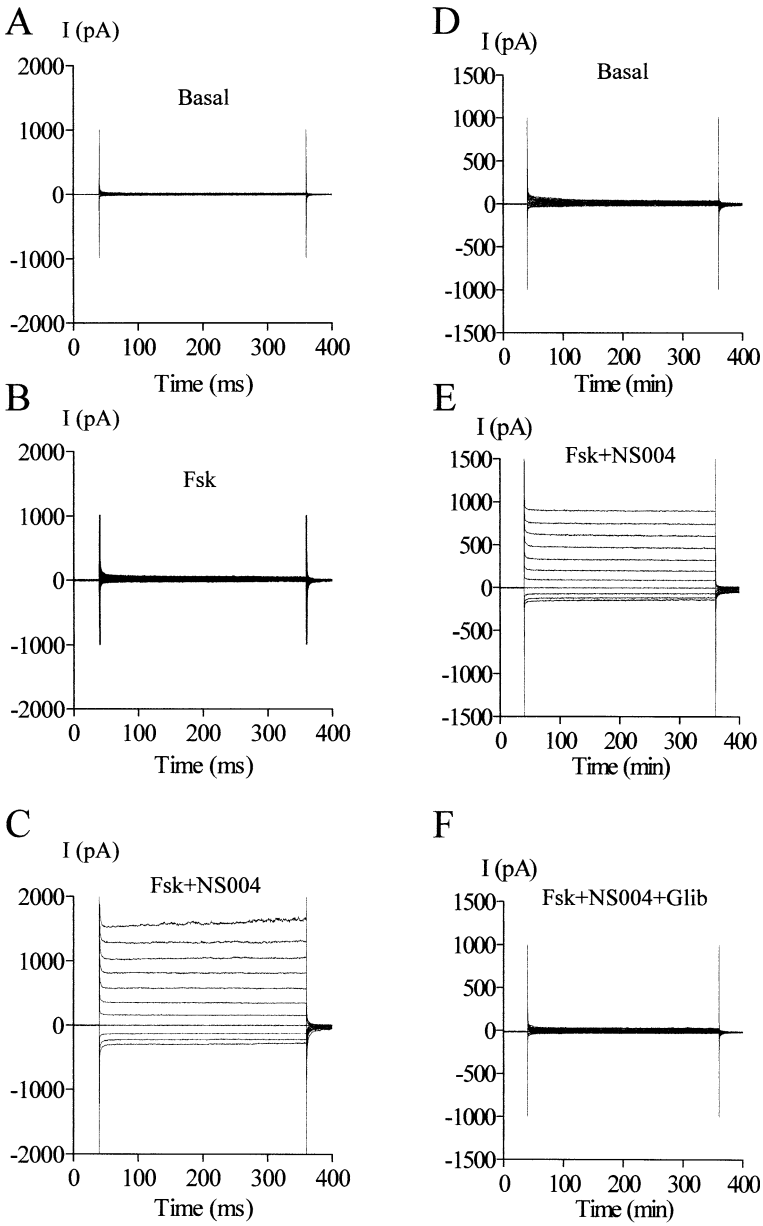

Fig. 5. Electrophysiological characteristics of G551D-CFTR chloride current. Whole-cell recordings using G551D-CFTR cells stimulated by 10 μM Forskolin (Fsk) and 20 μM NS004. Membrane currents were elicited by stepping from a holding potential of −40 mV to a series of test potentials from −100 to +100 mV in 20-mV increments. Conditions are indicated above the traces. (*A–C*) Representative currents showing the stimulation of G551D-CFTR chloride currents only with forskolin + NS004. Cell capacitance was 17 pF. (*D–F*) Effect of 100 μM glibenclamide on NS004-activated chloride conductance in G551D-CHO cells. Cell capacitance was 20 pF.

CFTR through a direct interaction with cryptic binding sites made accessible after phosphorylation. Dulhanty & Riordan (1994) indeed showed that phosphorylation of CFTR induced changes in the conformation of the R domain. Such modifications may allow the phosphorylated *R* domain to interact with parts of the CFTR protein unmasking NS004-sensitive sites. Direct interaction between NS004 and CFTR has been suggested from outside-out membrane patches (Gribkoff et al., 1994). In this study, we found that NS004 was able to stimulate forskolin-dependent phosphorylated G551D-CFTR activity. Interestingly, we also showed that, although up to 10 μM forskolin did not activate G551D-CFTR, only 1 μM was sufficient when 1.5 μM NS004 was also present. The maximum level of stimulation of G551D-CFTR was, however, achieved with higher concentrations of both agonists (*see* Fig. 3). These observations suggest a synergistic effect between the phosphorylation status of CFTR (mediated via forskolin) and the concentration of NS004.

Five classes of cystic fibrosis mutations have so far been described (Welsh & Smith, 1993). The glycine-to-aspartic acid missense mutation at codon 551 (G551D) is a class III mutation located within NBD1, which disrupts activation and regulation of CFTR at the plasma membrane (Cutting et al., 1990). The G551D mutated protein is, however, fully glycosylated and correctly located at the apical membrane. Importantly, G551D proteins have a decreased nucleotide binding and a reduced ATPase activity at NBD1 (Logan et al., 1994; Li et al., 1996; Howell

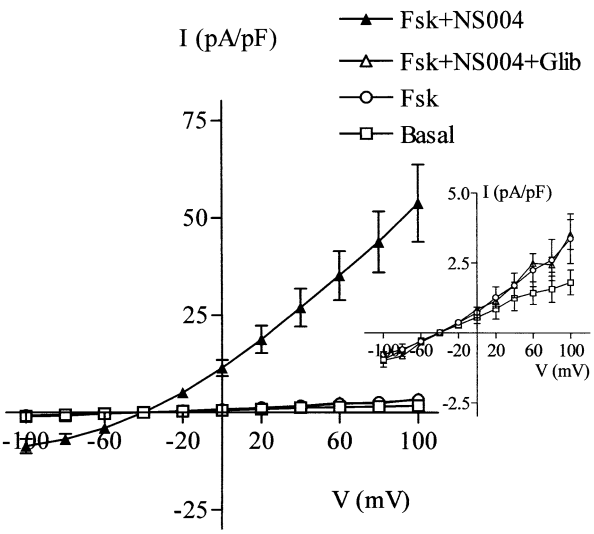

Fig. 6. Current-voltage relationships of G551D-CFTR chloride currents. The current-voltage (*I–V*) relationships are given for the following protocols: basal (squares, $n = 7$); Fsk (circles, $n = 7$); Fsk + NS004 (black triangles, $n = 7$); Fsk + NS004 + Glib (empty triangles, $n = 4$). In the inset, we magnify some *I–V* relationships for clarity. NS004: 20 μM; Fsk: 10 μM; Glib, glibenclamide, 100 μM. Error bars are SEM.

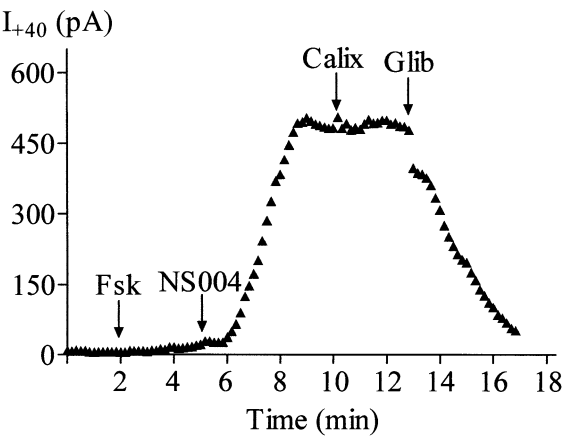

Fig. 7. Effect of calixarene and glibenclamide on NS004-activated chloride conductance in G551D-CHO cells. Representative experiments showing the time-dependent activation of G551D-CFTR chloride current in the presence of 10 μM forskolin and 20 μM NS004. Note that 100 nM calixarene (*Calix*) did not alter the current amplitude but that 100 μM glibenclamide (*Glib*) fully inhibited the current, which reversed to the basal level.

et al., 2000). It is important to note that this mutant form of CFTR is normally phosphorylated at the *R* domain by cAMP-dependent protein kinase, as shown by biochemical studies (Chang et al., 1993). Despite this apparent normal property, the channel activity of G551D mutant can not be stimulated by the classical pathway (Gregory et al., 1991). The importance of phosphorylation in the activation process of G551D-CFTR channel came from various studies. For example, it was found that phosphatase inhibitors activated G551D-CFTR as well as wt-CFTR (Becq et al., 1994). We and others were able to activate the mutant CFTR after co-application of cAMP agonists and drugs like genistein (He et al., 1998; Illek et al., 1999; Bulteau-Pignoux et al., 2002; Dérand et al., 2002), and benzoquinoliziniums (Dérand et al., 2001). Thus, phosphorylation of the G551D-CFTR protein appears to be essential before opening the channel by other means. Since the ATPase activity at NBD1 is abnormal for G551D, it can be hypothesized that both the altered ATPase activity and the unresponsiveness to PKA stimulation are linked. A1-Nakkash et al., (2001), reported that NS004 activates delF508-CFTR (a mutation that also disrupted the ATPase activity at NBD1) only in the presence of forskolin. Interestingly enough, we found similar behavior for NS004 with phosphorylated-wt-CFTR, suggesting a general and perhaps common mechanism of action of NS004.

Our knowledge of the pharmacological modulation of wild-type CFTR chloride channel activity is now on an exponential increase. In early studies (Drumm et al., 1991, Tabcharani et al., 1991), phosphodiesterase inhibitors (e.g., IBMX) were the only molecules found capable to stimulate CFTR channel activity. Now, a great number of molecules have been described as activators (*reviewed* in Schultz et al., 1999). These compounds are bromotetramisole (Becq et al., 1996), milrinone (Kelley, Al-Nakkash & Drumm, 1995), the isoflavone genistein (Illek et al., 1995), the benzimidazolones NS004 (5-trifluoromethyl-1-(5-chloro-2-hydroxyphenyl)-1,3-dihydro-2H-benzimidazole-2-one) (Gribkoff et al., 1994) and 1-EBIO (1-ethyl-2-benzimidazolinone) (Devor et al., 1996), the xanthine derivatives such as IBMX (Drumm et al., 1991; Becq et al., 1994), X-33 (Chappe et al., 1998; Bulteau et al., 2000) and CPX (Eidelman et al., 1992), and the benzoquinolizinium (Becq et al., 1999, Dérand et al., 2001). Most of these agents activate CFTR through cAMP-independent pathways (He et al., 1998; Chappe et al., 1998; Becq et al., 1999; Dérand et al., 2001) but the precise mechanism of activation remains unknown. Some of these compounds have also been found effective on delF508 CFTR (He et al., 1998; Devor et al., 2000; Al-Nakkash et al., 2001; Dormer et al., 2001). Genistein has been proposed as an activator of the trafficking-competent G551D mutant after forskolin exposure (Illek et al., 1999; Bulteau-Pignoux et al., 2002; Dérand et al., 2002). Previous work from our laboratory showed that benzo[c]quinolizinium derivatives (MPB-91 in particular) are also potent activators of G551D-CFTR (Derand et al., 2001).

NS004 was the first non-phosphodiesterase and non-phosphatase inhibitor presented as activator of wild-type CFTR through a cAMP-independent pathway (Gribkoff et al., 1994). Other groups reported similar activation in epithelia (Devor et al.,

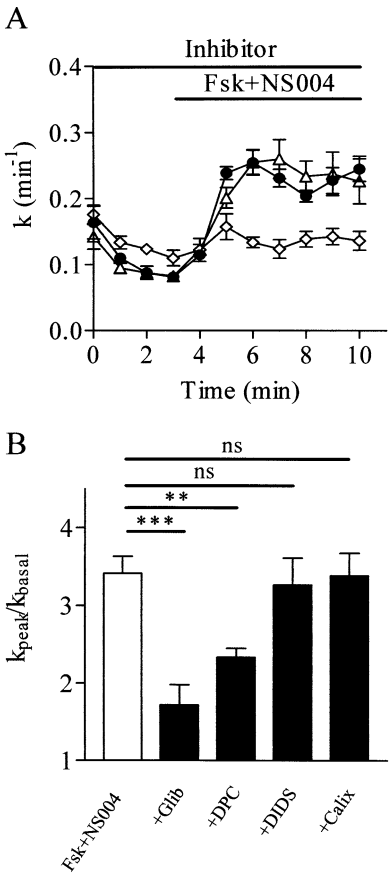

Fig. 8. Effect of various inhibitors on the iodide efflux stimulated in G551D-CFTR cells by NS004. (*A*) Averaged curves of ^{125}I efflux presenting the inhibitory profile of NS004-mediated ^{125}I efflux. 10 μM Fsk + 20 μM NS004 (•, $n = 8$); 100 μM glibenclamide (◇, $n = 8$), 200 μM DIDS (Δ, $n = 8$). (*B*) Histograms summarizing the inhibition of NS004-mediated efflux. Concentrations used are: glibenclamide (*Glib*, 100 μM), DPC (250 μM), DIDS (200 μM) and calixarene (*Calix*, 100 nM). All results are means ± SEM.

2000) or recombinant cells (He et al., 1998). Further studies have also demonstrated that NS004 is a modulator of P574H (Champigny et al., 1995), K1250A (Al-Nakkash et al., 2001) and delF508 (Gribkoff et al., 1994; He et al., 1998; Al-Nakkash et al., 2001). However, the contribution of the phosphorylation process to the NS004-activation of CFTR in Vero cells was not investigated in detail (Gribkoff et al., 1994). In particular the authors reported activation of the channel activity in the absence of cAMP-promoting agents. In the present report we showed, in contrast, that NS004 is a weak activator of wild-type CFTR expressed in CHO cells ($EC_{50} > 100$ μM) in the absence of a prior phosphorylation of CFTR. This discrepancy between both results may be explained by a different level of cAMP and/or adenylate cyclase activity in Vero and CHO cells as well as by the different level of protein expression in both systems.

When compared to wt-CFTR, the inhibitory profile of G551D was not altered, suggesting that the G551D mutation did not interfere with the pharmacological properties of the channel as we previously observed with genistein (Bulteau-Pignoux et al., 2002; Dérand et al., 2002). It is interesting to note that none of the CF mutations studied so far altered the inhibition of CFTR channel activity (Schultz et al., 1999). Most known CFTR inhibitors may act on different binding sites outside those involved in the activation (Schultz et al., 1999).

Despite the fact that it appears clear today that CFTR is a chloride channel gated by mechanisms involving phosphorylation of the *R* domain and ATP hydrolysis, both processes interacting with each other, it is also evident that alternative pathways should be investigated. We provided evidence in this report that NS004 and phosphorylation have a synergistic effect, which allows the stimulation of G551D-CFTR chloride channel activity.

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