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Capsaicin Potentiates Wild-Type and Mutant Cystic Fibrosis Transmembrane Conductance Regulator Chloride-Channel Currents

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ABSTRACT

To examine the effects of capsaicin on cystic fibrosis transmembrane conductance regulator (CFTR), we recorded wild-type and mutant CFTR chloride-channel currents using patch-clamp methods. The effects of capsaicin were compared with those of genistein, a well-characterized CFTR activator. In whole-cell experiments, capsaicin potentiates cAMP-stimulated wild-type CFTR currents expressed in NIH 3T3 cells or Chinese hamster ovary cells in a dose-dependent manner with a maximal response ~60% of that with genistein and an apparent K_d of 48.4 ± 6.8 μM. In cell-attached recordings, capsaicin alone fails to activate CFTR in cells that show negligible basal CFTR activity, indicating that capsaicin does not stimulate the cAMP cascade. The magnitude of potentiation with capsaicin depends on the channel activity before drug application; the lower the prestimulated P_o, the higher the potentiation. Single-channel kinetic analysis shows that capsaicin potentiates CFTR by increasing the opening rate and decreasing the closing rate of the channel. Capsaicin may act as a partial agonist of genistein because the maximally enhanced wild-type CFTR currents with genistein are partially inhibited by capsaicin. Capsaicin increases ΔR-CFTR, a protein kinase A (PKA)-independent, constitutively active channel, in cell-attached patches. In excised inside-out patches, capsaicin potentiates the PKA-phosphorylated, ATP-dependent CFTR activity. Both capsaicin and genistein potentiate the cAMP-stimulated G551D-CFTR, ΔF508-CFTR, and 8SA mutant channel currents. The binding site for capsaicin is probably located at the cytoplasmic domain of CFTR, because pipette application of capsaicin fails to potentiate CFTR activity. In conclusion, capsaicin is a partial agonist of genistein in activation of the CFTR chloride channel. Both compounds affect ATP-dependent gating of CFTR.

Cystic fibrosis transmembrane conductance regulator (CFTR) is a 169-kDa protein that shows topological homology to the members of ATP-binding cassette transport proteins (Riordan et al., 1989). Members of this family contain two membrane-spanning domains and two nucleotide-binding domains (NBD1 and NBD2) that couple the energy of ATP binding/hydrolysis to their functions. A unique feature of CFTR is that it has a regulatory (R) domain with multiple consensus sequences for phosphorylation by protein kinase A (PKA) and protein kinase C (PKC). The structural motifs that define NBDs include highly conserved Walker A and B motifs and a signature sequence that is believed to be involved in physical contacts between NBDs and the bound ATP molecules. For CFTR to function as a chloride channel, the R domain has to be phosphorylated first by PKA (perhaps by PKC). The phosphorylated channel, however, will not open unless hydrolysable nucleoside triphosphates are present (Gadsby and Nairn, 1999; Aleksandrov et al., 2000).

CFTR is a medically important transporter protein. Defective function of CFTR is responsible for cystic fibrosis (CF), the most common lethal autosomal recessive disorder in white persons (Riordan et al., 1989; Welsh and Smith 1993; Tsui and Durie, 1997). These malfunctions result in defective chloride transports across the epithelial cells in various tissues such as respiratory, gastrointestinal, hepatobiliary, and reproductive tracts (Quinton, 1990). Although years of functional characterization of CFTR have resulted in a better

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ABBREVIATIONS: CFTR, cystic fibrosis transmembrane conductance regulator; CF, cystic fibrosis; CHO, Chinese hamster ovary; PKA, protein kinase A; PKC, protein kinase C; R, regulatory; DMSO, dimethyl sulfoxide; CPT, 8-(4-chlorophenylthio); NMDG, *N*-methyl-D-glucamine; NBD, nucleotide-binding domain; VR1, vanilloid receptor; kb, kilobase(s); NS004, 5-trifluoromethyl-1-(5-chloro-2-hydroxyphenyl)-1,3-dihydro-2H-benzimidazole-2-one; I-V, current-voltage.

understanding of how CFTR activity is regulated (Gadsby and Nairn, 1999), a therapeutic strategy targeted to rectify CFTR dysfunction remains to be established. On the other hand, hyperactivity of CFTR causes secretory diarrhea, which debilitates millions of people in developing countries (Bhattacharya, 1995). Therefore, searching for reagents that can modify the activity of CFTR is an active area of research (Hwang and Sheppard, 1999).

Capsaicin, the main pungent ingredient in chili peppers, causes a sensation of burning pain (Caterina and Julius, 2001). Capsaicin binds to the vanilloid receptor (VR1) in the sensory neuron and activates ligand-gated cation channels producing an influx of cations, such as Na^+ and Ca^{2+}. Functional studies of VR1 expressed in cell lines or *Xenopus laevis* oocytes demonstrate that the receptor itself works as a nonselective cation channel that is stimulated by capsaicin or temperature in the noxious range (Oh et al., 1996; Caterina et al., 1997). In addition to these well-known effects on sensory nerves, capsaicin has been used as a diarrhea-inducing reagent in in vitro systems (Moriarty et al., 2001). This latter effect of capsaicin may be independent of its action on nerve cells (Miller et al., 2000). The topology of VR1 shows a characteristic transmembrane domain that contains six putative α-helices. It is interesting that its cytoplasmic domains contain Walker A and B sequences. Electrophysiological studies of the cloned VR1 indeed demonstrate that cytoplasmic ATP increases VR1 channel activity. Disrupting the Walker A or B sequence by site-directed mutagenesis abolishes this ATP-dependent modulation of VR1 function, indicating that ATP probably interacts with these structural motifs (Kwak et al., 2000).

Because CFTR and VR1 share structural similarities in the cytoplasmic domains and both channels are regulated by ATP, we hypothesized that capsaicin will activate CFTR-channel function. We examined the effects of capsaicin on wild-type and mutant CFTR expressed in mammalian cell lines using both whole-cell and single-channel patch-clamp techniques. Like genistein, a well-characterized CFTR activator, capsaicin only potentiates CFTR-channel activity after the channel is activated first via the cAMP signal-transduction cascade. Single-channel kinetic analysis of wild-type CFTR channels shows that the mechanism of capsaicin's potentiation is mediated by an increase of the opening rate and a decrease of the closing rate. Because capsaicin potentiates wild-type CFTR in excised inside-out patches and also increases the activity of a constitutively active ΔR-CFTR in the cell-attached mode, we conclude that capsaicin, like other known CFTR activators, directly affects ATP-dependent gating. Pharmacological implications, derived from the current model of CFTR gating, are discussed. Our exploration of the mechanisms involved in CFTR activation may contribute to a better understanding of CFTR pharmacology.

Materials and Methods

Cell Culture (Stable Cell-Line). NIH 3T3 cells stably expressing wild-type CFTR (NIH3T3/CFTR) or ΔF508-CFTR channels and C127 cells stably expressed ΔF508-CFTR channels were grown as described previously (Berger et al., 1991) at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. For whole-cell and cell-attached experiments, cell suspensions were prepared with trypsinization (0.25% trypsin and 1 mM EGTA in phosphate-buffered saline). For excised inside-out experiments, cells were grown on small glass chips in a 35-mm Petri dish 1 to 2 days before use. NIH 3T3 and C127-ΔF508 cells were incubated at 27°C for 2 to 3 days before use.

Construction of CFTR Mutants. The plasmid pTM 8SA CFTR was a gift from Dr. Michael Welsh (University of Iowa, Iowa City, IA). In the 8SA mutant construct, individual serine residues were substituted with alanine at amino acid positions 660, 686, 700, 712, 737, 768, 795, and 813. The plasmid pBQG551D CFTR was generously provided by Dr. Mitchell Drumm (Case Western Reserve University, Cleveland, OH). The 2.7-kb BspEI-PflMI fragments containing 8SA and G551D from the plasmids described above were exchanged with the corresponding ones in the pcDNA3.1 wild-type CFTR (Powe et al., 2002) to obtain the 8SA and G551D pcDNA3.1, respectively. The plasmids, pGEMHE-1-633 and pGEMHE-837-1480, were kindly provided by Dr. David Gadsby (Rockefeller University, New York, NY). To generate pBudCE4.1 CFTR1-633, the 2-kb PstI-XhoI fragment from pGEMHE1-633 was subcloned into PstI and SalI sites of pBudCE4.1 expression vector (Invitrogen, Carlsbad, CA). The 1.9-kb KpnI-XhoI fragment from pGEMHE837-1480 was then ligated to KpnI and XhoI sites of pBudCE4.1 CFTR1-633. The resultant construct, pBudCE4.1 split ΔR-CFTR, controls the expression of the N-terminal half of CFTR (amino acids 1–633) under CMV promoter and the expression of the C-terminal half of CFTR (amino acids 837-1480) under human elongation factor 1α promoter. All constructs were confirmed by automated sequencing (DNA Core, University of Missouri, Columbia, MO).

Transient Expression of Wild-Type and Mutant CFTR. To transiently express wild-type or mutant CFTR, CHO cells or NIH 3T3-naive cells were grown in 35-mm tissue culture dishes 1 day before transfection. The plasmid pBudCE4.1 split ΔR-CFTR and pcDNA3.1 constructs containing wild-type, 8SA, or G551D CFTR cDNA were cotransfected with pEGFP-C3 (BD Biosciences Clontech, Palo Alto, CA) encoding green fluorescent protein using SuperFect transfection reagent (QIAGEN, Valencia, CA) according to manufacturer's protocols. The cells were incubated at 27°C for 2 to 3 days before use.

Whole-Cell Experiments, Asymmetrical Cl^- Condition. Pipette electrodes were made from Corning 7056 glass capillaries (Warner Instrument, Hamden, CT). The pipette resistance was ~3 MΩ in the bath solution. The membrane potential was held with EPC9 amplifier (HEKA, Lambrecht/Pfalz, Germany) at 0 mV after break-in with suction and compensation of liquid junction potential (Figs. 1 and 6). Ramp voltages (±100 mV, 2 s in duration) were generated with Pulse software (HEKA) to create current-voltage (I-V) relationships. Currents traces were filtered at 1 kHz with a built-in four-pole Bessel filter (Warner Instrument) and then digitized to the computer at a sampling rate of 2 kHz. The currents were recorded at room temperature (~25°C). The pipette solution contained 85 mM aspartic acid, 10 mM EGTA, 20 mM tetraethylammonium-chloride, 10 mM MgATP, 2 mM $MgCl_2$, 5.5 mM glucose, and 10 mM HEPES, pH 7.4, with CsOH. The bath solution contained 119 mM NaCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM glucose, and 5 mM HEPES, pH 7.4 with NaOH. Sucrose (20 mM) was added to the bath solution to prevent the activation of swelling-induced currents.

Whole-Cell Experiments, Symmetrical Cl^- Condition. The membrane potential was held at 0 mV, and repetitive ramp pulses (±100 mV, 2 s in duration) were applied every 6 or 10 s (Figs. 4 and 7). The pipette solution contained 10 mM EGTA, 121 mM tetraethylammonium-chloride, 10 mM MgATP, 2 mM $MgCl_2$, 5.5 mM glucose, and 10 mM HEPES, pH 7.4, with CsOH. Other conditions were the same as in the protocols using the asymmetrical Cl^- condition.

Single-Channel Experiments. Single-channel CFTR currents were recorded at room temperature (~25°C) with a patch-clamp amplifier (EPC10, HEKA) and stored on videotapes. Data were filtered at 100 Hz with an eight-pole Bessel filter (Warner Instrument) and captured onto a hard disk at a sampling rate of 500 Hz. Patch-clamp electrodes were made from Corning 7056 glass capillaries (Warner Instrument). The pipette resistance was usually 3 to 5 MΩ, and seal resistance was at least 20 GΩ. The pipette solution contained 140 mM *N*-methyl-D-glucamine chloride (NMDG-Cl), 2 mM $MgCl_2$, 5 mM $CaCl_2$, and 5 mM HEPES, pH 7.4, with NMDG. For cell-attached experiments, the bath

solution contained 145 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 5 mM glucose, and 5 mM HEPES, pH 7.4, with NaOH. For excised inside-out patches, the bath solution contained 150 mM NMDG-Cl, 2 mM $MgCl_2$, and 5 mM Trizma base, pH 7.4, with NMDG.

Reagents. Forskolin, purchased from Alexis Corporation (Läufelfingen, Switzerland), was stored as 20 mM stock in dimethyl sulfoxide (DMSO) at 4°C. Capsaicin and genistein were purchased from Sigma Chemical (St. Louis, MO) and stored as 100 mM stock in DMSO at −20°C. DMSO did not affect the CFTR currents at a concentration lower than 0.05% (data not shown). 8-(4-chlorophenylthio) (CPT)-cAMP, purchased from Sigma, was stored as 50 mM stock in water at −20°C.

Statistics. In cell-attached patches, the steady-state mean current amplitudes and unitary amplitude were measured with Igor software (Wavemetrics, Lake Oswego, OR). Single-channel P_o was calculated from patches containing few channels (number of current steps <4) by dividing the mean current amplitude by the single-channel current amplitude and the number of maximal current steps in the presence of genistein. Current traces were baseline-corrected, idealized, and fitted to a three-state model:

$$C \underset{r_{OC}}{\overset{r_{CO}}{\rightleftarrows}} O \underset{r_{BO}}{\overset{r_{OB}}{\rightleftarrows}} B$$

where O and C are open and closed states, respectively, B is a blocked state induced by an intrinsic blocker (Zhou et al., 2001), and r_{CO}, r_{OC}, r_{OB}, and r_{BO} are corresponding rate constants.

This kinetic scheme was used previously by Csanady et al. (2000) to model CFTR gating. This model combines principal gating transitions into a closed-open step and incorporates flickery closures as a pore-blocking event (Ishihara and Welsh, 1997; Zhou et al., 2001). Rate constants r_{CO}, r_{OC}, r_{OB}, and r_{BO} are extracted by a simultaneous fit to the dwell-time histograms of all conductance levels. Mean interburst, burst durations, and channel P_o values were calculated as $\tau_{ib} = 1/r_{CO}$, $\tau_b = (1/r_{OC})(1 + r_{OB}/r_{BO})$, $P_o = 1/(1 + r_{OC}/r_{CO} + r_{OB}/r_{BO})$, respectively. All values are presented as mean ± S.E.M. Student's t test was performed with Sigma Plot (SPSS Science, Chicago, IL). $P < 0.05$ was considered significant.

Results

Enhancement of Wild-Type CFTR-Channel Currents with Capsaicin. The potency and efficacy of capsaicin on wild-type CFTR channel were quantified using whole-cell patch-clamp recordings. Because effects of genistein have been extensively studied, we used genistein as an internal reference. First, the cAMP-dependent chloride currents were elicited in NIH 3T3 cells stably expressing wild-type CFTR with a cocktail containing 10 μM forskolin and 100 μM CPT-cAMP, both of which are maximally effective concentrations for CFTR activation (Hwang et al., 1997; Al-Nakkash and Hwang, 1999). Effects of capsaicin on cAMP-stimulated

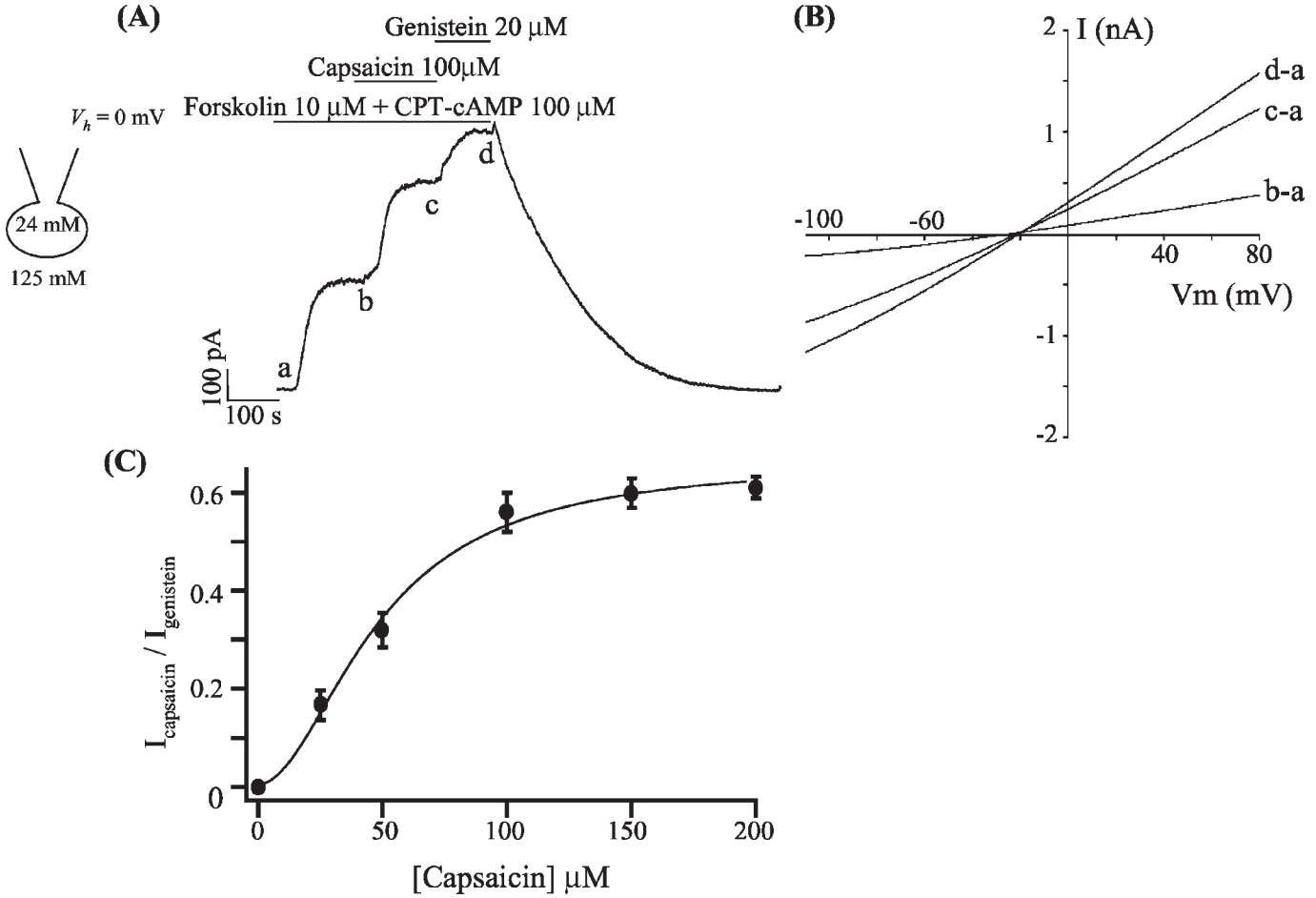

Fig. 1. Effects of capsaicin on cAMP-stimulated whole-cell CFTR currents. A, a continuous whole-cell current trace in the presence of various agonists. B, the I-V relationship of net CFTR currents as marked in A. C, a dose-response relationship of capsaicin on cAMP-stimulated currents. Whole-cell currents at each concentration of capsaicin were normalized to the current level with 20 μM genistein ($n > 5$ for each concentration). Solid line represents a fit with the Hill equation $y = y_{max}/[1 + (EC_{50}/x)^{n_H}]$, where y = response, y_{max} = maximal response; x = concentration of capsaicin; EC_{50} = half-maximal effective dose, and n_H = Hill coefficient. Error bars represent S.E.M. The numbers in the figure represent the Cl^- concentrations in the pipette solution or the perfusion solution.

CFTR currents were compared with those of 20 μM genistein, a maximally effective concentration for genistein on CFTR currents (Al-Nakkash et al., 2001). Figure 1A shows that 100 μM capsaicin increases the cAMP-stimulated CFTR currents by more than 2 fold (2.24 ± 0.11, n = 10). Replacement of capsaicin with 20 μM genistein further increased the currents by 33.5 ± 1.6% (n = 10). Even at a saturating concentration of capsaicin, 20 μM genistein always elicited higher CFTR currents in the same cell, indicating that the efficacy of capsaicin is lower than that of genistein. Similar results were obtained using CHO cells transiently transfected with wild-type CFTR (Fig. 4C). Figure 1B shows the effect of capsaicin and genistein on the I-V relationships. Capsaicin (100 μM) increased the amplitude of macroscopic CFTR currents over a ±100-mV range with negligible effect on the reversal potential.

Figure 1C shows the dose-response relationship of capsaicin on whole-cell cAMP-stimulated CFTR current. CFTR currents potentiated at each concentration of capsaicin were normalized to the current level obtained with 20 μM genistein in the same cell. A fit to the Hill equation yielded a concentration required for half-maximal potentiation of 48.35 ± 6.84 μM (EC_{50}) and a Hill coefficient of 1.92 ± 0.45. Thus, compared with genistein, capsaicin has ~10-fold lower potency and 40% lower efficacy.

Effects of Capsaicin Depends on Prior cAMP Stimulation. Because the CFTR is activated through the cAMP/PKA cascade and the cAMP production with forskolin is saturated at concentration around 10 μM (Illek et al., 1995), the potentiation of capsaicin on CFTR currents is not likely through an increased level of cellular cAMP. To further test whether capsaicin can stimulate the cAMP pathway and thereby promote CFTR phosphorylation, we examined effects of capsaicin in the absence of forskolin. It has been shown previously that NIH 3T3/CFTR cells exhibit a wide variation of basal CFTR activity in the absence of cAMP agonists presumably because of differences in basal production of cAMP among cells (Yang et al., 1997). Figure 2A shows a CFTR current trace in a cell-attached patch from a cell with significant basal activity in the absence of forskolin. Capsaicin (100 μM) enhanced the mean basal CFTR currents by ~3.0 fold (3.0 ± 1.4, n = 3). Removal of capsaicin returned the currents to the control level. On the contrary, capsaicin, when applied alone, had no effect if the cell showed negligible basal CFTR activity (n = 4) (Fig. 2B). However, in the same cell, once CFTR was activated with forskolin first, capsaicin increased the forskolin-dependent current further. These results thus indicate that capsaicin is not capable of raising cellular cAMP-level and only acts on CFTR that is already activated by PKA-dependent phosphorylation. Of note, this action of capsaicin is very similar to that of genistein.

One unique feature about genistein's effects on CFTR is that the magnitude of genistein's potentiation effect is determined by the level of cAMP stimulation. Genistein has a much larger effect when CFTR is activated with a submaximal concentration of forskolin (Hwang et al., 1997). This unique feature of pharmacological modulation of CFTR is also observed for capsaicin. Figure 3 shows two representative experiments carried out in cells that show minimal basal CFTR activity. The potentiation with 100 μM capsaicin in the presence of 10 nM forskolin is significantly greater than that activated with 10 μM forskolin (Fig. 3B).

Effects of Capsaicin on CFTR Mutated at PKA Consensus Serines. The inverse relationship between the magnitude of potentiation by capsaicin or genistein and the level of cAMP stimulation can be explained by a mechanism that these two reagents inhibit protein phosphatases involved in dephosphorylation of CFTR (Reenstra et al., 1996). On the other hand, these reagents may work through a phosphorylation-independent mechanism, but their actions on CFTR

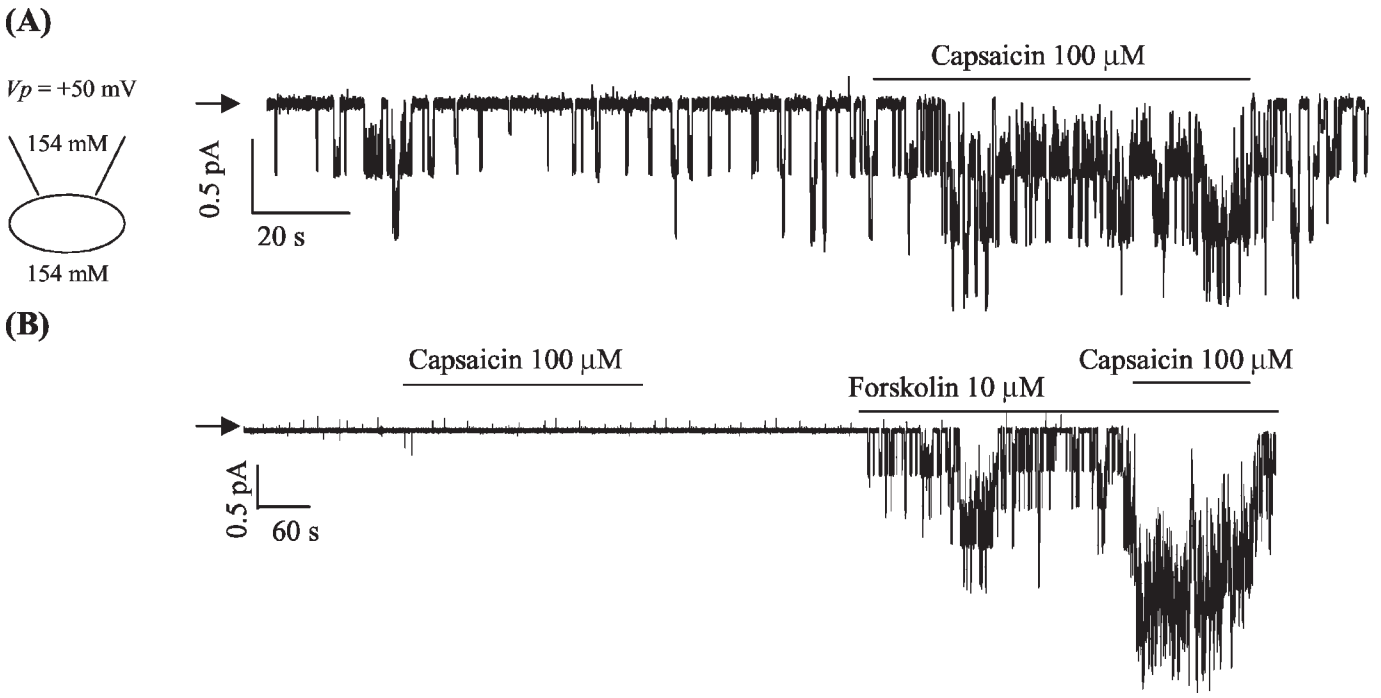

Fig. 2. Effects of capsaicin on CFTR activity with or without basal activity. A, capsaicin potentiated basal CFTR activity. B, capsaicin did not activate CFTR in a patch that shows negligible basal activity. In the same patch, capsaicin can enhance the cAMP-stimulated currents.

activation depend on the state of the channel (see *Discussion* for details). We further explored the action of capsaicin using CFTR mutants whose eight major PKA consensus serines are substituted with alanine (S660A, S686A, S700A, S712A, S737A, S768A, S795A, and S813A), so called S-oct-A or 8SA. This mutant has been shown to present lower channel activity than wild-type CFTR (Rich et al., 1993). We compared the fold increase of whole-cell currents with capsaicin or genistein in wild-type and 8SA-CFTR transiently expressed in CHO cells. Figure 4A shows that 8SA-CFTR can be activated with the cAMP cocktail, and subsequent applications of capsaicin or genistein further potentiate the currents. The I-V relationships of 8SA-CFTR currents in the presence of different reagents are shown in Fig. 4B. The magnitude of potentiation by capsaicin or genistein is significantly greater in 8SA than in wild-type CFTR (Fig. 4C). These results strongly suggest that protein dephosphorylation of these eight major PKA phosphorylation sites is not involved in the action of these two reagents.

Direct Effects of Capsaicin on CFTR Gating. Like genistein, capsaicin potentiates CFTR by directly affecting ATP-dependent gating. This conclusion is derived from two pieces of evidence. First, capsaicin increases the activity of ΔR-CFTR in cell-attached patches (Fig. 5, A and B). Because ΔR-CFTR is constitutively active and does not respond to cAMP stimulation (data not shown), the action of capsaicin (and also genistein) is probably on the step(s) downstream of PKA-dependent phosphorylation. Second, capsaicin increases the P_o of wild-type CFTR in excised inside-out patches (Fig. 5C). Wild-type CFTR-channel currents in excised inside-out patches were activated by initial application of exogenous PKA (25 U/ml) and ATP (1 mM). Once the channel activity reaches a steady state, the channel activity remains fairly stable after PKA is removed in the continued presence of 1 mM ATP, as reported previously (Zeltwanger et al., 1999). Subsequent application of capsaicin potentiated the CFTR activity. Replacement of capsaicin with genistein further increased the channel activity, again indicating that genistein is more effective than capsaicin. Figure 5D summarizes the fold increase of the mean current in the presence of 1 mM ATP in the excised inside-out patches.

Effects of Capsaicin on Single-Channel Kinetics. We next examined the effects of capsaicin on CFTR gating kinetics in the cell-attached mode. In patches containing fewer channels (maximal current steps ≤4) (Fig. 6A), we analyzed single-channel kinetics with a program developed by Dr. Csanady using the scheme under *Materials and Methods*. Calculated parameters are summarized in Fig. 6B. As reported previously (Hwang et al., 1997), 20 μM genistein prolonged the mean burst durations (τ_b) and shortened the mean interburst durations (τ_{ib}). Whereas capsaicin (100 μM) shortens the τ_{ib} to an extent similar to genistein, the increase of τ_b by capsaicin is smaller than that with genistein. Thus, like genistein, capsaicin increases the P_o of cAMP-stimulated CFTR-channel activity. However, the magnitude of enhancement is smaller than that of genistein, as predicted from whole-cell results (Fig. 1).

P_o was also estimated according the relationship $P_o = I/(N \times i)$, where I = mean currents, N = maximal number of current steps in the presence of genistein, and i = unitary current. Similar to previous results (Al-Nakkash and Hwang, 1999), genistein decreased the unitary current by 9.98 ± 1.16%. Capsaicin also decreased the unitary current by 6.2 ± 0.77%. The calculated P_o values were 0.20 ± 0.02, 0.47 ± 0.09, and 0.61 ± 0.01 for forskolin, forskolin plus capsaicin, and forskolin plus genistein, respectively (n = 5). These model-independent P_o values are very similar to those obtained from single-channel kinetic analysis based on the scheme above, suggesting that, although it is oversimplified, the scheme is a reasonable model for quantitative analysis.

Competition between Capsaicin and Genistein in Wild-Type CFTR. Because both capsaicin and genistein

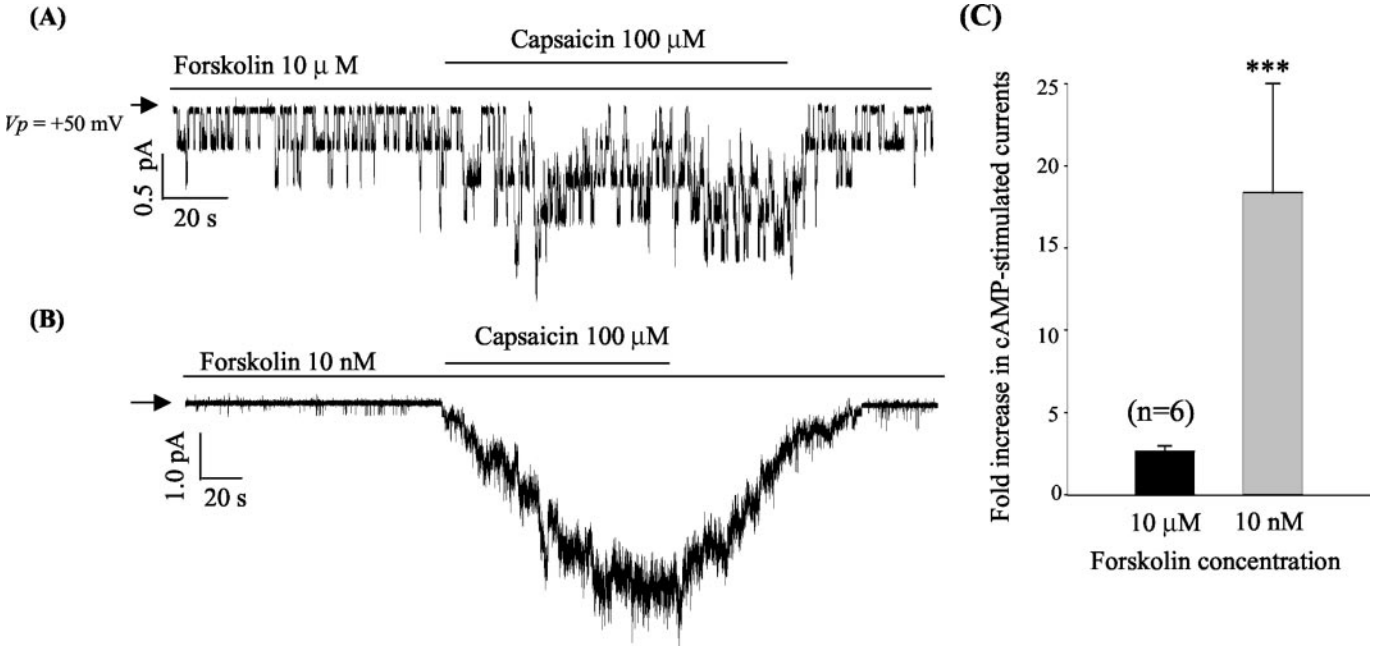

Fig. 3. Effects of capsaicin depend on the level of cAMP stimulation. A, effects of capsaicin on cAMP-stimulated currents with 10 μM forskolin. B, effects of capsaicin on cAMP-stimulated currents with 10 nM forskolin. C, summary of fold increase in cAMP-stimulated mean currents shown in A and B. Error bars represent S.E.M.; ***, $P < 0.001$.

affect wild-type and mutant CFTR in a fairly similar manner except for the extent of potentiation, we hypothesize that these compounds might act on a common binding site. In other words, capsaicin can be considered to be a partial agonist of genistein. If so, these compounds might exhibit competition when applied simultaneously. To test this idea, we carried out the following experiments. As shown in Fig. 7A, whole-cell CFTR-channel currents were first activated by cAMP stimulation. Subsequent application of 200 μM capsaicin or 20 μM genistein enhanced the cAMP-stimulated CFTR activity. However, once the current is maximally activated by cAMP stimulants plus genistein, additional application of capsaicin in the continued presence of genistein inhibited the whole-cell CFTR currents by 11.8 $\pm$ 1.9% (n = 5). Figure 7B shows the effect of capsaicin and genistein on the I-V relationships. It is interesting to note that the whole-cell CFTR conductance in the presence of 20 μM genistein plus 200 μM capsaicin is somewhat in between those in the presence of either compound alone. On the other hand, when the CFTR current is activated with a submaximal concentration of genistein (3 μM), additional application of capsaicin further increases the current (n = 5) (Fig. 7, C and D). These results support the idea that capsaicin and genistein bind to a common binding site but exert differential effects.

Effects of Intrapipette Capsaicin and Genistein. If capsaicin and genistein indeed bind to the same site, where is the binding site? Because, in the cell-attached recordings, a very tight seal between the glass pipette and cell membrane should prevent easy access of the extracellular domain of the channel to bath-applied reagents, we speculated that both capsaicin and genistein might act on a binding site that is located in the cytoplasmic side of the membrane. However, both capsaicin and genistein are fairly lipophilic, and they can easily partition into lipid bilayers to affect channel function (Hwang et al., 2003). Thus, it is possible that the binding site may be located in the bilayer or at the protein-lipid interface. We tested these possibilities by adding 100 μM capsaicin or 20 μM genistein directly into the pipette solution. The idea of a binding site at the protein-lipid interface predicts that capsaicin or genistein in the pipette solution will gain access to the binding site. Thus, application of a cAMP stimulant alone would maximally activate the channel, and further bath application of capsaicin or genistein should not affect the channel activity. This is not the case.

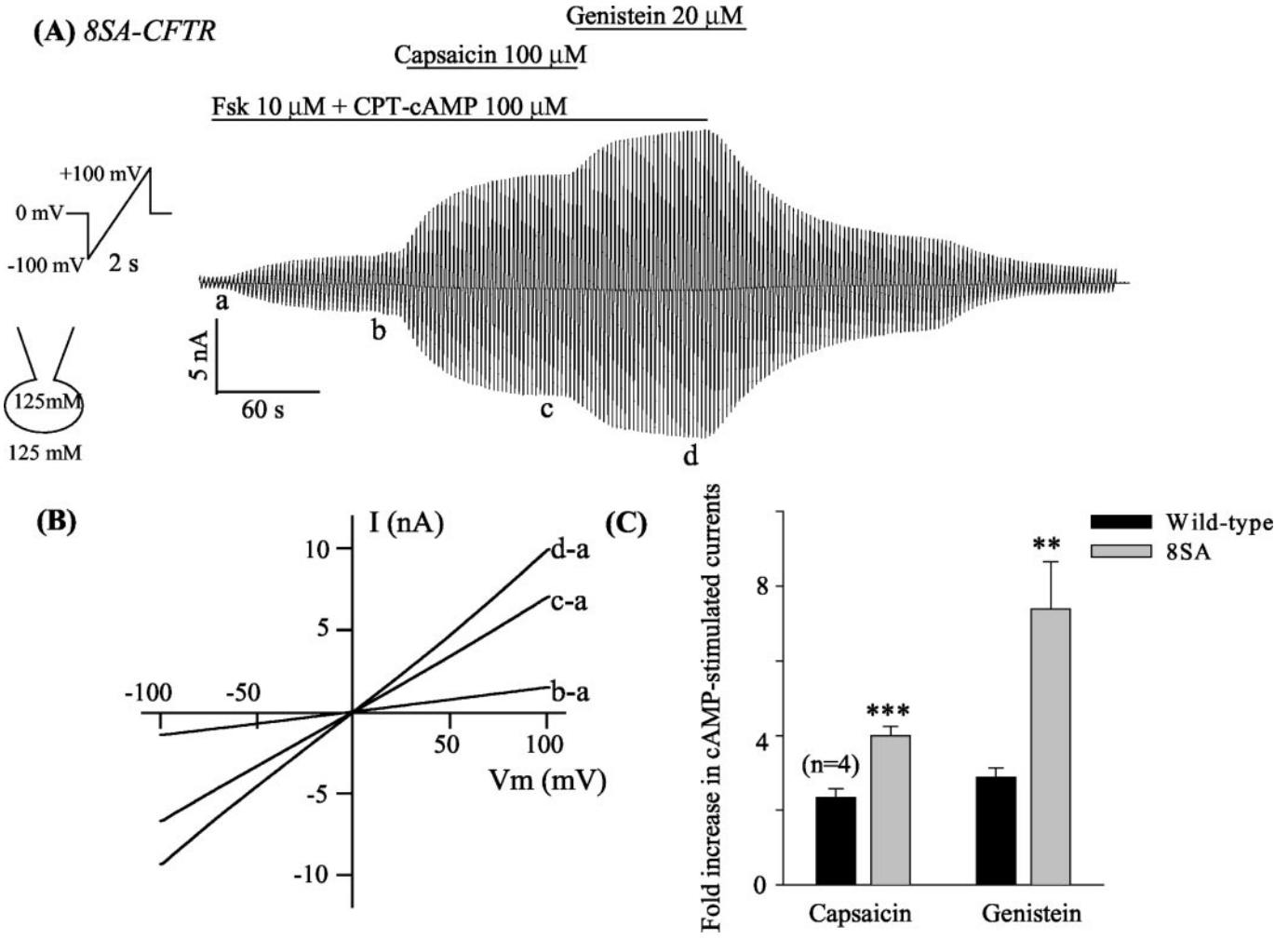

Fig. 4. Capsaicin potentiates mutant CFTR in the PKA-consensus sites (8SA). A, a continuous whole-cell current trace in the presence of various agonists. B, the I-V relationships of net CFTR currents in the presence of different agonists indicated in A. C, summary of fold increase in cAMP-stimulated currents in the presence of different agonists between wild-type and 8SA-mutant CFTR activity. Error bars represent S.E.M.; **, $P < 0.01$; ***, $P < 0.001$ versus wild-type.

After forming a tight seal using a glass pipette filled with pipette solution containing 100 μM capsaicin or 20 μM genistein, CFTR channel activity activated by extracellular perfusion of 10 μM forskolin is very similar to the activity observed in the absence of capsaicin or genistein in the pipette. The cAMP-dependent CFTR activity is further enhanced by bath application of capsaicin or genistein as if the channel has never "seen" these reagents (Fig. 8, A and B). Figure 8C shows that the fold increase of the cAMP-stimulated currents with extracellular-perfused capsaicin or genistein is the same as that without intrapipette reagents. These results suggest that both compounds may act on a site that is located on the cytoplasmic side of the membrane.

Effects of Capsaicin on Disease-Associated Mutant CFTR. The inverse relationship between the prestimulated P_o and the magnitude of potentiation makes many drugs, including genistein, ideal candidates for future therapeutic application because many disease-associated CFTR mutants exhibit extremely low P_o. We therefore tested the efficacy of capsaicin on some of these mutant channels. Figure 9A shows that 100 μM capsaicin increased the cAMP-dependent chloride currents by ~6 fold in NIH 3T3 cells transiently expressing G551D-CFTR. However, in the same cell, 20 μM genistein increased the currents by ~15 fold. On average, the enhancement of G551D-CFTR with capsaicin was ~50% of that with genistein (n = 6) (Fig. 9B). Figure 9C showed the effect of capsaicin and genistein on the I-V relationships. Similar results were obtained in the ΔF508-CFTR stably expressed in NIH 3T3 cells or C127 cells and in CHO cells transiently expressing ΔF508 mutants. Figure 9D shows a typical trace for ΔF508-CFTR, and the inset shows the corresponding I-V curves. The average ratio of ΔF508-CFTR currents with capsaicin over those with genistein was 60.5 ± 4.4% (n = 18). It is interesting to note that not only does the inverse relationship between the prestimulated P_o and the magnitude of potentiation hold for these CFTR mutants, the different extents of potentiation by these two reagents also are consistent with the results obtained in the wild-type CFTR (Fig. 1).

Discussion

In this study, we demonstrate that capsaicin potentiates the activity of wild-type and mutant CFTR chloride channels, including the two most common disease-associated mutants. Capsaicin's actions on CFTR are very similar to those of genistein, a well-characterized CFTR activator (Hwang et al., 1997). In particular, capsaicin acts only on CFTR channels that are activated through the cAMP-signaling pathway (or the constitutive ΔR-CFTR). The magnitude of potentiation is inversely proportional to the P_o of the channel before drug application. At the single-channel level, both capsaicin and genistein affect ATP-dependent gating of CFTR by increasing the opening rate and decreasing the closing rate. However, the maximal effect of capsaicin is ~60% of that of genistein. Experimental results obtained from competitive protocols suggest that these two compounds may share a common binding site on the cytoplasmic side of the membrane.

How Capsaicin Activates the CFTR. CFTR is regulated by phosphorylation/dephosphorylation of the R domain that contains multiple consensus sites for PKA- and PKC-dependent phosphorylation. PKA-dependent phosphorylation is a

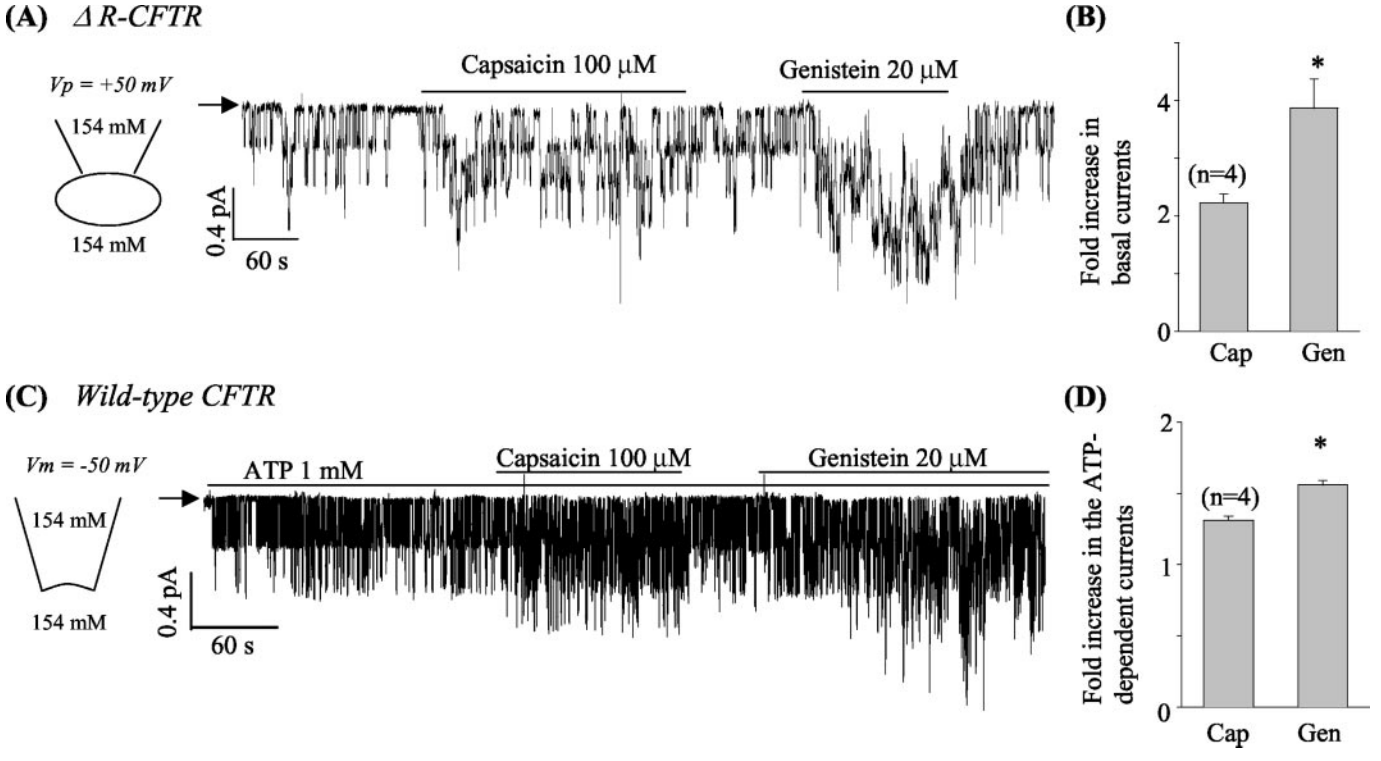

Fig. 5. Capsaicin affects CFTR gating. A, a cell-attached single-channel recording of ΔR-CFTR–channel currents in the presence of different agonists. Notice that ΔR-CFTR–channel currents were activated without any cAMP stimulants. B, summary of fold increase in basal ΔR-CFTR activity in the different agonists. C, an excised inside-out single-channel recording of wild-type CFTR in various conditions. CFTR-channel currents were initially activated by PKA (25 U/ml) and ATP (1 mM). D, summary of fold increase in the ATP-dependent CFTR-channel currents in the presence of different agonists. Error bars represent S.E.M.; *, $P < 0.05$.

prerequisite for channel opening and closing by ATP binding/hydrolysis at the NBDs (Gadsby and Nairn, 1999). In theory, CFTR activity in an intact cell can be enhanced by pharmacological tools that alter kinase activity such as forskolin, phosphodiesterase inhibitors, and CPT-cAMP, or agents that inhibit protein phosphatase activity (Hwang and Sheppard, 1999).

Genistein has been extensively studied as a potential candidate for a CFTR activator because it is a nature-occurring phytoestrogen and has been implied to have beneficial effects on human health (Dixon and Ferreira, 2002). Because genistein can potentiate ATP-dependent CFTR gating in excised inside-out patches, it has been proposed that the molecular target for genistein is the CFTR protein itself (French et al., 1997; Weinreich et al., 1997; Wang et al., 2000). Some biochemical evidence indeed supports the notion that genistein may interact at least with the NBD2 of CFTR (Randak et al., 1999; Howell et al., 2000).

In the present study, we showed that capsaicin and genistein share many similarities with regard to CFTR activation. First, neither reagent, by itself, activates CFTR before cAMP stimulation. Second, in the presence of cAMP agonists, capsaicin increases the P_o of CFTR through effects on both the opening rate and the closing rate. Third, the magnitude of capsaicin's potentiation effects is inversely proportional to the prestimulated activity of CFTR. Fourth, both compounds affect CFTR only from the cytoplasmic side of the membrane. Fifth, both compounds potentiate several different mutant CFTR channels including 8SA, G551D, ΔF508, and ΔR-CFTR. In all cases, the maximal effect of capsaicin is 50 to 60% of that with genistein. Thus, although these two compounds share little structural similarities, viewed from the perspective of CFTR activators, capsaicin can be considered a partial agonist for genistein. Our experiments using competitive protocols indeed show an apparent competition between capsaicin and genistein. Taken together, our results suggest that capsaicin and genistein may bind to a common binding site that can accommodate compounds with significant structural differences.

Where Is the Binding Site? Because hydrophobic compounds like genistein can easily partition into lipid bilayers (Arora et al., 2000; Hwang et al., 2003), it is conceivable that these compounds can change the physical property of lipid bilayers. Any functional perturbation of the membrane proteins is secondary to their effects on bilayer properties (Hwang et al., 2003). Because capsaicin is also fairly hydrophobic (log P values, 4.00), it may also diffuse readily into membrane lipids. Another possibility is that the drug binding site may be located at the protein-lipid interface. In either case, capsaicin and genistein should affect CFTR function when applied from either the extracellular or the cytoplasmic side of the membrane. Our data strongly suggest that the binding site is only accessible from the cytoplasmic side. The fact that both capsaicin and genistein have equivalent effects on the ΔR-CFTR whose R domain is completely removed indicates that the binding site cannot be in the R domain nor

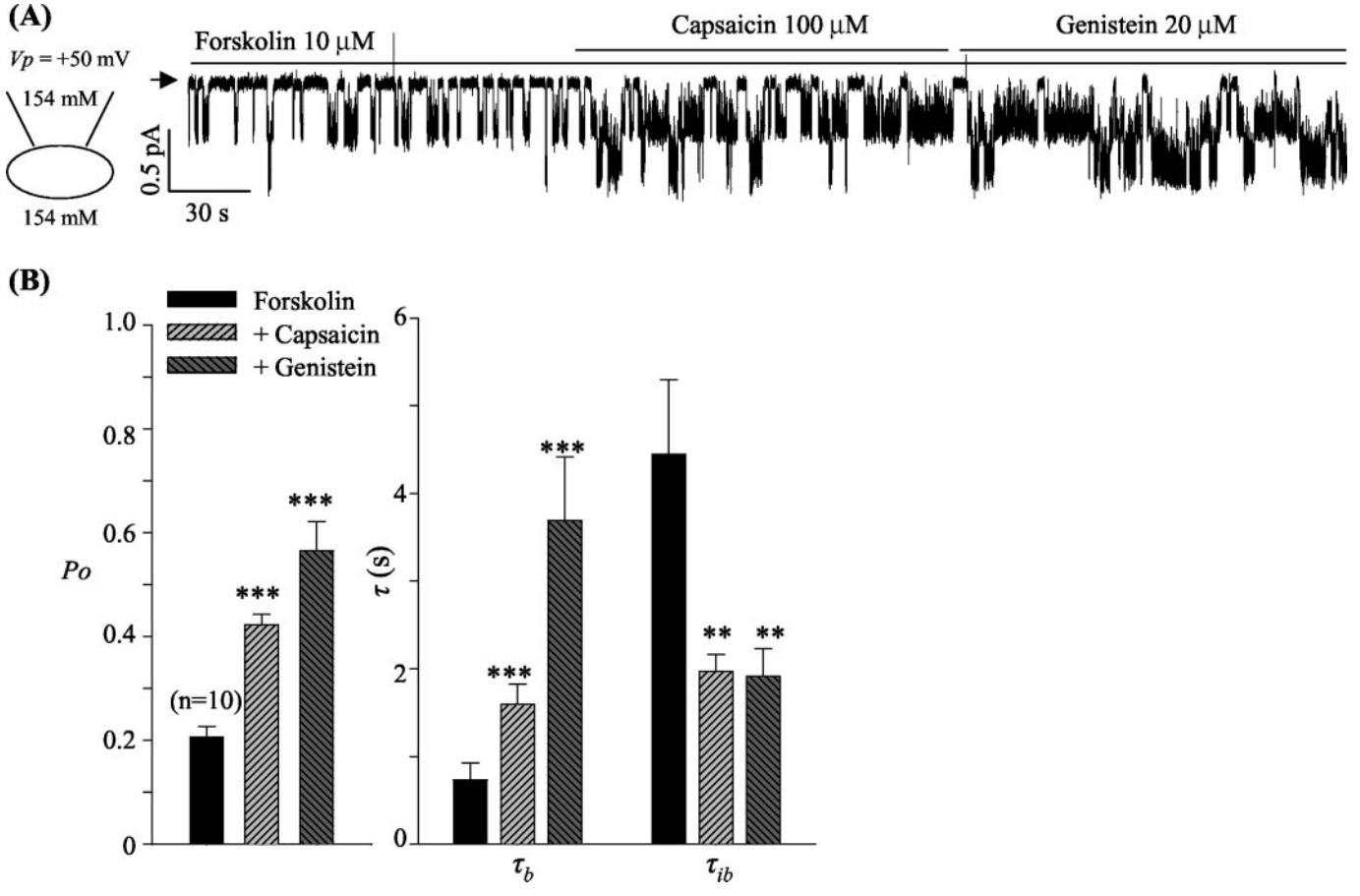

Fig. 6. Single-channel kinetic analysis of CFTR in the presence of capsaicin or genistein. A, a sample trace of CFTR activity in the presence of various agonists in the cell-attached patch. B, single-channel P_o, mean burst durations (τ_b), and mean interburst durations (τ_{ib}) were obtained with a program developed by Dr. Csanady (see *Materials and Methods* for details). All values are represented by mean ± S.E.M.; **, $P < 0.01$; ***, $P < 0.001$ versus forskolin.

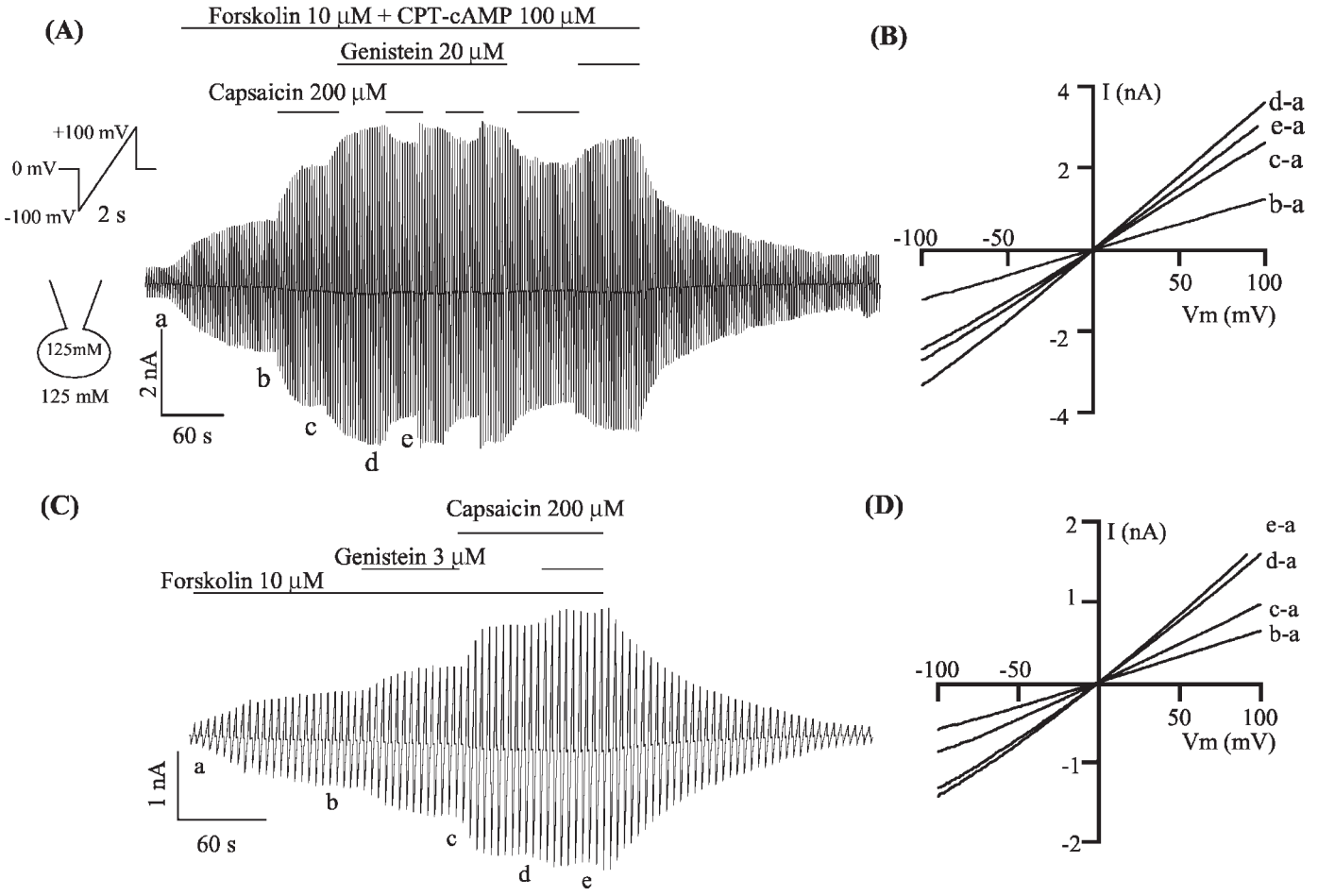

Fig. 7. Competition between capsaicin and genistein. A, a whole-cell wild-type CFTR activity in various conditions. B, the I-V relationships of net CFTR-channel currents in various conditions as indicated in A. C, capsaicin increases CFTR-channel currents in the presence of a submaximal concentration of genistein. D, the I-V relationships in the presence of various reagents as marked in C.

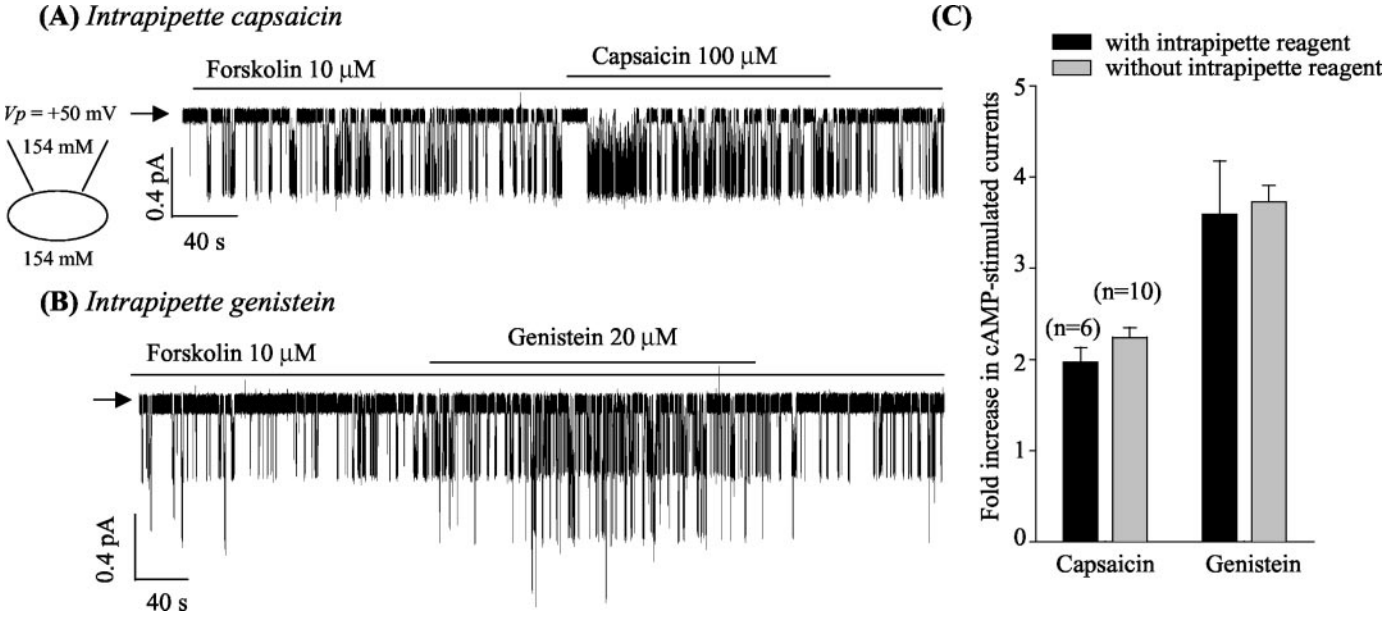

Fig. 8. Both capsaicin and genistein affect CFTR from the cytoplasmic side. Effects of intrapipette capsaicin (A) or genistein (B) on the single-channel wild-type CFTR activity in the cell-attached patch. C, summary of fold-increase of NP_o in the cAMP-stimulated CFTR-channel currents in different conditions. Error bars represent S.E.M. Notice that there is no significant difference between the fold increase with intrapipette reagents and that without intrapipette reagents ($P > 0.5$).

at the interface between the R domain and other parts of the CFTR molecule. Because these reagents clearly affect ATP-dependent gating and NBDs serve as the gating machinery for CFTR, we propose that the binding site is located in the NBDs (Randak et al., 1999).

From what we understand about how NBDs control the gating motion of CFTR, we may gain more insights into how gating modifiers like genistein and capsaicin work. Very recently, Vergani et al. (2003) proposed a mechanism linking the gating cycle and ATP-driven dimerization of CFTR's NBDs. This hypothetical model is derived from the observation that in some ATP-binding cassette transporters, two ATP molecules bind at the interface of the NBD dimer (Hung et al., 1998; Hopfner et al., 2000; Chen et al., 2003). Biochemical studies suggest that ATP binding itself facilitates the dimerization reaction (Smith et al., 2002). Crystal structures of the MalK protein reveal a tweezer-like motion between two NBDs upon ATP-binding (Chen et al., 2003). Although the exact molecular motion during CFTR's gating transitions is unknown, it is possible that this dimerization is indeed coupled to the opening and closing of the gate as proposed by Vergani et al. (2003). If one believes that this idea of coupling between gating and NBD dimerization is correct, then one mechanism by which capsaicin or genistein can affect gating is through an effect on NBD dimerization. One can imagine that those gating modifiers bind at the interface of the NBD dimer. By lowering the free energy of the transition state, they can increase the opening rate. Once two NBDs dimerize, these compounds will be sandwiched and could potentially provide extra free energy of interaction between NBDs. Thus, by stabilizing the dimer, these reagents can also decrease the closing rate. On the other hand, because ATP hydrolysis is proposed to be the rate-limiting step for channel closing (Hwang et al., 1994; Zeltwanger et al., 1999; Powe et al., 2002; Vergani et al., 2003), these compounds may reduce the closing rate by decreasing the rate of ATP hydrolysis. This idea of ligand binding at an extensive dimer interface, despite speculation, may explain why the "binding site" can accommodate structurally diverse compounds.

Taking one step further, because many disease-associated CFTR mutants and CFTR channels with insufficient phosphorylation have a slower opening rate that can be greatly increased by genistein and capsaicin, we speculate that the dimerization of NBDs may be the common molecular event that is affected by the mutations as well as by differential phosphorylation.

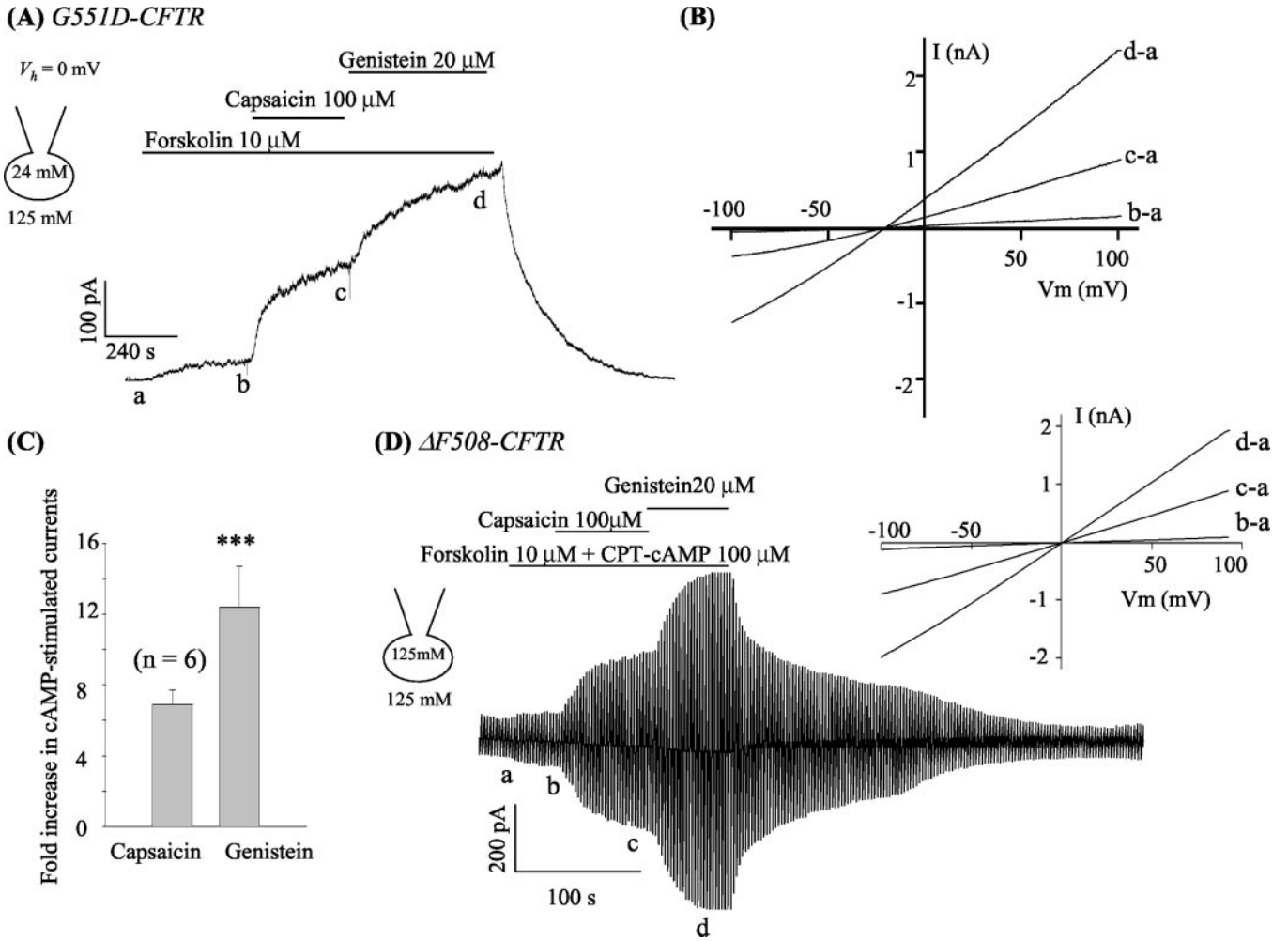

Fig. 9. Potentiation of G551D-CFTR and ΔF508-CFTR by capsaicin and genistein. A, a whole-cell G551D-CFTR activity in the presence of various agonists. B, the I-V relationships of net G551D-CFTR–channel currents in different conditions as indicated in A. C, summary of fold increase in cAMP-stimulated G551D-CFTR activity on different agonists. Error bars represent S.E.M. **, $P < 0.01$ versus capsaicin. D, a whole-cell recording of ΔF508-CFTR transiently expressed in a CHO cell. The inset shows the corresponding I-V relationships.

Our single-channel kinetic analysis indicates that the only difference between capsaicin and genistein is its effect on the closing rate. This difference must reflect the structural differences between these two compounds. Genistein consists of multiple ring structures, and capsaicin has one benzene ring and a very long flexible carbon chain. From the theory proposed above, we speculate that forming a more stable dimer may require those ring structures that are present in genistein but not in capsaicin; or those ring structures may affect the magnitude of inhibition of ATP hydrolysis. Regardless of the exact mechanism, it is interesting to note that NS004, which has been shown to have effects on CFTR gating identical with genistein (Al-Nakkash et al., 2001), also contains multiple rings. Future experiments with structural analogs of capsaicin and genistein and mutations at the NBDs may reveal the biophysical and biochemical mechanisms for the modulation of CFTR gating.

Clinical Implications. One of the important clinical manifestations in CF patients is constipation and its complications. Although those complications are mainly associated with steatorrhea resulting from the deficiency of pancreatic enzyme secretion to digest fat (Rubinstein et al., 1986), the impaired fluid secretion caused by defects of Cl^- transport in the intestinal epithelia also may play a pathophysiological role in gastrointestinal symptoms of CF patients (Gabriel et al., 1994; Binkovitz et al., 1999). On the other hand, capsaicin has been reported to cause diarrhea by increasing fluid secretion accompanied with stimulation of intestinal ion transport (Miller et al., 2000). It has been suggested that the mechanism for capsaicin-activated ion transport may involve stimulations of the sensory neurons to release neurotransmitters such as substance P and neurokinin (Vanner and MacNaughton, 1995; Moriarty et al., 2001). In our study, however, we demonstrate evidence that capsaicin, at the concentration range used for inducing diarrhea, can activate CFTR activity by a direct interaction with the channel protein. Although we cannot rule out other mechanisms involved, our studies suggest that a direct stimulation of CFTR could at least partly account for secretory diarrhea seen in patients with capsaicin intoxication (Snyman et al., 2001). The concept of direct stimulation of intestinal CFTR by orally ingested reagents may contribute to the development of a therapeutic strategy to remedy gastrointestinal disorders of CF patients.

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