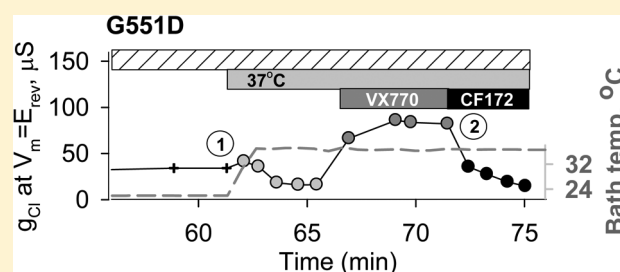


# Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) Potentiators Protect G551D but Not $\Delta$ F508 CFTR from Thermal Instability

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**ABSTRACT:** The G551D cystic fibrosis transmembrane conductance regulator (CFTR) mutation is associated with severe disease in ~5% of cystic fibrosis patients worldwide. This amino acid substitution in NBD1 results in a CFTR chloride channel characterized by a severe gating defect that can be at least partially overcome *in vitro* by exposure to a CFTR potentiator. In contrast, the more common  $\Delta$ F508 mutation is associated with a severe protein trafficking defect, as well as impaired channel function. Recent clinical trials demonstrated a beneficial effect of the CFTR potentiator, Ivacaftor (VX-770), on lung function of patients bearing at least one copy of G551D CFTR, but no comparable effect on  $\Delta$ F508 homozygotes. This difference in efficacy was not surprising in view of the established difference in the molecular phenotypes of the two mutant channels. Recently, however, it was shown that the structural defect introduced by the deletion of F508 is associated with the thermal instability of  $\Delta$ F508 CFTR channel function *in vitro*. This additional mutant phenotype raised the possibility that the differences in the behavior of  $\Delta$ F508 and G551D CFTR, as well as the disparate efficacy of Ivacaftor, might be a reflection of the differing thermal stabilities of the two channels at 37 °C. We compared the thermal stability of G551D and  $\Delta$ F508 CFTR in *Xenopus* oocytes in the presence and absence of CFTR potentiators. G551D CFTR exhibited a thermal instability that was comparable to that of  $\Delta$ F508 CFTR. G551D CFTR, however, was protected from thermal instability by CFTR potentiators, whereas  $\Delta$ F508 CFTR was not. These results suggest that the efficacy of VX-770 in patients bearing the G551D mutation is due, at least in part, to the ability of the small molecule to protect the mutant channel from thermal instability at human body temperature.



The recent demonstration of efficacy of a CFTR potentiator in patients carrying at least one copy of G551D CFTR was a quantum leap for CF therapy, being the first instance of a therapeutic intervention based on a small molecule that directly targets the mutant gene product.<sup>1–4</sup> The potentiator, VX-770, known as Ivacaftor or Kalydeco, did not exhibit similar efficacy in patients homozygous for the more common mutation,  $\Delta$ F508, however.<sup>5</sup> This difference could be attributed to the well-established difference in the molecular phenotypes of the two mutations, namely, a gating defect for G551D and a combined trafficking and gating defect for  $\Delta$ F508,<sup>6</sup> but we wondered if the two mutants might also differ with regard to the more recently established mutant CFTR phenotype of thermal instability.

Results from three laboratories provided strong evidence that the channel function of  $\Delta$ F508 CFTR exhibits severe thermal instability. In *Xenopus* oocytes<sup>7</sup> and HEK cells,<sup>8</sup> conductance due to  $\Delta$ F508 CFTR channels rescued at the surface by low temperature and activated by PKA and ATP rapidly decreased if the temperature was increased to 37 °C, an effect that could be traced to a reduction in open probability. A similar thermal instability was detected in  $\Delta$ F508 CFTR channels reconstituted in planar bilayers.<sup>9,10</sup> This severe gating defect, manifest at temperatures in excess of ~28 °C, was rescued to varying extents by single<sup>7</sup> and multiple<sup>7,8</sup> second-site suppressor

mutations. The apparent disparity in clinical efficacy of VX-770 in compound heterozygotes ( $\Delta$ F508/G551D) carrying one copy of G551D CFTR<sup>2,4</sup> and a G551D homozygote carrying two copies of G551D CFTR,<sup>3</sup> as well as the modest efficacy of VX-770 seen in  $\Delta$ F508 homozygotes, suggested to us that  $\Delta$ F508 CFTR channels and G551D CFTR channels might differ in their thermal stabilities. Might it be, for example, that the well-known trafficking defect seen with  $\Delta$ F508 CFTR is, at least in part, a reflection of thermal instability apparent in a channel function assay, a thermal instability that might be lacking in the normally trafficked G551D channels?

We compared the thermal stability of G551D CFTR channels expressed in *Xenopus* oocytes with that previously reported by us for  $\Delta$ F508 CFTR channels. We found, contrary to our initial expectations, that G551D CFTR channel function was thermally unstable at 37 °C, although G551D CFTR channel behavior differed from that of the  $\Delta$ F508 channels in several important respects. First, thermal deactivation was more rapid, although less complete, than that seen with  $\Delta$ F508 CFTR. Second, following a 37 °C thermal challenge, the conductance due to G551D channels recovered almost fully (85%), in contrast to that seen with  $\Delta$ F508 channels, which

Received: August 12, 2014

Published: August 13, 2014

although variable, was on average 43% of the original conductance. Most importantly, however, G551D CFTR channels appeared to be protected from thermal instability at 37 °C by CFTR potentiators, including VX-770. Furthermore, potentiators also provoked an increase in conductance due to G551D channels at 37 °C, following thermal deactivation, a condition more like that *in vivo*. These results provide a mechanistic basis for the differing efficacy of VX-770 in patients carrying G551D and  $\Delta$ F508 CFTR and have important implications for the design of assays for small molecule screening.

## MATERIALS AND METHODS

**Mutagenesis and *in Vitro* Transcription.** CFTR mutants were generated using a site-directed mutagenesis method similar to those reported previously.<sup>11–13</sup> The sequences in the region of the mutation were confirmed by direct DNA sequencing. CFTR cRNAs for *Xenopus* oocyte injection were synthesized using the mMessage mMachine T7 Ultra transcription kit (Ambion).

**Preparation and Microinjection of Oocytes.** *Xenopus laevis* oocytes were prepared using methods previously described in detail.<sup>11,12</sup> Briefly, oocytes were defolliculated by mechanical agitation (1–2 h) in a Ca<sup>2+</sup>-free solution containing 82.5 mM NaCl, 2 mM KCl, 1 mM MgCl<sub>2</sub>, and 5 mM HEPES (pH 7.5) with 0.2 Wünsch units/mL Liberase Blendzyme 3 (Roche Molecular Biochemicals, Indianapolis, IN) and maintained in a modified Barth's solution containing 88 mM NaCl, 1 mM KCl, 0.82 mM MgSO<sub>4</sub>, 0.33 mM Ca(NO<sub>3</sub>)<sub>2</sub>, 0.41 mM CaCl<sub>2</sub>, 2.4 mM NaHCO<sub>3</sub>, 10 mM HEPES-Hemi-Na, and 250 mg/L Amikacin with 150 mg/L Gentamicin (pH 7.5). Stage V to VI oocytes were injected with CFTR cRNA plus cRNA encoding the human  $\beta_2$ -adrenergic receptor. The CFTR RNA concentration was adjusted so that the maximal steady state-stimulated conductance was less than 200  $\mu$ S (~12.5–25 ng/oocyte).

**Whole-Cell Recordings.** Individual oocytes were continuously perfused with Frog Ringer's solution, which contained 98 mM NaCl, 2 mM KCl, 1 mM MgCl<sub>2</sub>, 1.8 mM CaCl<sub>2</sub>, and 5 mM HEPES-Hemi-Na (pH 7.4). CFTR channels were activated using 10  $\mu$ M isoproterenol (a  $\beta_2$ -adrenergic agonist) and 1 mM IBMX (a phosphodiesterase inhibitor) as the stimulating cocktail (Isop+IBMX) to increase the level of intracellular cAMP.<sup>14,15</sup> The data were acquired using an Oocyte 725 amplifier (Warner) and the pClamp 8 data acquisition program (Molecular Devices, Sunnyvale, CA). Oocytes were maintained under the open circuit condition, and the membrane potential was periodically ramped from –120 to 60 mV over 1.8 s. Data are reported as means  $\pm$  the standard error of the mean, and a *t* test was performed where appropriate.

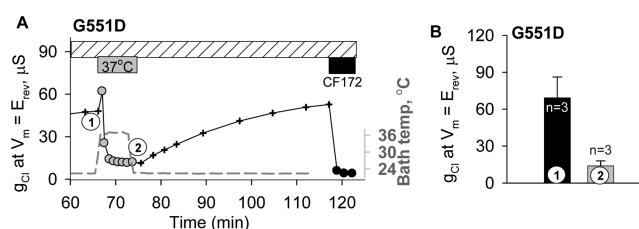
**Temperature Control.** The bath temperature was controlled using a Dual Automatic Temperature Controller (CL-200) and an in-line solution heater/cooler (SC-20) (Warner Instruments, Hamden, CT) and monitored in real time using a thermo probe positioned near the oocytes. The temperature was digitized and recorded using a USB Data Acquisition Device (DI-158, DATAQ Instruments, Inc., Akron, OH). All experiments were begun at room temperature (22–23 °C) unless otherwise specified.

**Reagents.** Isobutylmethyl xanthine (IBMX) and isoproterenol (Isop) were purchased from Sigma (St. Louis, MO). The following compounds were kindly provided by R. Bridges

(Rosalind Franklin University, Chicago, IL) and The Cystic Fibrosis Foundation (CFF): 4-[4-oxo-2-thioxo-3-(3-trifluoromethylphenyl)thiazolidin-5-ylidenemethyl]-benzoic acid (CFTR<sub>inh</sub>-172 or CF172), 2-[(2-1H-indol-3-ylacetyl)-methylamino]-N-(4-isopropylphenyl)-2-phenyl-acetamide (PG-01 or P2), and 4-methyl-2-(5-phenyl-1H-pyrazol-3-yl)-phenol (VRT-532 or P1). Genistein was purchased from TCI America (Portland, OR). N-(2,4-Di-*tert*-butyl-5-hydroxyphenyl)-4-oxo-1,4-dihydroquinoline-3-carboxamide (VX-770 or Ivacaftor) was purchased from Selleck Chemicals (Houston, TX).

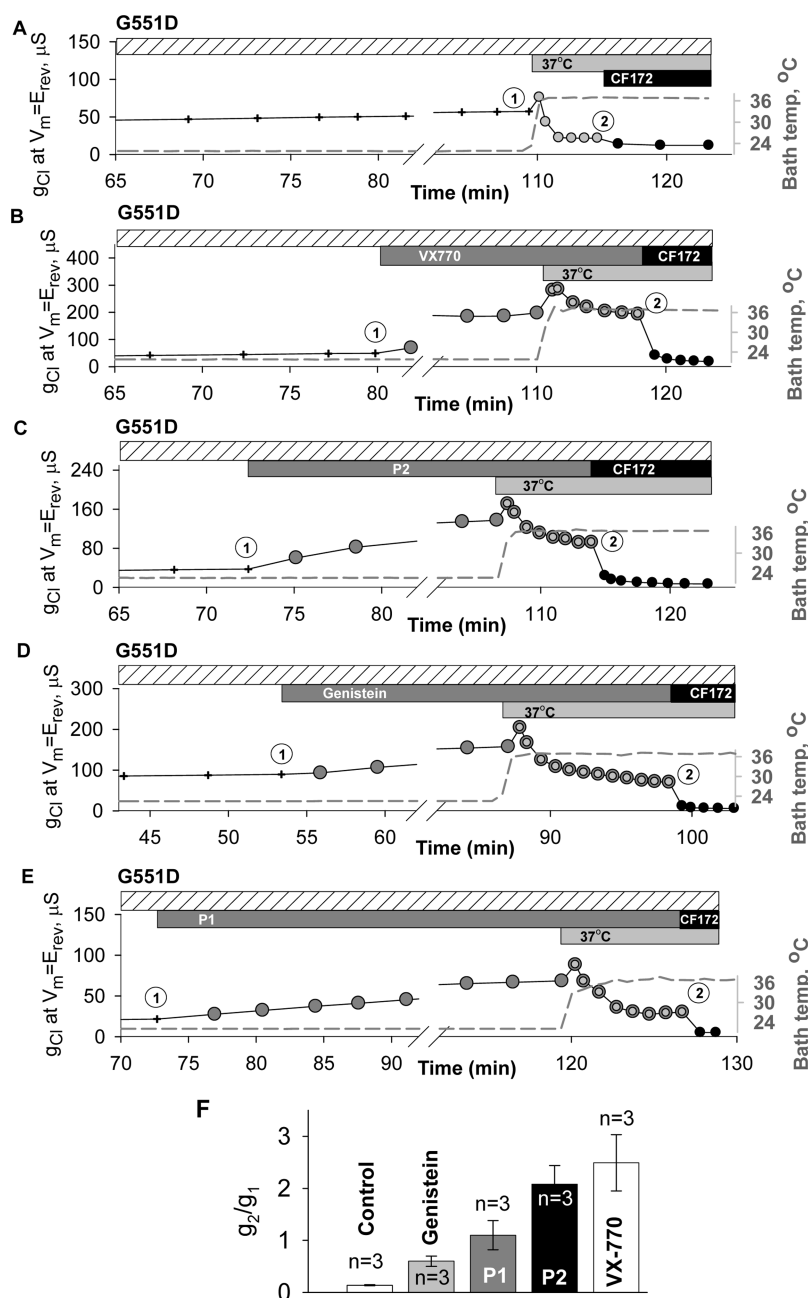
## RESULTS

**G551D Channels Exhibit Thermal Instability.** Figure 1A shows the results of a representative experiment in which an



**Figure 1.** Thermal instability of G551D CFTR channels. (A) Following stimulation [10  $\mu$ M isoproterenol and 1 mM IBMX (hatched bar and crosshairs)], an oocyte expressing G551D CFTR was warmed to 37 °C (gray bar and circles) for 10 min. After recovering at 22 °C, the oocyte was exposed to 10  $\mu$ M CF172. (B) Summary of G551D CFTR conductance before and after thermal deactivation ( $P < 0.05$ ). ① represents the initial conductance following stimulation. ② is the minimal conductance at 37 °C. To save space, the period prior to and during CFTR stimulation by the stimulatory cocktail is not shown. The half-time for G551D CFTR activation averaged  $37.2 \pm 4$  min ( $n = 18$ ), and the half-time for  $\Delta$ F508 CFTR activation under comparable conditions averaged  $7.0 \pm 2.0$  min ( $n = 18$ ). A prolonged half-time for activation by cAMP is a consistent feature of G551D channels and is compatible with the long closed times seen in single-channel recordings.<sup>25</sup>

oocyte expressing G551D CFTR channels was exposed to an increase in temperature from 22 to 37 °C for 10 min. The results of three such experiments are summarized in Figure 1B. The effect of the thermal challenge was qualitatively similar to that we previously reported for  $\Delta$ F508 channels expressed in *Xenopus* oocytes, a rapid, transient increase in conductance followed by a profound reduction, and partial recovery after the bath temperature was returned to 22 °C. However, there was a distinct, quantitative difference between the thermal deactivation of G551D CFTR and that we previously reported for  $\Delta$ F508 CFTR.<sup>7</sup> The rate of thermal deactivation was more rapid for the G551D channels [ $t_{1/2} = 0.3$  min ( $\pm 0.04$ ;  $n = 3$ ) vs  $3.7$  min ( $\pm 0.3$ ;  $n = 11$ ) for  $\Delta$ F508 channels] ( $P < 0.05$ ). More striking, however, was the greater extent of recovery following the thermal challenge,  $85 \pm 15\%$  ( $n = 3$ ) for G551D and  $43 \pm 15\%$  ( $n = 3$ ) for  $\Delta$ F508<sup>7</sup> ( $P < 0.05$ ). The relatively rapid and nearly complete recovery of the G551D channels was reminiscent of that seen with  $\Delta$ F508 channels when the phenylalanine deletion was combined with a nearby, second-site suppressor mutation like I539T ( $\Delta$ F508/I539T CFTR).<sup>7</sup> Upon comparison to contemporaneous experiments with  $\Delta$ F508 CFTR channels (see Figures 3 and 4), it also appeared that the extent of thermal deactivation was lower for G551D channels. The conductance remaining after thermal deactivation, expressed as a percent of the initial, activated steady state



**Figure 2.** Differential effects of CFTR potentiators on the thermal instability of G551D CFTR channels. (A) Following stimulation at 22 °C, an oocyte expressing G551D CFTR was warmed to 37 °C (gray bar and circles) and then exposed to 10  $\mu M$  CF172 at 37 °C. Note the break on the time axis. (B–E) Following stimulation, oocytes expressing G551D CFTR were exposed to 10  $\mu M$  VX-770, 10  $\mu M$  P2 (PG-01), 50  $\mu M$  Genistein, and 10  $\mu M$  P1, respectively, warmed to 37 °C (gray bar and circles) in the presence of potentiators, and then exposed to 10  $\mu M$  CF172 at 37 °C. (F) Summary of the ratio of conductance at the end of warming ( $g_2$ ) and the initial steady state conductance prior to exposure to the potentiators ( $g_1$ ). Doses of the potentiators were chosen on the basis of the maximal concentration reported in the literature as well as our previous study.<sup>7,16,26–28</sup> With respect to the activated conductance prior to the application of potentiator, the *P* values for stimulation by Genistein, P1, P2, and VX770 are all <0.05.

conductance at 22 °C, averaged  $40 \pm 8.1\%$  ( $n = 7$ ) for G551D and  $7 \pm 1.4\%$  ( $n = 6$ ) for  $\Delta F508$ . Taken together, these results suggested that G551D CFTR channels, despite their rapid, thermal deactivation, are less severely impacted by the elevated temperature than  $\Delta F508$  channels.

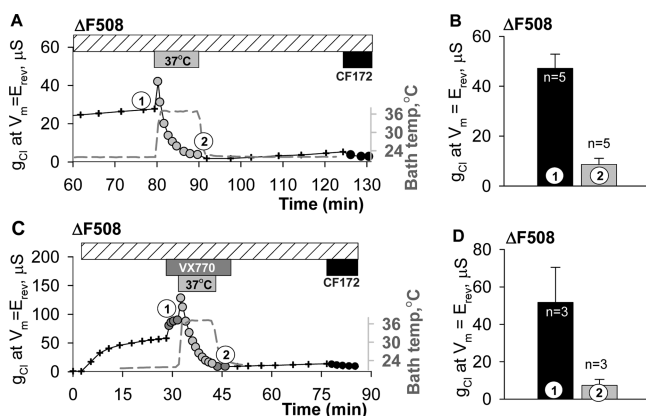
**CFTR Potentiators Protect G551D CFTR Channels but Not  $\Delta F508$  CFTR Channels from Thermal Deactivation.** The unexpected finding of thermal instability of G551D channel function prompted us to compare the effects of CFTR potentiators on the two constructs during a 37 °C

thermal challenge. In a previous report, we examined the effect of several potentiators obtained from CFF, VRT-532 (P1), PG-01 (P2), and Genistein, on the thermal stability of  $\Delta F508$  CFTR channel function.<sup>7</sup> Although each of these compounds increased conductance due to  $\Delta F508$  CFTR channels at 22 °C, none of them protected  $\Delta F508$  CFTR channels from thermal deactivation at 37 °C. In fact, one of the potentiators (P2) actually exacerbated both the rate and extent of thermal deactivation at 37 °C. As illustrated in Figure 2, however,

similar experiments employing oocytes expressing G551D channels produced dramatically different results.

Panels A–E of Figure 2 compare representative experiments in which we tested the effects of four CFTR potentiators, VX-770, P1, P2, and Genistein, on the thermal stability of conductance due to G551D CFTR channels. As summarized in Figure 2F via comparison of the conductance near the termination of the thermal pulse with that seen prior to exposure to a potentiator, all four potentiators protected G551D CFTR channels from thermal deactivation, albeit to varying extents. It can be seen that, at a predetermined maximal dose, VX-770 (10  $\mu$ M) and P2 (10  $\mu$ M) were the most potent as protectors, whereas P1 and Genistein (50  $\mu$ M) was less so. Also, note that the steady state-activated conductance due to G551D CFTR channels exhibited substantial variation from oocyte to oocyte.

**VX-770 Did Not Protect  $\Delta$ F508 CFTR from Thermal Deactivation.** Our previous study produced no evidence that potentiators could protect  $\Delta$ F508 CFTR channels from thermal instability.<sup>7</sup> VX-770 was not tested in this earlier study, however, as the compound was not available to us. Panels A and C of Figure 3 show the results of representative

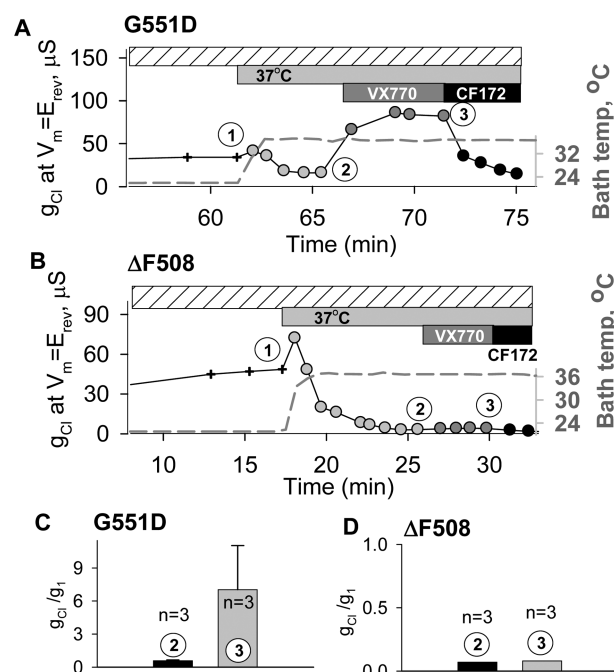


**Figure 3.** VX-770 did not protect  $\Delta$ F508 CFTR from thermal deactivation. (A) Following stimulation, an oocyte expressing  $\Delta$ F508 CFTR channels was warmed to 37 °C (gray bar and circles) for 10 min. It was then exposed to 10  $\mu$ M CF172 after being cooled to 22 °C. (B) Summary of conductance due to  $\Delta$ F508 CFTR before and after a temperature challenge ( $P < 0.05$ ). (C) Following stimulation, an oocyte expressing  $\Delta$ F508 CFTR was exposed to 10  $\mu$ M VX-770 and then warmed to 37 °C (gray bar and circles) in the presence of VX-770. It was then exposed to 10  $\mu$ M CF172 after being cooled to 22 °C. (D) Summary of VX-770-modified  $\Delta$ F508 CFTR conductance before and after the temperature challenge ( $P < 0.05$ ).

experiments that compared thermal deactivation of conductance due to  $\Delta$ F508 channels, with and without prior exposure to VX-770. Consistent with our previous report,<sup>7</sup> as summarized in panels B and D of Figure 3, VX-770, like VRT-532, P2, and Genistein, was without effect on the thermal instability of  $\Delta$ F508 CFTR channel function. Recently, Wang et al.<sup>24</sup> reported that  $\Delta$ F508 CFTR channels exhibited thermal instability in detached patches that was exacerbated by treatment with VX-770, an effect not detected in the studies presented here.

**Potentiator Efficacy following Thermal Deactivation of CFTR Channels.** The results described above point to an important difference in the effect of CFTR potentiators like VX-770 on G551D and  $\Delta$ F508 CFTR channels that may have a

counterpart in the efficacy of VX-770 in patients carrying these mutations. In a patient's first encounter with the drug, however, any G551D CFTR channels would presumably be partially deactivated, raising the question of the efficacy of potentiators in channels previously inactivated by exposure to 37 °C. Panels A and B of Figure 4 show the results of representative



**Figure 4.** VX-770 stimulated G551D but not  $\Delta$ F508 CFTR channels at 37 °C. (A) Following activation of conductance via an increase in intracellular cAMP concentration (see Materials and Methods), an oocyte expressing G551D CFTR was warmed to 37 °C (gray bar and circles). The oocyte was then exposed to 10  $\mu$ M VX770 and subsequently to 10  $\mu$ M CF172 at 37 °C. (B) Following stimulation, an oocyte expressing  $\Delta$ F508 CFTR was warmed to 37 °C (gray bar and circles) and then exposed to 10  $\mu$ M VX770 at 37 °C. (C) Summary of the conductance due to G551D CFTR at 37 °C before and after stimulation by 10  $\mu$ M VX770. The CF172-insensitive conductance was subtracted from the final analysis. (D) Summary of conductance due to  $\Delta$ F508 CFTR channels at 37 °C before and after stimulation by 10  $\mu$ M VX770.

experiments in which we investigated the efficacy of the most potent potentiator, VX-770, in oocytes expressing G551D CFTR channels when the drug was applied following the completion of thermal deactivation at 37 °C, to better mimic the *in vivo* condition. Exposure to VX-770 induced a robust increase in the conductance due to G551D channels at 37 °C (Figure 4A,C). Because of the small sample size and the large variance, this robust increase did not reach statistical significance, although the stimulatory effect of VX770 was unambiguous in each case. Comparison of the data summarized in Figure 4 with that in Figure 2 suggests that VX-770, when applied at 37 °C, not only “protected” the G551D CFTR channels but also increased conductance beyond that seen at 22 °C. Consistent with our previous report,<sup>7</sup> however, neither VX-770 nor any of the other potentiators previously tested [P1, P2, and Genistein (not shown)] produced significant stimulation of  $\Delta$ F508 channels when applied at 37 °C (Figure 4C,D). Figure 4D summarizes the efficacy of VX-770 in G551D and  $\Delta$ F508 CFTR channels at 37 °C.



## DISCUSSION

The introduction of a CF therapeutic that directly targets the CFTR chloride channel was an important validation of the promise of high-throughput screening and should invigorate the search for a new generation of compounds that target the more common mutation,  $\Delta F508$ . The results presented here have implications for such future drug discovery efforts. G551D channels, like  $\Delta F508$  channels, exhibit thermal instability. It would seem appropriate, therefore, to add assays of the thermal stability of channel function to those currently used to screen chemical libraries for efficacious compounds; that is, screening should include assays for “protection” from thermal instability, and compound efficacy should be compared at 37 °C. Yu et al.<sup>6</sup> reported that, in membrane patches detached from FRT cells at room temperature, Ivacaftor (VX-770) was effective in increasing the open probability of a variety of CFTR channel constructs bearing mutations that adversely affected channel gating, consistent with a previous report of an increase in the open probability of  $\Delta F508$  CFTR channels under similar conditions.<sup>16</sup> However, in FRT cell layers assayed in Ussing chambers at 37 °C, the efficacy of Ivacaftor on conductance due to  $\Delta F508$  CFTR channels was minimal, whereas a robust activation of conductance due to G551D channels was detected, despite a minimal conductance prior to exposure to Ivacaftor.<sup>6</sup> It seems clear that CFTR potentiators, compounds selected for their ability to increase CFTR channel open probability, can, among other things, stabilize the activated state of G551D CFTR channels at 37 °C, an effect that may provide clues about the mode of action of these compounds and ultimately their binding sites.

**Trafficking and Thermal Stability of Channel Function.** The finding that  $\Delta F508$  CFTR channels, rescued by a low temperature at the surface of *Xenopus* oocytes<sup>7</sup> or mammalian cells,<sup>8</sup> exhibit thermally induced decreases in open probability raised the question of whether the thermal instability of  $\Delta F508$  CFTR channel function is directly related to the well-established temperature sensitivity of  $\Delta F508$  CFTR trafficking.<sup>17–23</sup> The simplest model, perhaps, would hold that the decrease in open probability that is evident at 37 °C is a manifestation of the same temperature-dependent unfolding process that, within the cell, triggers a quality control mechanism that leads ultimately to the intracellular degradation of the misfolded protein. Our results, however, seem to argue against this simple model. Clearly, G551D CFTR channels, which are trafficked normally or nearly normally, nevertheless exhibit severe thermal instability of channel function. On the other hand, the difference between G551D and  $\Delta F508$  CFTR could simply be one of degree. It may be that the G551D CFTR channels, although thermally unstable, may not proceed as far along the unfolding pathway at 37 °C as do the  $\Delta F508$  channels. G551D CFTR channels would, thereby, escape intracellular degradation but nevertheless fail to contribute adequately to chloride conductance due to the thermal instability of channel function that seems likely to reduce channel open probability at 37 °C.

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### Funding

Supported by The Cystic Fibrosis Foundation [Dawson08G0 (D.C.D.)] and the National Institutes of Health [DK045880 (D.C.D.)].

### Notes

The authors declare no competing financial interest.

## ACKNOWLEDGMENTS

We gratefully acknowledge the gift of CFTR potentiators and inhibitors from Drs. Alan Verkman and Robert Bridges and CFE, without which this work would have been impossible.

## ABBREVIATIONS

CFTR, cystic fibrosis transmembrane conductance regulator; IBMX, isobutylmethyl xanthine; Isop, isoproterenol; P1 (VRT532), 4-methyl-2-(5-phenyl-1H-pyrazol-3-yl)phenol; P2 (PG-01), 2-[(2-1H-indol-3-ylacetyl)methylamino]-N-(4-isopropylphenyl)-2-phenylacetamide; VX-770 (Ivacaftor), N-(2,4-di-*tert*-butyl-5-hydroxyphenyl)-4-oxo-1,4-dihydroquinoline-3-carboxamide; CF172 (CFTRinh-172), 4-[4-oxo-2-thioxo-3-(3-trifluoromethylphenyl)thiazolidin-5-ylidenemethyl]-benzoic acid.

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