Phloxine B Interacts with the Cystic Fibrosis Transmembrane Conductance Regulator at Multiple Sites to Modulate Channel Activity*[^S]

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The fluorescent derivative phloxine B is a potent modulator of the cystic fibrosis transmembrane conductance regulator (CFTR). Low micromolar concentrations of phloxine B stimulate CFTR Cl⁻ currents, whereas higher concentrations of the drug inhibit CFTR. In this study, we investigated the mechanism of action of phloxine B. Phloxine B (1 μM) stimulated wild-type CFTR and the most common cystic fibrosis mutation, ΔF508, by increasing the open probability of phosphorylated CFTR Cl⁻ channels. At each concentration of ATP tested, the drug slowed the rate of channel closure without altering the opening rate. Based on the effects of fluorescent derivatives on transport ATPases, these data suggest that phloxine B might stimulate CFTR by binding to the ATP-binding site of the second nucleotide-binding domain (NBD2) to slow the dissociation of ATP from NBD1. Channel block by phloxine B (40 μM) was voltage-dependent, enhanced when external Cl⁻ concentration was reduced and unaffected by ATP (5 mM), suggesting that phloxine B inhibits CFTR by occluding the pore. We conclude that phloxine B interacts directly with CFTR at multiple sites to modulate channel activity. It is possible that related agents might be of value in the development of new treatments for diseases caused by the malfunction of CFTR.

The cystic fibrosis transmembrane conductance regulator (CFTR^[1]^[1]) is a novel member of the ATP-binding cassette (ABC) transporter family that forms a Cl⁻ channel with complex regulation (2, 3). It is predominantly expressed in epithelia, where it provides a pathway for the movement of Cl⁻ ions across the apical membrane and regulates the rates of transepithelial salt and water transport (4). Dysfunction of CFTR is associated with a wide spectrum of disease. The genetic disease cystic fibrosis (CF) is caused by loss of function of the CFTR Cl⁻ channel (4). CF affects ~1 in 2500 live births and is the most common fatal autosomal recessive disease to affect Caucasian populations (4). Other diseases, such as autosomal dominant polycystic kidney disease (ADPKD) and secretory diarrhea, likely involve increased activity of the CFTR Cl⁻ channel (5, 6). ADPKD affects more than 1 in 1000 live births and is the most common single gene defect associated with the loss of kidney function (5). Secretory diarrhea annually kills millions of infants in Africa, Asia, and Latin America (7). The prevalence of these diseases suggests that modulators of the CFTR Cl⁻ channel have significant therapeutic potential.

Several pharmacological strategies to manipulate the activity of the CFTR Cl⁻ channel have been identified. Some agents promote channel opening by modulating the activity of the protein kinases and phosphatases that regulate CFTR (2, 8). For example, the phenylimidazothiazole drug bromotetramisole inhibits the protein phosphatase that dephosphorylates CFTR (9). In contrast, other agents interact directly with CFTR to enhance channel activity (8, 10). The binding of the flavonoid genistein to the second nucleotide-binding domain (NBD2) prolongs dramatically the duration of channel openings (11–14), whereas cyclophilin A, a cis-trans peptidyl-prolyl isomerase, interacts with the R (regulatory) domain to stimulate greatly the activity of CFTR (15).

New inhibitors of the CFTR Cl⁻ channel have emerged from studies of agents that interact with other ABC transporters, including the sulfonylurea receptor (SUR) and multidrug-resistance associated proteins (MRPs, Ref. 16). SUR is the regulatory subunit of ATP-sensitive K⁺ channels (KATP channels) (17). It binds sulfonylureas, a class of hypoglycemia-inducing drugs used to treat non-insulin-dependent diabetes mellitus, to inhibit K⁺ flow through the pore-forming subunit of KATP (Kir6.2 (17)). Like their effects on KATP channels, the sulfonylureas glibenclamide and tolbutamide and the non-sulfonylurea hypoglycemic agents meglitinide and mitiglinide inhibit the CFTR Cl⁻ channel (18–20). MRPs hydrolyze ATP to export a wide range of large anions from cells (21). Lindsell and Hanrahan (22) demonstrated that two substrates of MRPs, tauroliotholate-3-sulfate and β-estradiol 17-((β-D-glucurono)-nide), inhibit the CFTR Cl⁻ channel. The data indicate that hypoglycemia-inducing drugs and substrates of MRPs block the CFTR Cl⁻ channel by occluding the intracellular end of the CFTR pore (19, 22).

In the search for new modulators of CFTR, we tested the effect on the CFTR Cl⁻ channel of fluorescein derivatives, a...
group of drugs used to investigate the function of transport ATPases and K\textsubscript{ATP} channels (23–26). Like its effect on K\textsubscript{ATP} channels (25), the fluorescein derivative phloxine B both stimulated and inhibited channel activity (27, present study). To understand better the mechanism of action of phloxine B, we studied CFTR Cl\textsuperscript{−} channels in excised inside-out membrane patches from cells expressing human CFTR.

**EXPERIMENTAL PROCEDURES**

**Cells and Cell Culture**—For these studies, we used mouse mammary epithelial cells (C127 cells) stably expressing either wild-type human CFTR or ΔF508, the most common CF-associated mutation (28). C127 cells expressing wild-type CFTR were cultured as previously described (19). C127 cells expressing ΔF508 CFTR were cultured at 28 °C to overcome the processing defect of this mutation and promote its delivery to the cell membrane (29). Cells were seeded onto glass coverslips and used within either 2 days (wild-type CFTR) or 1 week (ΔF508 CFTR).

**Electrophysiology**—CFTR Cl\textsuperscript{−} channels were recorded in excised inside-out membrane patches using an Axopatch 200A patch-clamp amplifier (Axon Instruments Inc., Union City, CA) and pClamp data acquisition and analysis software (version 6.03, Axon Instruments Inc.) as previously described (19, 30). The established sign convention was used throughout; currents produced by positive charge moving from intra- to extracellular solutions (anions moving in the opposite direction) are shown as positive currents.

The pipette (extracellular) solution contained (in millimolar): 140 N-methyl-d-glucamine (NMDG), 140 aspartic acid, 5 CaCl\textsubscript{2}, 2 MgSO\textsubscript{4}, and 10 N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES), pH 7.3, with Tris (ICl\textsubscript{o}, 10 mM). The bath (intracellular) solution contained (millimolar): 140 NMDG, 3 MgCl\textsubscript{2}, 1 CaEGTA, and 10 TES, pH 7.3, with HCl (ICl\textsubscript{i}, 147 mM, free [Ca\textsuperscript{2+}], <10\textsuperscript{−11} M) and was maintained at 37 °C.

After excision of inside-out membrane patches, CFTR Cl\textsuperscript{−} channels were activated by the addition of the catalytic subunit of protein kinase A (PKA, 75 nm) and ATP (1 mM) to the intracellular solution within 5 min of patch excision. Unless otherwise specified, the ATP concentration was reduced to 0.3 mM before the addition of phloxine B to the intracellular solution as previously described (31); PKA was added to all intracellular solutions. Membrane patches were voltage-clamped at −50 mV.

In this study, we used membrane patches containing large numbers of active channels for time-course studies and voltage ramp protocols and membrane patches containing five or less active channels for single-channel studies. The number of channels in a membrane patch was determined from the maximum number of simultaneous channel openings observed during the course of an experiment as described previously (32). Because the effects of phloxine B on CFTR Cl\textsuperscript{−} channels were only partially reversible (Fig. 7C), specific interventions were not bracketed by control periods. However, we have previously shown that in the continuous presence of PKA and ATP, rundown of CFTR Cl\textsuperscript{−} channels in excised membrane patches from C127 cells is minimal (19).

To investigate whether phloxine B inhibition of CFTR was voltage-dependent and enhanced when the external Cl\textsuperscript{−} concentration was reduced, we used voltage ramp protocols to acquire macroscopic current-voltage (I–V) relationships as previously described (31). Basal currents recorded before the addition of PKA and ATP were subtracted from those recorded in the absence and presence of phloxine B to determine the effect of phloxine B on CFTR Cl\textsuperscript{−} currents.

CFTR Cl\textsuperscript{−} currents were initially recorded on digital audiotape using a digital tape recorder (Biologic Scientific Instruments, model DTR-1204, Intracel Ltd., Rostock, UK) at a bandwidth of 10 kHz. On playback, records were filtered with an eight-pole Bessel filter (Frequency Devices, model 900LPF2, Scenessys Ltd., Aylesbury, UK) at a corner frequency of 500 Hz and acquired using a Digidata 1200 interface (Axon Instruments, Inc.) and pClamp at sampling rates of either 2.5 kHz (time-course studies) or 5 kHz (single-channel studies). For the purpose of illustration, single-channel records were filtered at 500 Hz and digitized at 1 kHz.

In time-course studies, each data point is the average current for a 4-s interval with data points collected continuously; no data were collected while solutions were changed. Average current (I) for a specific intervention was determined as the average of all the data points collected during the intervention. The relationship between drug concentration and CFTR inhibition was fitted to the Hill equation,

\[
I_{\text{drug/Control}} = 1/(1 + ([\text{Drug}]/K_{p}^{n}))
\]

(1)

where [Drug] is the concentration of drug, \(I_{\text{drug/Control}}\) is the fractional current at the indicated drug concentration relative to that in the same solution in the absence of added drug, \(K_{p}\) is the drug concentration causing half-maximal inhibition, and \(n\) is the slope factor (Hill coefficient). Mean data were fitted to a linear form of Equation 1 using linear least-squares regression to yield \(K_{p}\) and \(n\) values.

To measure single-channel current amplitude (i), Gaussian distributions were fit to current amplitude histograms. For open probability (\(P_{o}\)) and burst analyses, lists of open- and closed-times were created and analyzed as previously described (31). Burst analysis was performed as described by Curson et al. (33), using a \(t_{b}\) (the time that separates interburst closures from interburst reopening) of 15 ms. Mean interburst interval (\(T_{b}\)) was calculated using the equation,

\[
P_{o} = T_{b}/(T_{b} + T_{c})
\]

(2)

where \(T_{b}\) is (mean burst duration) \times (open probability within a burst). Mean burst duration and open probability within a burst were determined directly from experimental data; \(P_{o}\) was calculated from open and closed times. Only membrane patches that contained a single active channel were used for burst analysis.

To perform maximum likelihood analysis and develop kinetic models to quantify the interaction of phloxine B with CFTR, we used the QuB software suite (www.qub.buffalo.edu). In brief, digitized current records generated by pClamp software were imported with no further filtering and baseline corrected (program PRE). Using a recursive Viterbi algorithm (program SKM), idealized currents were produced. Finally, rate constants for kinetic models were calculated from the idealized current dwell time sequence using a maximum likelihood approach (program MIL). For consistency with analyses using pClamp software, transitions of <1 ms were excluded. With the exception of the ADP data (Fig. 5 and supplementary information), only membrane patches that contained a single active channel were used for maximum likelihood analysis and kinetic modeling.

**Drugs and Chemicals**—PKA was purchased from Promega Corp. (Southampton, UK). ATP (dissodium salt), glibenclamide, phloxine B (2’, 4’, 5’, 7-tetramethoxy-4,5,6,7-tetrahydrofluorescein, Fig. 1A), and TES were obtained from Sigma-Aldrich Company Ltd. (Gillingham, UK). All other chemicals were of reagent grade.

Stock solutions of phloxine B were prepared in Me\textsubscript{2}SO and stored at −20 °C. Immediately before use, stock solutions were diluted to achieve final concentrations. Me\textsubscript{2}SO did not affect the activity of CFTR (19).

**RESULTS**

**Phloxine B Stimulates and Inhibits the Activity of CFTR**—To examine the effect of the fluorescein derivative phloxine B on the activity of CFTR, we studied CFTR Cl\textsuperscript{−} currents in excised inside-out membrane patches from C127 cells expressing wild-type human CFTR. In the absence of either ATP (\(n = 4\)) or PKA (\(n = 4\)), phloxine B was without effect on CFTR Cl\textsuperscript{−} channels (data not shown). However, in the presence of both PKA (75 mM) and ATP (0.3 mM), addition of phloxine B to the intracellular solution altered channel activity (Fig. 1, B and C). Phloxine B (0.1–5 μM) stimulated CFTR Cl\textsuperscript{−} current whereas, phloxine B (20–50 μM) inhibited channel activity. The inset of Fig. 1C shows the fit of the Hill equation to the relationship between phloxine B concentration and current inhibition (\(K_{i} = 17 \mu\text{M} \pm 2\); correlation coefficient, \(r^{2} = 0.943 \pm 0.043\) at −50 mV).

These data suggest that phloxine B is a potent modulator of the CFTR Cl\textsuperscript{−} channel: it stimulates CFTR with greater efficacy than genistein, and it inhibits channel activity with equipotency to glibenclamide (13, 19). The Hill plot also suggests that phloxine B might inhibit CFTR by binding at two or more sites that interact co-operatively. Modulation of CFTR by phloxine B was partially reversible (see Fig. 7C, below).

**Mechanism of Phloxine B Stimulation of CFTR**—In principle, phloxine B might stimulate CFTR Cl\textsuperscript{−} currents in one of three ways: first, by increasing the number of active channels; second, by enhancing current flow through open channels; third, by increasing \(P_{o}\). To discriminate between these different possibilities, we investigated the effect of phloxine B (1 μM) on...
the single-channel activity of CFTR (Fig. 2). Visual inspection of single-channel records suggests that phloxine B (1 μM) did not stimulate CFTR by enhancing the number of active channels present in a membrane patch (n = 15, Fig. 2A). Similarly, phloxine B (1 μM) did not stimulate CFTR by increasing the amount of current flowing through an open channel. On the contrary, the drug produced a small, but significant (p < 0.05), reduction in i (Fig. 2B). Instead, phloxine B (1 μM) caused a large increase in P_o (p < 0.0001; Fig. 2C).

To determine how phloxine B (1 μM) increased P_o, we investigated its effects on the gating kinetics of phosphorylated CFTR Cl^- channels using membrane patches that contained only a single active channel. Using maximum likelihood analysis, Winter et al. (34) demonstrated that the simplest model to describe the gating kinetics of single phosphorylated wild-type CFTR Cl^- channels is a linear three-state model (Fig. 3A). In this model, C_1 represents the long duration closed state separating bursts of channel activity and C_2 ↔ O represents the bursting state in which channel openings (O) are interrupted by brief flickery closures (C_2). Transitions between the three states are described by the rate constants β_1, β_2, α_1, and α_2. Winter et al. (34) demonstrated that intracellular ATP regulates CFTR at the transition between C_1 and C_2; as the ATP concentration increases, β_1 increases. In contrast, the other transitions were not altered significantly by ATP (34). Similar analyses of our data support this model of ATP-dependent regulation of CFTR channel gating (see supplementary material).

Fig. 3B summarizes the effect of phloxine B (1 μM) on the rate constants. The major effect of phloxine B (1 μM) was to decrease α_1 to about 40% of the control value. The drug also increased β_2, but did not change either β_1 or α_2. The decrease in α_1 delays the exit from the bursting state to the long-lived closed state and, hence, increases burst duration. The increase in β_2, the transition rate between the short-lived closed state and the open state, further increases the duration of bursts. These changes explain the observed increase in P_o caused by phloxine B (1 μM, Fig. 2C).

Effect of ATP and ADP on Phloxine B Stimulation of CFTR—The effect of phloxine B (1 μM) on channel gating resembles that of adenosine 5’-(β,γ-iminoh)triphosphate (AMP-PNP) and genistein, two activators of the CFTR Cl^- channel. Previous work suggests that these agents interact directly with NBD2, which regulates channel closure (11–14, 35). Based on these data and the observation that fluorescein derivatives prevent
the hydrolysis of ATP by transport ATPases (23, 24), we speculated that phloxine B might compete with ATP for a common binding site on NBD2. To test this hypothesis, we examined the effect of ATP concentration on phloxine B (1 μM) stimulation of CFTR Cl\(^-\) channels. Fig. 4 (A and B) shows that, as the ATP concentration increased, CFTR activity in both the absence and presence of phloxine B (1 μM) increased. With the exception of ATP (3 mM), at each concentration of ATP tested, \(P_o\) values in the presence of phloxine B (1 μM) were significantly greater than those recorded in the absence of the drug (\(p < 0.05\); Fig. 4B). In both the absence and presence of phloxine B (1 μM), mean data were best fitted by a Michaelis-Menten function (control: \(K_m = 197 \mu M\), maximum \(P_o\) (\(P_o,max\)) = 0.59, \(r^2 = 0.963\); phloxine B: \(K_m = 33 \mu M\), \(P_o,max\) = 0.73; \(r^2 = 0.983\)). These data suggest that phloxine B (1 μM) increases the affinity of CFTR for ATP. They also raise the possibility that phloxine B might not compete with ATP for a common binding site.

To quantify the effect of phloxine B (1 μM) on channel gating in the presence of different concentrations of ATP, we performed an analysis of bursts using a burst delimiter (\(t_o\)) of 15 ms.\(^2\) Under control conditions, both interburst interval and burst duration exhibited ATP dependence. Interburst interval was highly ATP-dependent, decreasing 11-fold between 0.03 and 5 mM ATP (Fig. 4D). In contrast, burst duration was weakly ATP-dependent at low ATP concentrations, increasing 1.4-fold between 0.03 and 1 mM ATP, but ATP-independent at higher ATP concentrations (Fig. 4C). Phloxine B (1 μM) was without effect on the ATP dependence of the interburst interval (Fig. 4D). However, the drug caused a marked increase in burst duration at each concentration of ATP tested (Fig. 4C). These data correlate well with the results of maximum likelihood analysis of phloxine B (1 μM) stimulation of CFTR (Fig. 3 and supplementary material).

\(^2\) Similar results were observed using a \(t_o\) of 80 ms (\(n = 6\)).
CFTR (Fig. 6A). Visual inspection of single-channel records suggests that phloxine B (20 μM) altered channel gating in several ways. First, it greatly prolonged the interburst interval (Fig. 6A). Second, it caused a large increase in flickery closures interrupting bursts of channel activity (Fig. 6A). Third, it appeared to prolong the duration of bursts (Fig. 6A). To begin to quantify channel block, we measured \( i \) and \( P_o \). Fig. 6 (B and C) demonstrates that phloxine B (20 μM) significantly decreased both \( i \) and \( P_o \) (\( p < 0.05 \)).

Fluorescein derivatives inhibit glibenclamide binding to SUR (25, 26). Based on these data, we were interested to learn the effect of phloxine B on glibenclamide inhibition of CFTR. Stimulation of CFTR with phloxine B (1 μM) failed to prevent channel block by glibenclamide (50 μM, \( n = 5 \), data not shown). Moreover, in the presence of glibenclamide (50 μM) and phloxine B (20 μM) channel inhibition was greater than that observed in the presence of either drug alone (\( p < 0.001 \), Fig. 7A and B (31)). These data suggest that glibenclamide and phloxine B might interact with CFTR at distinct sites.

Elevated concentrations of the CFTR activator genistein inhibit channel activity by slowing greatly the rate of channel opening (13, 31). Like genistein, phloxine B prolonged the duration of long closures separating channel openings. This suggests that genistein and phloxine B might inhibit the CFTR Cl\(^-\) channel by similar mechanisms. To test this idea, we investigated whether high concentrations of ATP attenuate phloxine B inhibition of CFTR. In contrast to genistein block of the CFTR Cl\(^-\) channel (31), ATP (5 mM) failed to relieve phloxine B (20 μM) inhibition of CFTR Cl\(^-\) currents (\( p > 0.05 \), Fig. 7, C and D).

The prolonged channel openings interrupted by flickery closures induced by inhibitory concentrations of phloxine B (Fig. 6A) suggest that phloxine B might be an open-channel blocker of CFTR. To investigate this idea, we examined the voltage dependence of channel inhibition. Membrane patches were bathed in symmetrical 147 mM Cl\(^-\) solutions, and CFTR Cl\(^-\) currents were recorded in the absence and presence of phloxine B (40 μM) over the voltage range ±100 mV using a voltage ramp protocol. Consistent with previous data (31), under control conditions, CFTR Cl\(^-\) currents exhibited weak inward rectification (Fig. 8A). This rectification of CFTR is caused by a reduction in \( i \) and changes in gating behavior at voltages above 50 mV (38).

Fig. 8A demonstrates that phloxine B inhibition of CFTR is voltage-dependent. Phloxine B (40 μM) decreased CFTR Cl\(^-\) currents at negative voltages. However, at positive voltages inhibition was relieved. Using current values recorded in the presence and absence of phloxine B, we calculated the voltage-dependent dissociation constant (\( K_d \)) for phloxine B inhibition of CFTR from the equation,

\[
K_d(V) = [\text{drug}]III_e - I
\]

(Eq. 3)

where \( K_d(V) \) is the voltage-dependent dissociation constant at voltage \( V \), and \( I \) and \( I_e \) are the current values in the presence and absence of drug, respectively. Fig. 8C demonstrates that \( K_d \) values showed weak voltage dependence at negative voltages.
However, at positive voltages $K_d$ values were strongly voltage-dependent. The data also suggest that the potency of phloxine B inhibition of CFTR is comparable to glibenclamide and greater than that of genistein (phloxine B, $K_d(0 \text{ mV}) = 49 \pm 7 \mu M$ (n = 5); glibenclamide, $K_d(0 \text{ mV}) = 37 \pm 6 \mu M$ (19); genistein, $K_d(0 \text{ mV}) = 87 \pm 11 \mu M$ (31)).

The electrical distance across the membrane sensed by blocking ions can be calculated using the Woodhull relationship (39),

$$K_d(V) = K_d(0 \text{ mV}) \exp\left(-z'FV/RT\right) \quad (\text{Eq. 4})$$

where $z'$ is the apparent valency of the blocking ion (defined as the actual valency of the blocking ion $z$) multiplied by the electrical distance across the membrane experienced by the blocking ion ($\delta$), and $F$, $R$, and $T$ are the Faraday constant, gas constant, and absolute temperature, respectively. Using the data in Fig. 8C, $z' = 0.04 \pm 0.01$ (n = 5) measured from the inside of the membrane over the voltage range $-100$ to $-40 \text{ mV}$.

Because phloxine B inhibition of CFTR was voltage-dependent and unaffected by elevated ATP concentrations, we speculated that phloxine B might bind within the CFTR pore. To test this hypothesis, we reduced the external Cl$^-$ concentration to $10 \text{ mM}$ and measured CFTR Cl$^-$ currents in the absence and presence of phloxine B ($40 \mu M$) over the voltage range $-100$ to $0 \text{ mV}$ using a voltage ramp protocol. Fig. 8C demonstrates that reducing the external Cl$^-$ concentration enhances the potency of phloxine B inhibition of CFTR (external [Cl$^-$/H11005$]$ = $147 \text{ mM}$, $K_d(0 \text{ mV}) = 49 \pm 7 \mu M$ (n = 5); external [Cl$^-$/H11005$]$ = $10 \text{ mM}$, $K_d(0 \text{ mV}) = 26 \pm 5 \mu M$ (n = 4); p < 0.05). However, reducing the external Cl$^-$ concentration was without effect on the electrical distance sensed by phloxine B ($z' = 0.06 \pm 0.01$ (n = 4) measured from the inside of the membrane over the voltage range $-100$ to $-40 \text{ mV}$; p > 0.05). Thus, these data indicate that phloxine B inhibition of CFTR is voltage-dependent and enhanced when the external Cl$^-$ concentration is reduced. This

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**Fig. 7. Effect of glibenclamide and ATP on phloxine B inhibition of CFTR.** A, time-course of CFTR Cl$^-$ current in an excised inside-out membrane patch. ATP (0.3 mM), PKA (75 nM), and glibenclamide (Glib, 50 $\mu M$) were present in the intracellular solution during the times indicated by the filled bars. Other details as in Fig. 1B. B, effect of glibenclamide (50 $\mu M$) on phloxine B (20 $\mu M$) inhibition of CFTR Cl$^-$ current. Columns and error bars indicate means ± S.E. (n = 5). Values represent the average current recorded during the indicated interventions normalized to that measured under control conditions at the start of the experiment. Other details as in A. C, time-course of CFTR Cl$^-$ current in an excised inside-out membrane patch. ATP (0.3 or 5 mM), PKA (75 nM), and phloxine B (20 $\mu M$) were present in the intracellular solution during the times indicated by the filled bars. D, effect of ATP concentration on phloxine B (20 $\mu M$) inhibition of CFTR Cl$^-$ currents. Columns and error bars indicate means ± S.E. (n = 6). Other details are as in B and C.

**Fig. 8. Phloxine B inhibition of CFTR is voltage-dependent and enhanced when the external Cl$^-$ concentration is reduced.** A, $I$-$V$ relationships of CFTR Cl$^-$ currents recorded in the absence and presence of phloxine B (40 $\mu M$) when the membrane patch was bathed in symmetrical 147 mM Cl$^-$ solutions. ATP (1 mM) and PKA (75 nM) were continuously present in the intracellular solution; holding voltage was $-50 \text{ mV}$. B, effect of voltage on the fraction of CFTR Cl$^-$ current inhibited by phloxine B (40 $\mu M$). Values are means ± S.E. (n = 5) at each voltage. The continuous line is the fit of a second-order regression to the data. C, relationship between the voltage-dependent dissociation constant ($K_d$) and voltage when the external Cl$^-$ concentration was either 147 mM (filled circles) or 10 mM (open circles). Data are means ± S.E. (n = 4–5) at each voltage. The continuous lines are the fits of first-order regressions to the data.
shows the effect of phloxine B (1 μM) on the activity of a single ΔF508 CFTR Cl⁻ channel. ATP (1 mM) and PKA (75 mM) were continuously present in the intracellular solution. B, C, and D, effect of phloxine B (1 μM) on Pₒ, mean burst duration, and interburst interval. Columns and error bars indicate means ± S.E. (n = 3–5). The asterisks indicate values that are significantly different from the control value (p < 0.05). Other details as in Fig. 2A.

suggests that phloxine B might inhibit CFTR by occluding the CFTR pore.

**Phloxine B Stimulates the CF-associated Mutant ΔF508 CFTR**—To begin to evaluate the therapeutic potential of phloxine B, we investigated whether phloxine B (1 μM) stimulates the activity of ΔF508 CFTR, the most common CF-associated mutant. ΔF508 CFTR causes a loss of Cl⁻ channel function in two ways: first, it disrupts the biosynthesis of CFTR and second, it perturbs channel gating (12, 40). To investigate whether phloxine B stimulates the activity of ΔF508 CFTR Cl⁻ channels, we grew cells at 28 °C to overcome the defective processing of ΔF508 CFTR and facilitate its delivery to the cell membrane (29). Fig. 9A shows the effect of phloxine B (1 μM) on the activity of a single ΔF508 CFTR Cl⁻ channel following phosphorylation by PKA. ΔF508 CFTR Cl⁻ channels are characterized by dramatically prolonged closures separating bursts of channel openings. As a result, the Pₒ of ΔF508 CFTR is much less than that of wild-type CFTR (Figs. 2 and 9). Phloxine B (1 μM) increased the Pₒ of ΔF508 CFTR 1.5-fold by enhancing burst duration (p < 0.05) without altering the interburst interval (p > 0.05, Fig. 9). These data demonstrate that phloxine B (1 μM) stimulates ΔF508 CFTR Cl⁻ channels.

**DISCUSSION**

The fluorescein derivative phloxine B is a potent modulator of CFTR with complex effects on channel activity. Low micromolar concentrations of phloxine B stimulate CFTR by prolonging channel openings. In contrast, higher concentrations of the drug inhibit CFTR by impeding Cl⁻ permeation.

Activators of CFTR enhance channel activity either by regulating the intracellular signaling pathways that control CFTR activity or interacting directly with CFTR (8, 10). Several lines of evidence suggest that phloxine B acts by the latter mechanism. First, PKA and ATP were required for channel stimulation (27, present study). This indicates that cAMP-dependent phosphorylation of CFTR is a prerequisite for phloxine B to augment channel activity. Second, phloxine B enhanced channel activity in excised inside-out membrane patches (27, present study). This suggests that phloxine B might bind directly to CFTR. Third, phloxine B stimulated channel activity by prolonging the duration of channel openings (present study). Based on previous work (for review see Ref. 2), this result suggests that phloxine B might inhibit channel closure. These characteristics of phloxine B stimulation of CFTR resemble those of genistein, a CFTR activator that interacts directly with CFTR (11–14, 41). However, they differ markedly from those of bromotetramisole, a CFTR activator that inhibits protein phosphatases (9). Consistent with these data, phloxine B (1 μM) and bromotetramisole (3 mM) stimulation of CFTR Cl⁻ currents was additive (n = 4, data not shown).

Al-Nakkash et al. (42) proposed that genistein and benzimidazolones stimulate CFTR by a common mechanism. These drugs inhibit ATP hydrolysis at NBD2 to stabilize the open channel configuration. Our observation that phloxine B inhibits its channel closure suggests that phloxine B might stimulate CFTR by a similar mechanism. However, the observation that phloxine B, but not genistein, altered the relationship between ATP concentration and channel activity (13, 27, 43, present study) argues that phloxine B stimulates CFTR by a distinct mechanism. Given that fluorescein derivatives inhibit ATP hydrolysis by transport ATPases (23, 24), that CFTR possesses two NBDs that bind and hydrolysis ATP (44), and that phloxine B enhances the affinity of CFTR for ATP (27, present study), we speculate that phloxine B stimulates CFTR by interacting with the NBDs.

A variety of models have been proposed to account for the regulation of channel gating by the NBDs (for discussion see Ref. 45). In the model of Zeltwanger et al. (46), brief channel openings in the presence of low ATP concentrations reflect ATP binding and hydrolysis at NBD1, whereas prolonged channel openings in the presence of high ATP concentrations reflect ATP binding and hydrolysis at NBD2. To explain how phloxine B stimulates CFTR, we propose that phloxine B binding to one NBD (we suggest NBD2) slows the rate of ATP dissociation at NBD1. Consistent with our data, this interpretation predicts that phloxine B would increase burst duration without altering the interburst interval.

Fluorescein derivatives interact with several sites on transport ATPases, with the principal site being the high affinity ATP-binding site of the catalytic subunit (23). These data suggest that phloxine B might prevent ATP dissociation from NBD1 by binding at the ATP-binding site of NBD2. This interpretation predicts that phloxine B and ATP compete for a common binding site on NBD2. To test this idea, we used phloxine (1 μM), whereas Bachmann et al. (27) used phloxine B (300 nM). Because competition was only observed using phloxine B (300 nM), phloxine B and ATP might only compete for a common binding site at non-saturating (nanomolar) concentrations of the drug. At saturating (micromolar) concentrations of the drug, phloxine B might interact with a site distinct from the ATP-binding site. Given that fluorescein derivatives are hydrophobic analogues of AMP (24) and AMP interacts with a site distinct from that of ATP to enhance the affinity of NBD2 for ATP (14), we speculate that high concentrations of phloxine B might stimulate CFTR by binding at or near the AMP-binding site of NBD2.

Like genistein (13, 31), elevated concentrations of phloxine B inhibit the CFTR Cl⁻ channel. Both genistein and phloxine B
caused a flickery block that prolonged the duration of bursts and lengthened dramatically the interburst interval (31, present study). However, ATP (5 mM) and Cl− concentration was reduced, and unaffected by ATP (5 mM, present study). The failure of ATP (5 mM) to relieve phloxine B inhibition of CFTR was not a consequence of the slow dissociation of the drug from the channel.3

Previous work has demonstrated that large anions cause a voltage-dependent block of the CFTR Cl− channel by binding to sites located 15–60% of the way through the transmembrane electric field from the inside (19, 22, 36, 37, 47–49). In contrast, the phloxine B-binding site is located only 4% of the way through the transmembrane electric field from the inside. This suggests that phloxine B interacts with a site outside the pore to block CFTR. For two reasons, we do not favor this interpretation. First, phloxine B inhibition of CFTR was voltage-dependent and enhanced when the external Cl− concentration was reduced. These data suggest strongly that phloxine B enters the CFTR pore from the intracellular end, binds at or near Cl−-binding sites, and blocks Cl− flow. Second, calculation of the electrical distance that is sensed by phloxine B is complex. The Hill coefficient for phloxine B inhibition of CFTR is 2. This suggests that phloxine B might block the CFTR Cl− channel by binding to two or more sites. Consistent with this idea, phloxine B possesses two negative charges located on different parts of the molecule (Fig. 1A). This suggests that these charges might experience different fractions of the transmembrane electric field when phloxine B inhibits CFTR. However, the relationship between electrical distance and physical distance within the CFTR pore is unknown. Based on our experience with genistein (31), it is feasible that phloxine B might bind to a site deep within the CFTR pore. Alternatively, the drug might interact with a site at the extremity of the intracellular vestibule. Given the location of the gibbenclamide-binding site (19, 50) and our observation that channel block by phloxine B and gibbenclamide was additive, we favor this possibility.

In conclusion, our data indicate that phloxine B is a potent modulator of the CFTR Cl− channel. They also suggest that phloxine B interacts directly with CFTR at multiple sites to modulate channel activity. Based on the present results, future studies might identify the phloxine B-binding sites.

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