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Potent stimulation and inhibition of the CFTR Cl^- current by phloxine B

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1 The effects of the fluoresceine derivative, phloxine B, on the Cl^- current through the cystic fibrosis transmembrane conductance regulator (CFTR) were examined in *Xenopus* oocytes expressing human CFTR.

2 In whole oocytes, the CFTR Cl^- current (I_{CFTR}) was activated by superfusion with isobutylmethylxanthine and forskolin. I_{CFTR} was stable during activation and deactivated rapidly upon washout of the activation solution. Phloxine B slowed deactivation and, at high concentrations, inhibited I_{CFTR} weakly.

3 In excised inside-out macropatches, I_{CFTR} was activated by the catalytic subunit of protein kinase A (cPKA) and MgATP. Phloxine B (0.01–3 μM), applied after activation, increased I_{CFTR} within 30 s followed by a slow decrease which became dominant at high concentrations. Slowing of deactivation of the CFTR was observed at all concentrations.

4 The effect of phloxine B after 30 s had a bell-shaped concentration-dependence with midpoints at 45 and 1600 nM for the stimulatory and the inhibitory limb, respectively; maximum stimulation was about 1.8 times. The slow inhibitory component, measured after 6 min, occurred with an IC_{50} value of $\sim$1 μM.

5 In the absence of cPKA, phloxine B did not stimulate I_{CFTR}. In the presence of cPKA and MgATP, the effects of phloxine B were more prominent at low (0.02 mM) than at high ATP (2 mM).

6 The data show that phloxine B modulates I_{CFTR} by increasing channel activity and slowing channel deactivation; at high concentrations inhibition dominates. The effects may be mediated by direct interactions with CFTR from the inside of the cell.

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Abbreviations: ABC protein, ATP binding cassette protein; AMP-PNP, 5′-adenosine(β,γ-imido)triphosphate; CFTR, cystic fibrosis transmembrane conductance regulator; cPKA, protein kinase A catalytic subunit; IBMX, isobutylmethylxanthine; I_{CFTR}, CFTR Cl^- current; NBF, nucleotide binding fold; SUR, sulphonylurea receptor

Introduction

The cystic fibrosis transmembrane conductance regulator (CFTR) belongs to the family of ATP binding cassette (ABC-) proteins, containing on the intracellular side two nucleotide binding folds (NBFs) and a regulatory domain (Riordan *et al*., 1989). After phosphorylation of the regulatory domain by protein kinase A (PKA) and in the continued presence of ATP, CFTR functions as a small conductance non-rectifying Cl^- channel (Anderson *et al*., 1991; Cheng *et al*., 1991; Bear *et al*., 1992). Several models for the gating of the channel have been established (for review see Sheppard & Welsh, 1999) and there is some agreement that hydrolysis of ATP at one NBF (presumably NBF1) opens the channel and subsequent hydrolysis of ATP at the other NBF induces channel closure. In addition to its function as a Cl^- channel, CFTR modulates various other ion channels like the outwardly rectifying Cl^- channel, the epithelial Na^+ channel and the renal outer medulla K^+ channel (ROMK; inwardly rectifying K^+ channels Kir1.1a and 1b) (review: Schwiebert *et al*., 1999). Defects of CFTR which lead to a loss of function cause cystic fibrosis (Riordan *et al*., 1989; Drumm *et al*., 1991; Welsh & Smith, 1993); in contrast, exaggerated activity of CFTR is involved in secretory diarrhoea (Gabriel *et al*., 1994; Prince, 1998). Modulators of CFTR activity are therefore of great interest and a number of compounds which directly interact with CFTR to modulate its channel activity have been described (Schultz *et al*., 1999; Hwang & Sheppard, 1999).

ATP-sensitive K^+ channels (K_{ATP} channels) are a group of K^+ channels which are closed by intracellular ATP and opened by the Mg^{2+} salts of nucleoside diphosphates, e.g. MgADP. Pharmacologically, K_{ATP} channels are closed by the hypoglycaemic sulphonylureas and activated by the K^+ channel openers, a heterogenous group of compounds which induce vasorelaxation (Ashcroft & Ashcroft, 1990; Edwards & Weston, 1993; Quast, 1996). K_{ATP} channels are heteromeric complexes composed of pore-forming α-subunits belonging to the family of inwardly rectifying K^+ channels (Kir6.x) and sulphonylurea receptors (SURs) as β-subunits (reviews: Seino, 1999; Ashcroft & Gribble, 1998; Aguilar-Bryan & Bryan, 1999). SUR is a member of the ABC protein superfamily like CFTR (Aguilar-Bryan *et al*., 1995; Tusnády *et al*., 1997). Sulphonylureas and openers bind to SUR to modulate channel activity whereas the high affinity block by ATP of the K_{ATP} channels in the pancreatic β-cell and in heart and skeletal muscle is mediated by binding of ATP to Kir6.2 (Seino, 1999; Ashcroft & Gribble, 1998; Aguilar-Bryan & Bryan, 1999).

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Sheppard & Welsh (1992) showed that both blockers and openers of the K_{ATP} channel inhibited I_{CFTR}, albeit with low potency; the most potent inhibitor was the sulphonylurea, glibenclamide, with an $IC_{50} \sim 20$ μM. This shows that SUR and CFTR share some pharmacological properties.

Fluoresceine derivatives like phloxine B have long been used as probes for various ATPases (De Weille *et al*., 1992; Gatto *et al*., 1995). Following from there, their role as modulators of K_{ATP} channels has been investigated (De Weille *et al*., 1992). At μM concentrations, phloxine B inhibited pancreatic K_{ATP} channels; at 100 μM, the compound also transiently reactivated channels which had reached the inactivated state in the absence of cytoplasmic ATP (De Weille *et al*., 1992). In addition, phloxine B has been shown to inhibit ^{3}H-glibenclamide binding in membranes from insulinoma cells (De Weille *et al*., 1992; Schwanstecher *et al*., 1995) and binding of the K_{ATP} channel opener ^{3}H-P1075 in membranes from skeletal (Dickinson *et al*., 1997) and cardiac muscle (Löffler-Walz & Quast, 1998); in all cases IC_{50} values were in the low μM range. Using the recombinant system we have recently shown that the binding site for phloxine B was located on SUR (Hambrock *et al*., 2000).

In consideration of the structural and pharmacological relationship between SUR and CFTR we have examined the effects of phloxine B on CFTR. Human CFTR was expressed in *Xenopus* oocytes and I_{CFTR} was measured in excised macropatches and in the whole cell configuration. The data show that at low concentrations ($\geqslant$10 nM), phloxine B potentiates I_{CFTR} and slows inactivation of the channel; at higher concentrations ($\geqslant$300 nM), it also produces inhibition.

Methods

Expression of CFTR in Xenopus *oocytes*

A 4.7 kb sequence encoding human epithelial CFTR including exon 5 (accession number P13569) was subcloned into the p-Bluescript vector (Stratagene, La Jolla, California, U.S.A.) using the restriction enzymes *Kpn*I and *Not*I (Hipper *et al*., 1995) and amplified in *E. coli* (XL1-Blue, Stratagene). For *in vitro* transcription of cRNA, the plasmid was linearized using *Kpn*I and cRNA was synthesized using the T3 promotor (T3 RNA polymerase and rNTP's, Boehringer Mannheim, Mannheim, Germany) and a 5′ cap (mCAP RNA, Boehringer Mannheim). Adult female *Xenopus laevis* frogs were anaesthetized with 3-aminobenzoic acid ethyl ester solution (1 g l^{-1}) and intact ovary lobes were removed. Oocytes were prepared and injected with the CFTR cRNA as described previously (Bachmann *et al*., 1999). Oocytes were stored under gentle shaking at 16–18°C in a buffer containing (in mM): NaCl 96, KCl 2, $CaCl_2$ 1.8, $MgCl_2$ 1, HEPES 5, Na-pyruvate 2.5, theophylline 0.5, gentamycin 50 μg ml^{-1}, titrated to pH 7.5 with NaOH. The solution was changed after 2 days. Oocytes were used for experiments 1–3 days after injection.

Whole oocyte voltage clamp

Two electrode voltage clamp recordings (Hodgkin *et al*., 1952) were performed in a medium containing (in mM): NaCl 96, KCl 2, $CaCl_2$ 1.8, $MgCl_2$ 1, HEPES 5, pH 7.0 with NaOH. Microelectrodes were drawn from filament borosilicate glass capillaries (GC 150TF, Clark Electromedical Instruments, Pangbourne, U.K.) using a horizontal microelectrode puller (Zeitz, Augsburg, Germany). After filling with 3 M KCl, pipettes had a resistance of 200–500 kΩ. Oocytes were clamped at −25 mV for 30 s and the holding potential was stepped from −90 mV to +10 mV in 10 mV steps lasting 1 s. I_{CFTR} was induced by addition of forskolin (3 μM) and isobutylmethylxanthine (IBMX, 1 mM) to the bath.

Excised macropatches

The patch clamp technique (Hamill *et al*., 1981) was used in the inside-out configuration excising macropatches as described by Hilgemann (1995). After shrinking the oocytes in a hypertonic solution containing 200 mM K^+-aspartate at pH 7.0, the vitelline layer of the oocytes was removed with sharpened watchmaker's forceps. Oocytes were then placed in a bath solution containing (in mM): NaCl 96, KCl 2, $MgCl_2$ 1, EGTA 2, HEPES 5, titrated to pH 7.4 with NaOH. Patch pipettes were drawn as described above and heat polished. After filling with (in mM) NaCl 96, KCl 2, $CaCl_2$ 1.8, $MgCl_2$ 1, HEPES 5, pH 7.4, pipettes had a resistance of 250–400 kΩ. Patches were excised and clamped at −30 mV. Every 30 s, voltage was stepped from −90 mV to +50 mV in 10 mV intervals lasting 1 s. I_{CFTR} was activated by addition of protein kinase A catalytic subunit (cPKA, 30 u $ml^{-1} \sim 17$ nM) and Na_2ATP (0.2 mM) to the bath solution.

Data analysis

Data were recorded with a Geneclamp 500 amplifier (Axon Instruments, Foster City, CA, U.S.A.) using a MacLab interface and the Chart software (AD Instruments, Castle Hill, Australia). Current and voltage signals were filtered at 20 Hz, digitized online at 100 Hz, and stored on a Power Macintosh 8200/120 for later analysis. Data are shown as mean ± s.e.mean. Concentration dependencies were analysed by fitting the logistic form of the Hill equation

$$y = b + (a - b) * (1 + 10^{n*(px-pK)})^{-1} \quad (1)$$

or the sum of two such components to the data. Here b denotes the starting level of the curve and a the level at saturation so that a − b represents the extent of the effect (amplitude); n ($= n_H$) is the Hill coefficient, x the concentration of the compound under study and K the midpoint of the curve with $px = -\log x$ and $pK = -\log K$. Fitting was done according to the Marquardt-Levenberg algorithm using the program SigmaPlot 4.01 (Statistical Product and Service Solutions Inc., IL, U.S.A.). Errors in the parameters derived from the fit to a single curve were estimated using the univariate approximation (Draper & Smith, 1981) and assuming that amplitudes and pK values are normally distributed (Christopoulos, 1998). In the text, generally the K ($= IC_{50}$, EC_{50}) values are given followed by the 95% confidence interval in brackets.

Drugs and solutions

Phloxine B, Na_2ATP, Li_4AMP-PNP (5′-adenosine(β,γ-imido)-triphosphate) and IBMX were from Fluka (Deisenhofen, Germany) and cPKA from Promega (Heidelberg, Germany). Forskolin was a generous gift from Aventis Pharma (Frankfurt, Germany). IBMX (dissolved in diluted NaOH), Li_4AMP-PNP and Na_2ATP were freshly prepared every day. Forskolin was dissolved in ethanol as a 10 mM stock solution and stored at −20°C for 6 months; phloxine B was dissolved in DMSO as 50 and 30 mM stock solutions and kept at 4°C for up to 1 week.

Results

Whole oocyte voltage clamp

Initial experiments were performed with whole oocytes expressing CFTR. Figure 1a shows that superfusion with IBMX (1 mM) and forskolin (3 μM) activated a current which was stable for at least 30 min; upon washout of the activation solution the current decreased to ~15% of the activation level within 15 min. No activation of current was seen in control experiments using diethylpyrocarbonate-H_2O-injected oocytes (not shown), suggesting that the current observed after superfusion of IBMX and forskolin was indeed the CFTR Cl^- current, I_{CFTR} (Drumm *et al*., 1991; Bear *et al*., 1991). Addition of phloxine B (0.3 μM) in the presence of activation solution had no effect on I_{CFTR} during the activation period; during washout, however, the current decayed slowly, indicating that phloxine B slowed deactivation of CFTR (Figure 1b). At 30 μM, phloxine B inhibited I_{CFTR} slightly; washout with control buffer produced little change and a substantial activation of the current remained (Figure 1c). At 100 μM, phloxine B inhibited I_{CFTR} to ~60%; during washout, the current rapidly increased to ~70% and remained approximately stable thereafter (Figure 1d).

Figure 2a shows I_{CFTR} after 6 and 15 min of washout normalized with respect to the current immediately before washout. In the absence of phloxine B, the current deactivated with a half-time of ~6 min and had decayed to 15% of the activation level after 15 min. After application of 0.3 μM phloxine B, deactivation the current occurred with a half-time of 17 min, indicating that the drug slowed deactivation by ~3 times. At 30 and 100 μM, phloxine B inhibited I_{CFTR} and, during washout, both slowing of deactivation and partial reversal of inhibition must be considered (see Figure 1d). The latter explains that at 100 μM phloxine B, the current at 6 and 15 min was higher than at the beginning. At both concentrations of phloxine B, the currents did not change significantly between 6 and 15 min of washout.

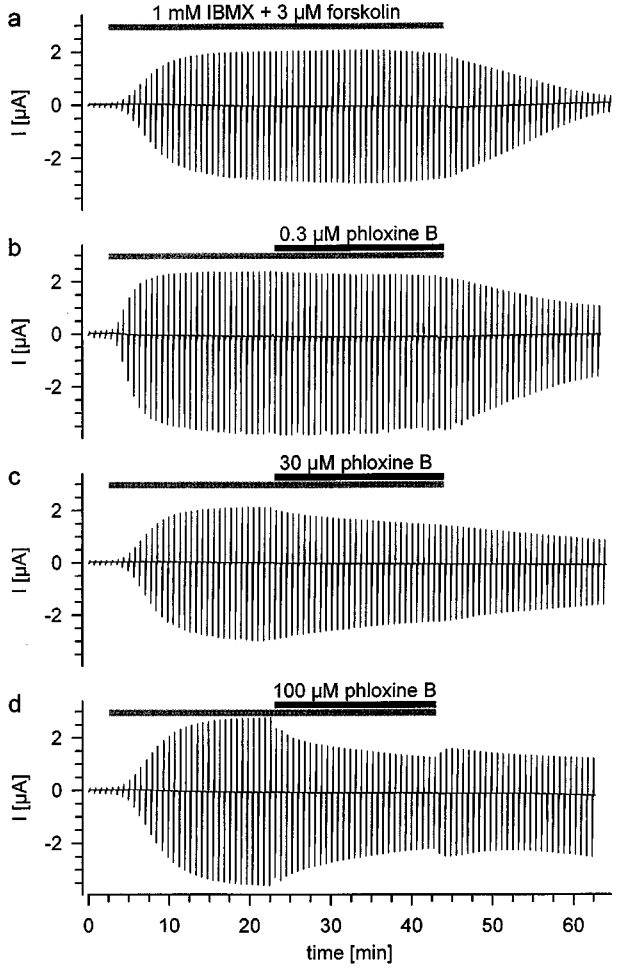

Figure 1 Effect of phloxine B on the CFTR Cl^- current, I_{CFTR}, in the whole cell configuration. Two electrode voltage clamp recordings from *Xenopus* oocytes injected with hCFTR-mRNA are shown. Oocytes were clamped at −25 mV and voltage was stepped every 30 s from −90 mV to +10 mV in 10 mV steps of 1 s duration. CFTR was activated by superfusion of IBMX + forskolin. (a) Control experiment showing activation of I_{CFTR} and washout of activation solution. (b–d) Addition of phloxine B to the bath after activation of CFTR followed by washout with control buffer. Note the concentration-dependent inhibition of I_{CFTR} and the persistent activation of CFTR during washout of the activation solution.

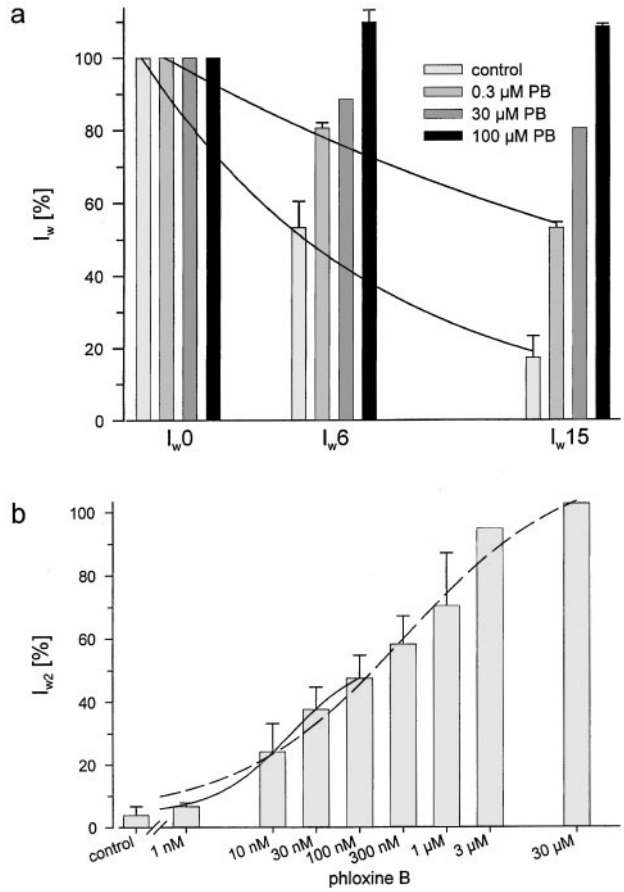

Figure 2 Effect of phloxine B on I_{CFTR} during washout. The current (I_w) at −70 mV is normalised with respect to the current immediately prior to washout; means ± s.e.mean from 2–5 experiments. (a) Current in whole oocytes at the beginning (I_{w0}) and after 6 (I_{W6}), and 15 min (I_{W15}) of washout of activation solution + phloxine B with control buffer. The solid curves show fits of an exponential to the data giving half-times of 6.3 ± 0.3 and 17 ± 1 min at 0 and 0.3 μM phloxine B, respectively. The current at the beginning of washout was 100, 98, 79, and 60% of the activation current prior to addition of phloxine B = 0, 0.3, 30 and 100 μM phloxine B, respectively. (b) Current in macropatch experiments after 2 min washout. The broken curve shows fit of the Hill equation to all data, the solid curve to the data up to 100 nM phloxine B; for fitting parameters see text.

Stimulatory and inhibitory effects of phloxine B on I_{CFTR} in macropatches

A more detailed investigation of phloxine B was performed in macropatches in the inside-out configuration. Addition of cPKA (30 u ml^{-1}) + MgATP (0.2 mM) to the bath induced, at −30 mV, a negative holding current and increased the current response to the voltage step protocol (Figure 3a). After ~5 min the response reached a stable level and faded rapidly upon washout of the activating solution. In control (diethylpyrocarbonate-H_2O^- injected) oocytes, this was not observed, indicating that the conductance activated by cPKA + ATP represented I_{CFTR} (Gadsby & Nairn, 1999).

The effects of phloxine B, applied after activation of I_{CFTR}, are shown in Figure 3b–e. At 30 nM, the substance increased I_{CFTR} rapidly to 140% (value at −70 mV). The current remained stable during the activation period; upon washout with control buffer, substantial activation of I_{CFTR} remained (Figure 3b). At 300 nM (Figure 3c), stimulation (179%) was transient. During the activation period in the presence of phloxine B (6 min), I_{CFTR} decreased to 112% and remained unchanged during 3 min of washout. At 3000 nM, a small increase was followed by a rapid and strong decline; again the current remained unaltered during washout (Figure 3d). At 30 μM, phloxine B showed only an inhibitory effect (Figure 3e).

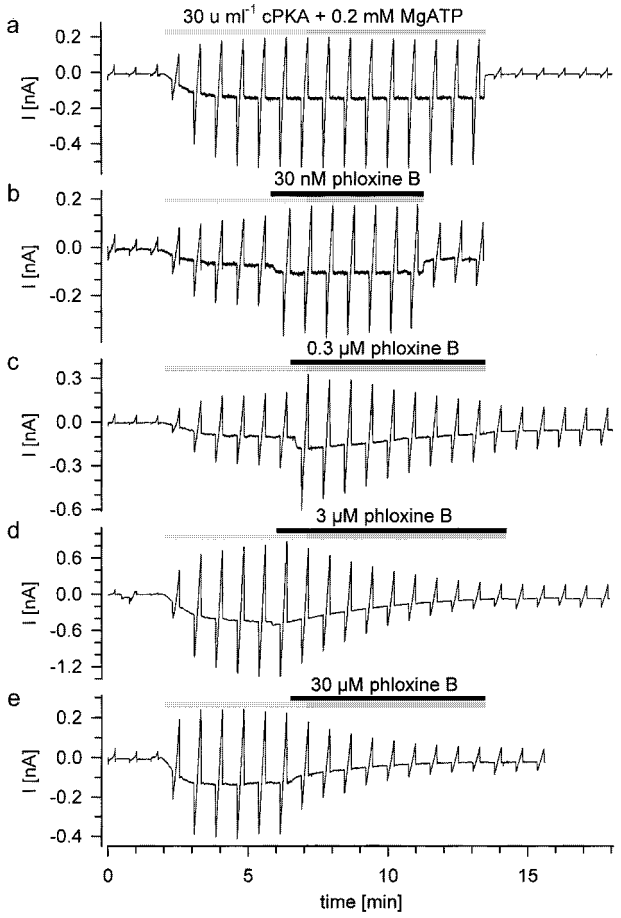

Figure 3 Effects of phloxine B on I_{CFTR} in inside-out macropatches in a symmetrical high Cl^- solution. Patches were excised from oocytes injected with hCFTR-mRNA, held at −30 mV and stepped every 30 s from −90 mV to +50 mV in 10 mV steps of 1 s duration. CFTR was activated by addition of cPKA + ATP in the presence of 1 mM Mg^{2+}. (a) Activation of CFTR and washout of cPKA + MgATP. (b–e) Addition of phloxine B to the bath after activation of CFTR followed by washout with control buffer.

Figure 4 illustrates the concentration-dependent stimulatory and inhibitory effects of phloxine B on I_{CFTR} at −70 mV, measured at 0.5 and 6 min after addition of phloxine B. At both time points, I_{CFTR} showed a bell-shaped concentration-dependence and an equation with two logistic terms was fitted to the data. At 0.5 min (Figure 4a), the ascending limb, which reflects potentiation, extrapolated to a maximum value of 220 ± 23% with midpoint at 45 (9,102) nM. The descending limb extrapolated to ~60 ± 22% reflecting the fact that high concentrations of phloxine B induced immediate inhibition; the midpoint was at 1.6 (0.8,3.2) μM. Due to the overlap of the two limbs, maximal stimulation of I_{CFTR} is calculated to ~180%. This value was indeed reached at 300 nM phloxine B in seven out of 11 experiments (179 ± 10%); in four patches only a small increase was found (112 ± 3%). Figure 4b shows the concentration-dependence of the current 6 min after application of phloxine B. The ascending limb was small with an amplitude of 34 ± 8% and a midpoint at 4 (0.4,40) nM. The prominent inhibitory limb extrapolated to ~16% of control with midpoint at 0.8 (0.6,1.1) μM and Hill coefficient 1.9 ± 0.4.

The phloxine B-dependence of the current measured after 2 min washout is presented in Figure 2b. The current, normalized with respect to the value immediately prior to

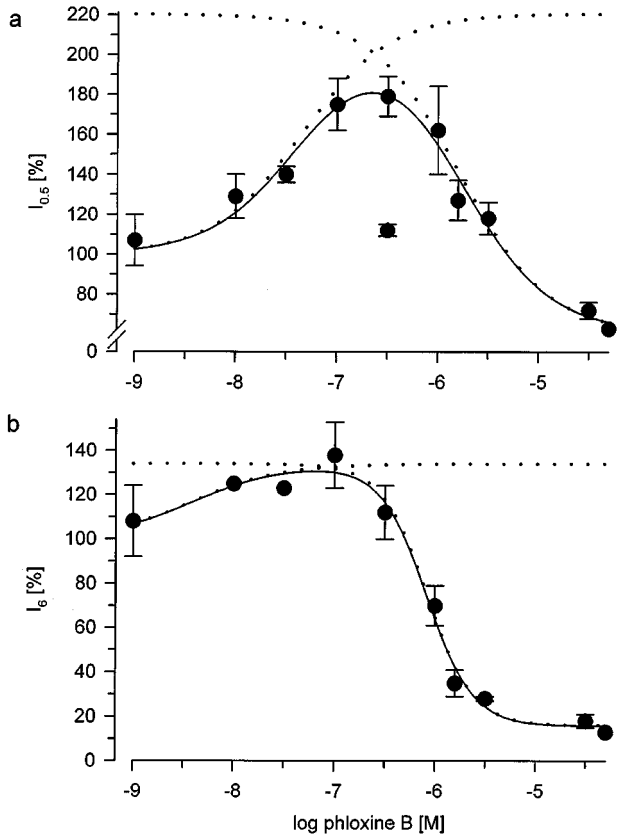

Figure 4 Concentration-dependent effects of phloxine B on I_{CFTR} at −70 mV. Experiments were performed as in Figure 3, basal currents were subtracted and the resulting currents normalized with respect to the activation current prior to drug addition. (a) Current at 0.5 min after addition of phloxine B ($I_{0.5}$); $n = 3–14$ per point. A superposition of two logistic equations with Hill coefficients set to 1 was fitted to the data (solid curve); the dotted curves show the individual components. The parameters of the fit were: b = 100, amplitudes: 120 ± 23/−158 ± 22% and pK values: 7.35 ± 0.18/5.79 ± 0.15 for the two components, respectively. (b) Current at 6 min after addition of phloxine B (I_6); $n = 2–5$ per point. The fitting parameters were b = 100, amplitudes: 34 ± 8/−118 ± 10%, pK values: 8.40 ± 0.49/6.08 ± 0.07, and Hill coefficients (n): 1.0/1.9 ± 0.4 for the two components, respectively.

washout, extrapolates to 111±14% at saturation with midpoint at 0.3 (0.1,1.1) μM phloxine B and Hill coefficient 0.44±0.08. The flatness of the curve reflects the fact that the current after 2 min washout of phloxine B is determined by several processes, i.e. deactivation of the channel, (partial) reversal of potentiation and (partial) reversal of inhibition. Indeed, if analysis is restricted to the values at ⩽100 nM phloxine B, where inhibition does not yet come into play, the curve extrapolates to 50±10% with midpoint at 15 (5,50) nM and Hill coefficient ~1 (Figure 2b). In an alternative analysis, currents were normalized with respect to the I_{CFTR} prior to addition of phloxine B. In this case, the concentration-dependence has a bell-shaped appearance (not shown). The ascending limb, which reflects deactivation and the residual stimulation after 2 min washout, extrapolates to 100±39% of the predrug current with midpoint at 30 (10,76) nM.

The traces shown in Figure 3 suggest that the effects of phloxine B did not depend significantly on voltage. Indeed, the concentration dependent effects at 40 mV (not illustrated) were similar to those shown in Figure 4. The fit to the data at 0.5 min showed that the ascending/descending limb extrapolated to 340±210%/90±110% with midpoint at 100 (20,560) nM/0.6 (0.1,3.2) μM, respectively. After 6 min, the ascending/descending component extrapolated to 152±40%/16±46% with midpoint at 30 (3,300) / 600 (420,1000) nM and Hill coefficients 1.0/1.4±0.5, respectively. The large errors in these parameters mainly reflected the fact that the midpoints of the two components were lying more closely together without that this difference from the parameters at −70 mV became significant.

Dependence of phloxine B effects on CFTR activation conditions

In order to gain more insight into the complex effects of phloxine B, the activation conditions of CFTR were varied and phloxine B was applied at 0.3 μM, a concentration where both stimulation and inhibition were prominent (Figure 3c). When applied prior to activation of I_{CFTR} in the presence of buffer (Figure 5a) or MgATP (0.2 mM; not shown), phloxine B did neither affect basal current nor prevent a subsequent activation. However, phloxine B was able to increase channel activity when CFTR was in a state of very low phosphorylation (Figure 5b). In these experiments, CFTR was first activated by superfusion with cPKA + ATP and the activation solution then washed out, leaving CFTR in a state of residual phosphorylation with the channel closed in the absence of ATP. Subsequent superfusion with ATP induced some channel activity and addition of phloxine B (0.3 μM) rapidly increased I_{CFTR}; upon washout with control buffer, the current decreased slightly below the level in the presence of ATP prior to the addition of phloxine B (Figure 5b). In seven experiments, phloxine B (0.3 μM) increased I_{CFTR} to 178±11% ($n=7$), i.e. an augmentation similar to that seen in the presence of cPKA (Figure 4, Table 1). Figure 5c illustrates that under the routine activation conditions (cPKA = 30 u ml^{-1} ~17 nM, MgATP = 0.2 mM), the non-hydrolyzable ATP analogue AMP-PNP did not increase I_{CFTR}, instead a slight inhibition was seen.

In other experiments, the ATP concentration used for activation was varied; free Mg^{2+} was kept at ~0.8 mM and cPKA at 30 u ml^{-1} (Figure 6, Table 1). Figure 6a shows the ATP-dependence of I_{CFTR} in the absence of phloxine B. Increasing [ATP] from 0.02 to 0.2 mM doubled the current, further increase to 2 mM ATP had little effect. When these experiments were performed in the continued presence of phloxine B (0.3 μM), maximum stimulation was already observed at 0.02 mM ATP; higher ATP concentrations did not increase the current but prevented the inhibitory effect of phloxine (Figure 6b and Table 1). For comparison, phloxine B was also given after activation of CFTR. At 0.02 mM ATP, the

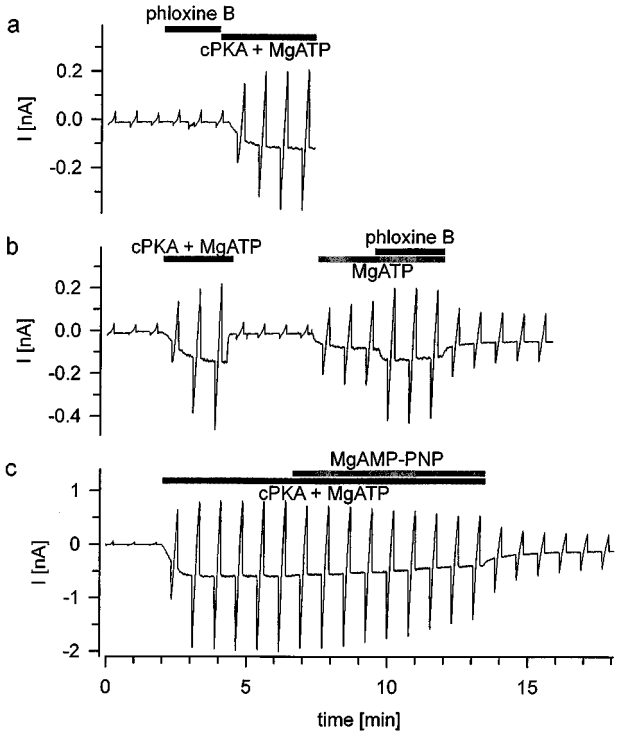

Figure 5 Different activation conditions of CFTR and effects of phloxine B and AMP-PNP. (a) Phloxine B (0.3 μM) alone does neither activate CFTR nor prevent subsequent activation of I_{CFTR} by cPKA + MgATP. (b) Effects in a low phosphorylation state of CFTR: After activation with MgATP + cPKA and washout of activation solution, the CFTR channel was opened again with MgATP, followed by additional application of 0.3 μM phloxine B. (c) Superfusion of MgAMP-PNP (0.5 mM) after activation. In all experiments, cPKA was 30 u ml^{-1} and MgATP 0.2 mM.

Table 1 ATP-dependence of CFTR Cl^- current

Current	*Phloxine B* (μM)	*MgATP* (mM), (n) 0.02	0.2	2
I_A/I_A at 2 mM MgATP (%)[a]	—	55±5 (3)	90±5 (9)	100
I_A/I_A at 2 mM MgATP (%)[b]	0.3	101±5 (5)	94±3 (5)	100
$I_{0.5}/I_A$ (%)[c]	0.3	217±21 (7)	179±10 (7)	111±4 (8)
$I_6/I_{0.5}$ (%)[c]	0.3	45±3 (3)	61±4 (5)	76±6 (3)
$I_{0.5}/I_A$ (%)[c]	3.0	—	—	97±5 (4)

$CFTR\ Cl^-$ current was activated by protein kinase A catalytic subunit (30 u ml^{-1}) and the ATP concentration indicated. I_A denotes the current after activation of CFTR; $I_{0.5}$ and I_6 are the currents 0.5 and 6 min after addition of phloxine B, repectively. The experimental protocols are identical or analogous to those shown in [a]Figure 6a, [b]Figure 6b, [c]Figure 3. Note that in Figure 3 phloxine B is given after, in Figure 6b during activation.

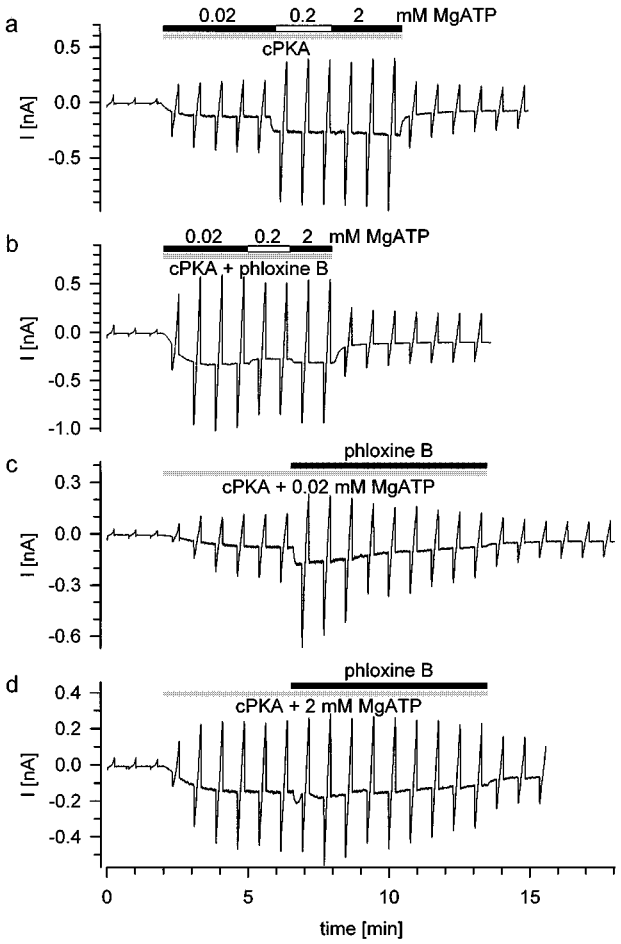

Figure 6 Effects of phloxine B on I_{CFTR} under different activation conditions. (a) ATP-dependence of activation in the presence of cPKA (30 u ml^{-1}). (b) Additional application of 0.3 μM phloxine B. (c, d) Effects of 0.3 μM phloxine B after activation with cPKA + MgATP (0.02 or 2 mM).

stimulatory and inhibitory effects of phloxine B were very pronounced (Figure 6c). At 2 mM ATP, the stimulatory effect of phloxine B (0.3 μM) was again absent and the inhibitory effect weakened (Figure 6d). When in the presence of 2 mM MgATP, the phloxine B concentration was increased to 3 μM, there was again no stimulation (see Table 1); however, inhibition was much faster and stronger than at 0.3 μM phloxine B (similar to Figure 3d).

Discussion

In macropatch experiments, phloxine B potentiated I_{CFTR} and slowed deactivation of the CFTR chloride channel with EC_{50} values of 15–45 nM; at concentrations $\geqslant$0.3 μM, inhibition was also apparent.

Potentiation

The potentiation of I_{CFTR} by phloxine B could be caused by activating kinases or inhibiting phosphatases, thereby increasing the phosphorylation state of CFTR, and/or by an interference with MgATP binding and hydrolysis at the NBFs of CFTR. When given alone or in the presence of MgATP, phloxine B was unable to stimulate I_{CFTR} (Figure 5a). This showed that at least partial phosphorylation of the regulatory domain was required before phloxine B could act. In addition, the stable activation of weakly phosphorylated CFTR by MgATP alone showed that phosphatase activity in the patch was low and the immediate effect of phloxine B suggested that it did not act as phosphatase inhibitor (Figure 5b). A key observation is that the potentiating effect depended strongly on ATP concentration. As shown in Table 1, phloxine B (0.3 μM) shifted the ATP-dependence of I_{CFTR} towards the left and, at 0.2 mM MgATP, increased I_{CFTR} beyond the level reached at saturating MgATP (2 mM). This suggests that the compound directly interacted with (the phosphorylated state of) CFTR to sensitize the NBFs for MgATP and to increase the open probability of the channel. AMP-PNP did not increase the current under routine activation conditions (Figure 5c). Within the framework of the gating model established by Hwang *et al.* (1994), this would suggest that, even in the presence of cPKA, CFTR was in a state of partial phosphorylation and that only NBF1 was available for ATP binding and hydrolysis (reviews: Gadsby & Nairn, 1999; Sheppard & Welsh, 1999). Considering the ability of phloxine B to modify ATP binding of many ATPases (see Introduction) one may assume that phloxine B increases the sensitivity of NBF1 for MgATP by a direct interaction with NBF1 or NBF2. Furthermore, potentiation was observed only at non-saturating MgATP. Since the ATP concentration in the oocyte is in the mM range (Gribble *et al.*, 1997), this explains the absence of the stimulatory effect of phloxine B on I_{CFTR} in the whole cell configuration.

In the macropatch experiments, potentiation occurred with an EC_{50} value of 45 (19,102) nM and was complete in less than 30 s. At low concentrations of phloxine B, ($\leqslant$30 nM), the current level was well maintained during the activation period. At concentrations $\geqslant$100 nM, however, the current faded during activation with a time course which became faster with increasing concentrations of phloxine B. A plausible explanation is that fading reflects the inhibitory effect of phloxine B discussed below. Alternatively, it may be that potentiation of I_{CFTR} by phloxine B is an intrinsically transient process. It is difficult to distinguish between these alternatives since at low concentrations of phloxine B (where inhibition does not yet occur, see below), the current in the presence of the drug is well maintained over many minutes, contrasting with the limited life-time of the patch.

Slowing of deactivation

Phloxine B slowed the decay of I_{CFTR} upon washout of the activation solution in both oocytes and macropatches. Washout leads to fast release of the hydrolysis products of ATP from the NBFs and slow dephosphorylation of CFTR, thereby inducing deactivation of the channel (Gadsby & Nairn, 1999). How could phloxine B slow down channel deactivation? It is tempting to speculate that phloxine B locks the channel in the open state like several other compounds, amongst them the phosphate analogues orthovanadate and BeF_3 (Baukrowitz *et al.*, 1994), the non-hydrolyzable ATP analogue AMP-PNP (Hwang *et al.*, 1994) and the tyrosine kinase inhibitor genistein (Illek *et al.*, 1995; for review see Gadsby & Nairn, 1999; Hwang & Sheppard, 1999; Schultz *et al.*, 1999). Whereas AMP-PNP induces the locked open state of CFTR only if the channel is in a high phosphorylation state (cardiomyocytes: Hwang *et al.*, 1994; CFTR expressed in oocytes: Weinreich *et al.*, 1999), genistein is most effective when CFTR is weakly phosphorylated (Hwang & Sheppard, 1999).

In the macropatch experiments, phloxine B slowed down channel deactivation with an EC_{50} value of 15–30 nM, i.e. a value close to that determined for potentiation of I_{CFTR}. One

might speculate that the two phenomena actually reflected the same process; indeed, locking CFTR in the activated state would both increase I_{CFTR} during the activation period and delay the decay of the current during washout. In the oocyte, however, slowing of deactivation was observed at all phloxine B concentrations tested, but potentiation did not occur (Figures 1 and 2). This suggests that the two phenomena are separate; however, they may be mediated *via* binding of phloxine B to a single site on CFTR, possibly at a NBF (see above).

Figure 2 shows that in the whole cell configuration, higher concentrations of phloxine B were required to slow deactivation as compared to the concentrations effective in the inside-out patch. A plausible explanation is that in the intact oocyte, phloxine B may not reach its binding site easily. This would be expected if the binding site was located intracellularly and/or if absorption of the amphiphilic compound by the oocyte yolk would occur. Indeed, phloxine B, available as the disodium salt, carries two negative charges at neutral pH (the closely related dye eosin has pK_a values of 3.25 and 3.8 (Levillain & Fompeydie, 1985)) and is not expected to cross the membrane well.

Inhibition of I_{CFTR}

Inhibition of I_{CFTR} by phloxine B was observed in the whole cell configuration and in macropatches. In oocytes, higher concentrations were again required to produce the effect which may reflect the difficulties of phloxine B to reach its target site as discussed above. In addition, a small part of the inhibition observed in the oocyte experiments at 100 μM phloxine B (Figure 1d) was rapidly washed out whereas the major part remained unchanged, suggesting that inhibition was a heterogeneous process.

In the macropatch experiments, inhibition occurred with IC_{50} values of 1.6 (0.8,3.2) and 0.8 (0.6,1.1) μM, when measured at 0.5 and 6 min of drug application (Figures 3 and 4). Since at low concentrations of phloxine B ($\leqslant 1$ μM), inhibition is a slow process, it had not yet reached its final level after 6 min; hence, the inhibitory limbs in Figure 4 are not at equilibrium. This may explain the steepness of the curve after 6 min (Hill coefficient 1.9 ± 0.4). It is likely that the true IC_{50} value for inhibition is ~ 1 μM, which is 10–30 times higher than the EC_{50} values for potentiation and slowing of deactivation. Hence, inhibition is mediated by binding of phloxine B to a second site with low affinity.

The mechanism(s) by which phloxine B inhibits I_{CFTR} remain(s) to be defined. In the macropatch, however, inhibition was weakened at increasing ATP concentrations and development of inhibition was slow. This suggests that phloxine B inhibited I_{CFTR} by interfering with channel gating and not by blocking the CFTR channel pore from the inside. Furthermore, since ATP hydrolysis at NBF1 opens the channel, one may speculate that phloxine B interferes with this process at NBF1 either by a direct or an indirect (allosteric) interaction. In the oocyte, a major part of the inhibition at 100 μM phloxine B occurred slowly and remained during 20 min washout, rendering it unlikely that a pore block from the outside was responsible for this component. However, the minor part which was rapidly reversed upon washout may be caused by blocking the CFTR pore (see also below: genistein; Lansdell *et al*., 2000).

Comparison with genistein

The effects of phloxine B on I_{CFTR} resemble those of genistein in that both compounds potentiate I_{CFTR} once CFTR is phosphorylated (Reenstra *et al*., 1996; Yang *et al*., 1997; Wang *et al*., 1998) and that both inhibit CFTR in a manner that is counteracted by ATP (Lansdell *et al*., 2000). Since both compounds interfere with ATP-using enzymes (De Weille *et al*., 1992; Akiyama & Ogawara, 1991), this similarity may not be too surprising. However, there are also striking differences. First, potentiation of I_{CFTR} by phloxine B was due, at least in part, to a leftward shift in the ATP-dependence of activation; in contrast, genistein does not alter the ATP-dependence (Weinreich *et al*., 1997). Second, phloxine B slowed deactivation of the channel upon washout of activation solution whereas genistein does not (Weinreich *et al*., 1997; Wang *et al*., 1998). Third, the concentration regions of potentiation and inhibition were quite separated for phloxine B (45 nM vs 1 μM) whereas for genistein, they are closely together (20 μM *vs* 60 μM; Wang *et al*., 1998). The inhibitory effect of genistein is due to two mechanisms, an inhibition of channel opening, possibly by an interaction with NBF1 and a weak block of the CFTR Cl^- channel (Lansdell *et al*., 2000).

In conclusion, we have shown here that the fluoresceine derivative, phloxine B, is a potent modulator of CFTR. At low concentrations, it potentiates I_{CFTR} and slows channel deactivation, at higher concentrations channel inhibition occurs. At least some of these effects may be mediated by direct interactions with CFTR from the inside of the cell, possibly at the NBFs. More work, including single channel recordings and appropriate mutations of CFTR, is required to elucidate the complex mechanisms of action of this compound.

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