PKC-mediated Stimulation of Amphibian CFTR Depends on a Single Phosphorylation Consensus Site. Insertion of This Site Confers PKC Sensitivity to Human CFTR

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ABSTRACT   Mutations of the CFTR, a phosphorylation-regulated Cl⁻ channel, cause cystic fibrosis. Activation of CFTR by PKA stimulation appears to be mediated by a complex interaction between several consensus phosphorylation sites in the regulatory domain (R domain). None of these sites has a critical role in this process. Here, we show that although endogenous phosphorylation by PKC is required for the effect of PKA on CFTR, stimulation of PKC by itself has only a minor effect on human CFTR. In contrast, CFTR from the amphibians *Necturus maculosus* and *Xenopus laevis* (*X*CFTR) can be activated to similar degrees by stimulation of either PKA or PKC. Furthermore, the activation of *X*CFTR by PKC is independent of the net charge of the R domain, and mutagenesis experiments indicate that a single site (Thr^{665}) is required for the activation of *X*CFTR. Human CFTR lacks the PKC phosphorylation consensus site that includes Thr^{665}, but insertion of an equivalent site results in a large activation upon PKC stimulation. These observations establish the presence of a novel mechanism of activation of CFTR by phosphorylation of the R domain, i.e., activation by PKC requires a single consensus phosphorylation site and is unrelated to the net charge of the R domain.

KEY WORDS: chloride channel • PKA • ABC proteins • R domain

INTRODUCTION

Cystic fibrosis results from mutations in the CFTR, a 1,480–amino acid protein that functions as a Cl⁻-selective ion channel in a variety of epithelial cells. CFTR has two topologically equivalent halves, each consisting of a transmembrane domain (six membrane-spanning helices) followed by a nucleotide-binding domain. The cytoplasmic regulatory domain (R domain)[1] links these two halves. The membrane-spanning helices form the anion-conductive pore, the nucleotide-binding domains bind and hydrolyze ATP and are involved in channel gating, and the R domain contains consensus phosphorylation sites for PKA and PKC (for review see Seibert et al., 1997).

CFTR channels are gated by exposure to PKA and ATP, both in excised patches and after purification and reconstitution in planar lipid bilayers (for review see Seibert et al., 1997). Although it is well established that phosphorylation of the R domain is essential for activation of CFTR, the precise molecular mechanism of this activation has proven elusive, and is thought to involve

several sites and complex interactions (for reviews see Chang et al., 1993; Gadsby and Nairn, 1999; Sheppard and Welsh, 1999; Ma, 2000).

PKA-mediated activation of human CFTR (hCFTR) requires endogenous phosphorylation by PKC (Cohn et al., 1991; Jia et al., 1997; Vankeerberghen et al., 1999). However, PKC-mediated phosphorylation, per se, has only a small effect on hCFTR activity. In contrast, we discovered that *Necturus* CFTR can be activated by either PKA- or PKC-mediated phosphorylation (Copello et al., 1993). The same phenomenon occurs with *Xenopus* CFTR (*X*CFTR), as shown in this study.

The goal of the present work was to determine the molecular bases for the differences in the activation of hCFTR and *X*CFTR by PKC stimulation. Our results show that, in contrast with the complexity of PKA-mediated activation, the stimulation of *X*CFTR by PKC depends on a single PKC consensus phosphorylation site in the R domain. Confirming the importance of the critical site in *X*CFTR, engineering an equivalent site in hCFTR makes this molecule responsive to PKC stimulation.

MATERIALS AND METHODS

cRNA Preparation

Full-length hCFTR and *X*CFTR cDNAs were subcloned into the oocyte expression vector pOCYT7 (gift from Dr. Nancy Wills, University of Texas Medical Branch; Mo et al., 1999). This expression vector contains the *Xenopus* 5′ and 3′ β-globin untranslated regions to boost expression in oocytes (Mo et al., 1999). In brief,

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[1]*Abbreviations used in this paper:* hCFTR, human CFTR; H*X*H-CFTR, CFTR chimera containing the transmembrane domains and nucleotide-binding domains of hCFTR and the regulatory domain of *Xenopus* CFTR; R domain, regulatory domain; *X*CFTR, *Xenopus* CFTR.

human CFTR (a gift from Dr. Lap-Chee Tsui, The Hospital for Sick Children, Toronto, Canada), was cut with XmaI and SalI and ligated into pOCTY7 cut with the same restriction enzymes. *Xenopus* CFTR (a gift from Drs. Margaret Price and Michael Welsh, University of Iowa, Iowa City, IA; Price et al., 1996), was cloned into the KpnI and SalI sites of pOCYT7. The resulting vectors were linearized with XhoI and used as templates for T7-directed capped cRNA synthesis (mMessage mMachine; Ambion).

Oocyte Preparation and cRNA Injection

Xenopus laevis oocytes were isolated and prepared using a well-documented protocol (Sharon et al., 1997). In brief, ovarian lobes were surgically removed from frogs anesthetized with tricaine methanesulfonate (1 g/liter). Stage-V and -VI oocytes were isolated by manual dissection and defolliculated using enzymatic treatment with 1 mg/ml type I collagenase in Barth's solution ([in mM] 88 NaCl, 1.0 KCl, 1.0 CaCl$_2$, 1.0 MgCl$_2$, and 10 HEPES/NaOH, pH 7.40) containing 10 μg/ml penicillin, 10 μg/ml streptomycin, and 100 μg/ml gentamicin sulfate, for 16 h (all incubations were performed at 16°C). The enzymatic treatment was followed by a 2-h incubation in calcium-free Barth's solution. Treated oocytes were transferred to Barth's solution. Oocytes were injected with 47 nl of CFTR cRNA (5–10 ng) or sterile water 24–72 h before analysis, using a Nanoject autoinjector (Drummond Scientific). The 8-Br-cAMP–activated currents in the mutant H667R-hCFTR were very high; thus, cRNA injection was reduced to 1–2.5 ng/oocyte.

Two-electrode Voltage Clamp

Oocytes were bathed in the HEPES-buffered solution ND96 ([in mM] 96 NaCl, 2 KCl, 1.0 MgCl$_2$, 1.8 CaCl$_2$, and 5 HEPES/NaOH, pH 7.40). All experiments were performed at 22–24°C. Borosilicate microelectrodes were pulled with a horizontal puller (P-97; Sutter Instruments), filled with 3 M KCl, and had tip resistances of 0.5–1.5 MΩ when immersed in ND96 solution. A voltage-clamp amplifier (model OC-725C; Warner Instruments) was used to measure whole-oocyte conductance. Voltages were referenced to the bath. Membrane currents were filtered at 1.0 kHz, digitized, stored, and analyzed with pCLAMP version 8.0 (Axon Instruments). I-V relationships were obtained by current measurements 400 ms after changing the potential from a holding value of -30 mV to test values ranging from -100 to $+30$ mV, in 10-mV steps, with 100-ms intervals between pulses. Oocyte conductance was determined in the consistently linear range between the reversal potential and 30 mV. The membrane conductances of unstimulated oocytes (i.e., no 8-Br-cAMP or PMA) after cRNA injection were not different from those of water-injected oocytes (Table I). In hCFTR- and *XCFTR*-expressing oocytes, membrane conductances were directly proportional to the amount of cRNA injected (not shown) and time after injection (Table I). In all experiments detailed here, supra-maximal concentrations of either cAMP cocktail (250 μM 8-Br-cAMP and 25 μM forskolin) or PMA (250 nM) were used to stimulate maximally PKA or PKC, respectively. For the time course experiments, differences between the current at -30 mV (the holding potential) and 0 mV were measured every 5 s. Halide selectivity sequences were determined from the changes in reversal potential after substituting NaCl with the corresponding sodium-halide salt for 2 min before the I-V plots. Corrections for liquid junction potentials were as previously described (Vanoye et al., 1997).

Whole-cell Patch Clamp

Whole cell patch-clamp studies were performed essentially as previously described (Vanoye et al., 1997). COS-1 cells transiently transfected with hCFTR or *XCFTR* cDNA into the pTracer-CMV vector (Invitrogen) were studied after 2–3 d. The bath solution contained (in mM): 140 NMDG, 0.5 MgCl$_2$, 1.3 CaCl$_2$, and 10 HEPES/NaOH, pH 7.4. The pipet solution contained (in mM): 140 NMDG, 1.2 MgCl$_2$, 1 EGTA, 2 ATP 10 mM HEPES/NaOH, pH 7.2. Patch pipets (2–4 MΩ tip resistance in nearly symmetric NMDG-Cl) were pulled with a horizontal pipet puller (model P-97; Sutter Instruments). Negative pressure was used to rupture the membrane patch after obtaining a gigaohm seal, and currents were measured in the whole-cell configuration with an amplifier (model Axopatch 200A; Axon Instruments). The holding voltage (V_m) was -60 mV. I-V relationships were constructed using 400-ms voltage pulses between -80 to $+80$ mV, at 10-mV intervals. PClamp6 (Axon Instruments) was used for generation of the pulses, data collection, and analysis.

Human-Xenopus R Domain Chimera Construction

A chimera, in which residues 607–811 of the hCFTR were replaced by the corresponding *XCFTR* residues, was engineered by a combination of site-directed mutagenesis and PCR. Site-directed mutagenesis was used to introduce MluI and KasI unique sites at the 5′ and 3′ ends of the R domain hCFTR cDNA sequence with silent mutations. The QuickChange site-directed mutagenesis kit (Stratagene) was used to insert the unique restriction sites. Two mutagenic oligonucleotide primers, each complementary to opposite strands of the vector, were used for each mutagenesis reaction. The wild-type hCFTR was used as a template. The primers used were as follows (only sense primer is shown, restriction sites underlined): 5′-TGGCTAACAAAACGCG-TATTTTGGTCAC-3′ (MluI site) and 5′-GGATATATATTCAAG-GCGCCTATCTCAAGAAAC-3′ (KasI site). The reaction mixture was heated at 95°C for 45 s and subjected to 12 cycles for 30 s at 95°C, 30 s at 55°C, and 10 min at 72°C. Cycling was finished by a 10-min incubation at 70°C. The PCR reaction was treated with DpnI to digest the DNA template, and then used to transform *Escherichia coli*. The *XCFTR* R domain cDNA was amplified by PCR, introducing MluI and KasI sites that were used to exchange the hCFTR and *XCFTR* R domain sequences. The PCR primers were as follows (MluI and KasI sites underlined): 5′-GGGACGCG-TATTTTAGTTACATCT AAAGTCG-3′ (forward) and 5′-TTT-GAAGTGGATATATATAATAGGCGCCGCG-3′ (reverse).

PKC Consensus Site Mutagenesis

The QuickChange site-directed mutagenesis kit was used, as described above, to make point mutations. To remove consensus phosphorylation sites, Ser or Thr were substituted with Ala. The primers also contained silent mutations to introduce unique restriction sites for primary screening. The following primers were used (only sense primer is shown, with mutations underlined): 5′-TAATAACTGAGGCCCTGAGACGATGCT-3′ (Thr[665] to Ala,

TABLE I

Conductances of Xenopus Oocytes Expressing CFTR

Unstimulated		cAMP-stimulated		
		24 h	48 h	72 h
Mock	2.9 ± 1.7 (11)	2.7 ± 1.4 (4)	2.9 ± 1.6 (4)	3.2 ± 2.1 (3)
hCFTR	4.2 ± 3.8 (35)	426 ± 60 (16)	685 ± 131 (13)	1,147 ± 217 (6)
XCFTR	3.1 ± 2.1 (34)	214 ± 50 (14)	1,009 ± 268 (14)	2,028 ± 217 (6)

Xenopus laevis oocytes were injected with water (mock), hCFTR cRNA, or *XCFTR* cRNA, and their membrane conductances were measured at 24, 48, or 72 h after injection with and without stimulation with cAMP cocktail. Conductances are expressed in microSiemens. Each result represents the mean ± SEM of *n* experiments (numbers in parentheses).
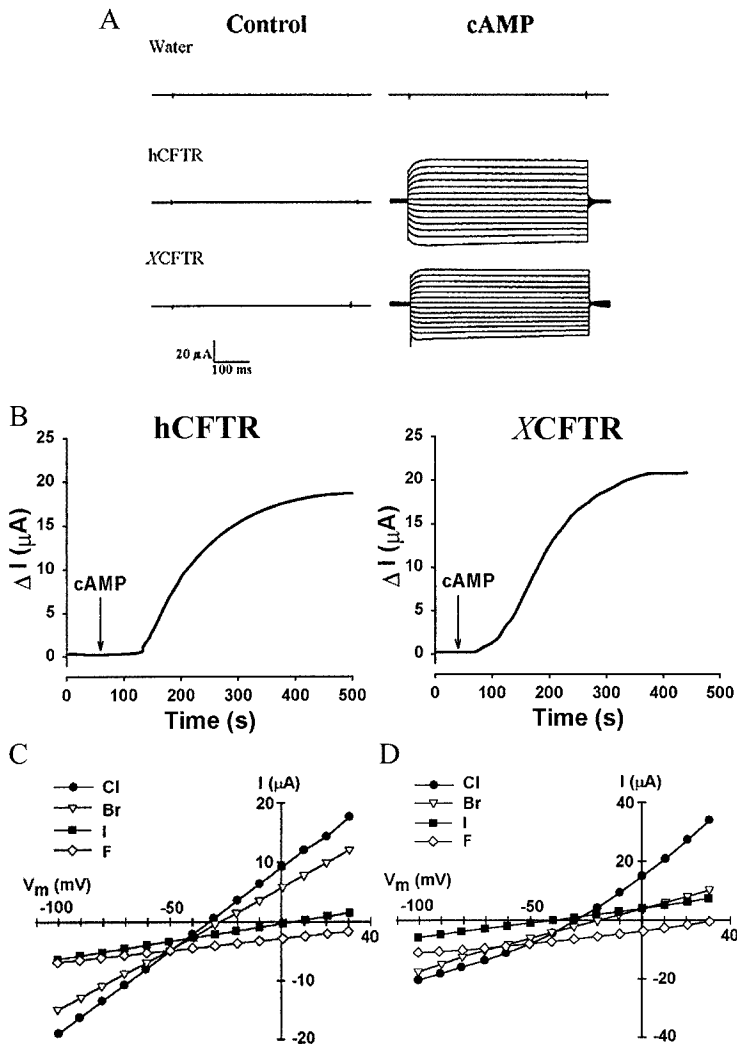
FIGURE 1. Activation of CFTR Cl⁻ conductance by PKA stimulation. The two-electrode voltage-clamp technique was used to measure ionic currents before and ∼30 min after exposure to a cAMP cocktail consisting of 250 μM 8-Br-cAMP and 25 μM forskolin (cAMP). (A) Representative currents obtained in water-injected oocytes and oocytes expressing hCFTR or XCFTR. (B) Time course of hCFTR and XCFTR activation by cAMP. ΔI is the current measured at 0 mV minus the current at −30 mV (holding potential). (C) Halide-selectivity sequence of cAMP-activated hCFTR. I-V relationships were obtained from an oocyte in the constant presence of cAMP cocktail by substituting NaCl in each solution with the corresponding sodium halide salt for 2 min before the I-V plots. Currents were measured at 400 ms after the start of voltage pulses ranging from −100 to 30 mV at 10-mV intervals. (D) Halide-selectivity sequence obtained from a XCFTR-expressing oocyte. Injection of hCFTR and XCFTR cRNAs elicits anion currents with the expected halide selectivity.

plus addition of HaeIII site), 5′-GTCAAGAATA<u>AAGCTT</u>TTAAG-CAGG-3′ (Ser[686] to Ala, plus addition of HindIII site), 5′-TGGG-GATTTC<u>GCTGAG</u>AAAAGAAAGAG-3′ (Ser[694] to Ala, plus addition of DdeI site), and 5′-CAAGAAAAA<u>CTGCAG</u>TTCG-TAAAATG-3′ (Ser[790] to Ala, plus addition of PstI site). For construction of the H667R-hCFTR mutant, the primer used was 5′-AGACCTT<u>GCGCC</u>GTTTCTCA-3′ (construct screened with HhaI, underlined). The wild-type XCFTR or hCFTR in the pOCYT7 vector (see above) was used as a template.

Oocyte cAMP Levels

Intracellular cAMP was determined 24–48 h after cRNA injection. Oocytes were incubated for 20 min in the presence of either 250 nM PMA to activate PKC or 25 μM forskolin to activate PKA. At the end of the incubation period, cells were lysed by sonication. All samples were heated to 95°C for 5 min and centrifuged at 12,000 *g* for 15 min at 4°C. The supernatants were assayed using a cAMP enzyme immunoassay kit (Amersham Pharmacia Biotech).

Western Blot Analysis

Western blot analysis was performed with the affinity-purified rabbit anti-CFTR antibody α1468 (provided by Dr. Jonathan Cohn, Duke University, Durham, NC; Cohn et al., 1991; Torres et al., 1996). The epitope consists of the COOH-terminal 13 amino acids of human CFTR. The last 12 amino acids are identical in hCFTR and XCFTR. Enriched plasma membranes were prepared by membrane biotinylation and streptavidin affinity purification (Pajor et al., 1998). In brief, membrane proteins were labeled by incubating the oocytes with 0.5 mg/ml biotin (EZ-Link Sulfo-NHS-Biotin; Pierce Chemical Co.) for 30 min at room temperature in Barth's solution. Oocytes were washed three times with 4 ml of ice-cold PBS to remove unbound biotin, resuspended in Barth's solution, and lysed at 4°C by sonication with an ultrasonic processor (model GE501; Sonics and Materials). Yolk and debris were collected by centrifugation at 1,000 *g* for 5 min at 4°C. The supernatant was gently mixed for 1 h at 4°C with 50 μl of streptavidin attached to beads (ImmunoPure immobilized streptavidin; Pierce Chemical Co.). Bound membranes were collected by centrifugation at 1,000 *g* for 2 min at 4°C. The pellet was washed twice with PBS, resuspended in electrophoresis sample buffer, and subjected to 10% SDS-PAGE electrophoresis (NuPage Bis-Tris; Novex). Western blots were performed as previously described (Han et al., 1996). The primary antibody concentration was 1 μg/ml, and the secondary antibody was a horseradish peroxidase goat anti–rabbit. CFTR detection was performed by enhanced chemiluminescence (Amersham Pharmacia Biotech).

To activate PKA, intracellular cAMP was elevated using a cAMP cocktail containing 250 μM 8-Br-cAMP and 25 μM forskolin (Sigma-Aldrich). To activate PKC, 250 nM PMA (Sigma-Aldrich) was added to the bath. Stock solutions of these compounds were prepared in water (8-Br-cAMP), dimethylsulfoxide (PMA), or ethanol (forskolin), and diluted to the desired final concentration in ND96 solution immediately before use. At the concentrations used, the vehicles had no effects on the CFTR currents.

Statistical Analysis

Data are expressed as the means ± SEM. Differences between means were compared by either paired or unpaired two-tailed t tests, as appropriate. Statistical significance was ascribed to $P < 0.05$.

RESULTS

Differential Activation of Human and Xenopus CFTR by PKC

We expressed human and *Xenopus* CFTR orthologues in *Xenopus* oocytes because these cells are capable of appropriate posttranslational processing, express CFTR at high levels, and have no significant endogenous cAMP- or PMA-activated Cl⁻ currents (Cunningham et al., 1992). Injection of either human or *Xenopus* CFTR cRNA into *Xenopus* oocytes resulted in functional CFTR expression in the plasma membrane, as shown by the stimulation of CFTR chloride currents in response to 8-Br-cAMP and forskolin (Fig. 1). The current in water-injected oocytes was insensitive to PKA agonists (Fig. 1 A; Bear et al., 1991; Yamazaki et al., 1999). The currents are mediated by CFTR based on their halide selectivities (i.e., $Br^- \approx Cl^- > I^- > F^-$ for hCFTR and $I^- > Cl^- > Br^- > F^-$ for XCFTR [Fig. 1, C and D]), and their reversal potentials (in NaCl-based bath solution) near the Cl⁻ equilibrium potential (about −30 mV; Cunningham et al., 1992).

To determine the effect of PKC-mediated phosphorylation on the activation of the two CFTR orthologues, oocytes expressing hCFTR or XCFTR were exposed to PMA. I-V relationships, current time course, and conductance data in Fig. 2 show that, in hCFTR-expressing oocytes, PMA alone elicits only a small fraction of the conductance observed after the subsequent application of the cAMP cocktail. Exposure to PMA resulted, as previously observed by others (Berger et al., 1993; Yamazaki et al., 1999), in a transient hCFTR conductance (Fig. 2 C) that amounted to 28 ± 7%, followed by a sustained conductance of only 13 ± 3% of the total conductance observed with stimulation of both PKC and PKA (Fig. 2 A, C, and E). Higher PMA concentrations did not increase the currents further. Relative to the current elicited by 250 nM PMA, the current after 500 nM PMA was 103 ± 5% ($n = 4$), and after 1 μM it was 89 ± 9% ($n = 2$).

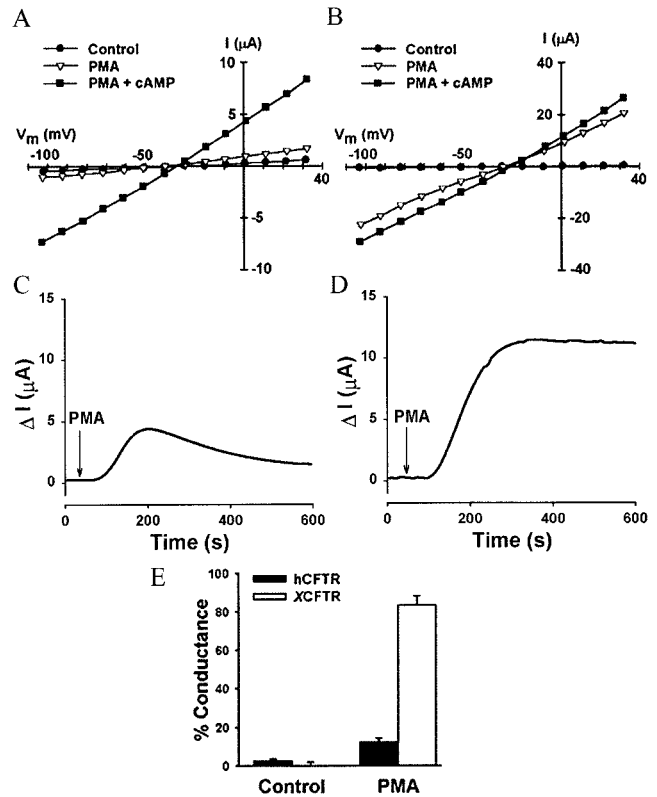In contrast, in XCFTR-expressing oocytes, PMA activated 79 ± 8% of the conductance seen after stimula-

FIGURE 2. Activation of CFTR Cl⁻ conductance by PKC. (A) Representative I-V plot obtained from an oocyte injected with hCFTR. (B) I-V plot obtained from an oocyte injected with XCFTR. (C) Time course of hCFTR activation by PMA. (D) Time course of XCFTR activation by PMA. (E) Summary of the maximal conductance elicited by PMA. Data were normalized to the maximal conductance obtained with PMA + cAMP. Data shown are from 10 to 12 hCFTR- and XCFTR-expressing oocytes, respectively. I-V plots in PMA-treated oocytes were obtained approximately 15 min after exposure to 250 nM PMA. Next, the cells were exposed to the cAMP cocktail in the continuous presence of PMA, and I-V plots were recorded approximately 10 min later. The results show that PMA is highly effective in activating XCFTR, but not hCFTR. See Fig. 1 for additional details.
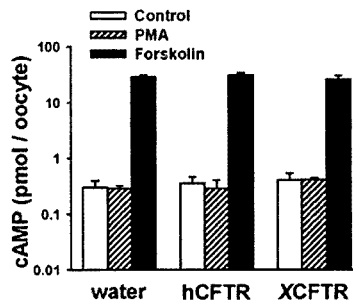

FIGURE 3. Effects of PMA and forskolin on cAMP levels. Data are from unstimulated oocytes and oocytes exposed for 20 min to either PMA (250 nM) or forskolin (25 μM). Forskolin, but not PMA, increased oocytes cAMP ($P < 0.01$, $n = 3$ for each group of oocytes).
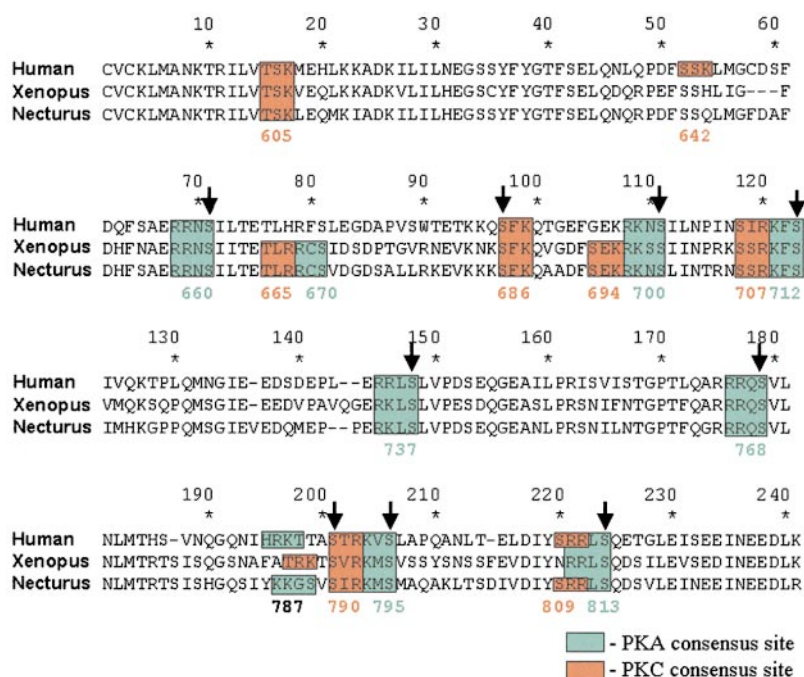
FIGURE 4. Alignment of the sequences of human, *Xenopus* and *Necturus* CFTR R domains. The number below each consensus sequence refers to the Ser/Thr position of the full-length hCFTR sequence. Arrows above indicate that the labeled residue has been shown to be phosphorylated by either PKA or PKC in human CFTR (Cheng et al., 1991; Picciotto et al., 1992).

tion of both PKA and PKC (Fig. 2, B, D, and E). The PMA-activated conductance was sustained for over 30 min, in the continued presence of PMA, and was reversible after extended washout, like the *X*CFTR conductance elicited by PKA stimulation (not shown). In these experiments, higher 8-Br-cAMP concentrations did not increase the currents further. Relative to the current elicited by 250 μM 8-Br-cAMP, the current after 500 μM 8-Br-cAMP was 98 $\pm$ 12% ($n = 3$), and after 1 mM 8-Br-cAMP it was 96 $\pm$ 2% ($n = 3$).

The above results demonstrate that exposure to PMA activates *X*CFTR to a level similar to that obtained after PKA stimulation. To determine whether the effect of PMA is due to activation of PKC, we tested the inactive phorbol analogue 4α-PMA on *X*CFTR-expressing oocytes. Application of 4α-PMA (250 nM) did not change oocyte membrane conductance, whereas subsequent exposure to 4β-PMA caused an increase in conductance to 86 $\pm$ 11% of the value after exposure to the cAMP cocktail and 4β-PMA combined, similar to the results in Fig. 2. Further, application of 4β-PMA to water- or hCFTR-injected oocytes, as well as application of 4α-PMA after 4β-PMA in the *X*CFTR-injected oocytes, had no effect on the oocyte conductances (data not shown).

Another possibility is that PMA increases *X*CFTR conductance via an elevation of cAMP. Cross-talk between the PKA and PKC signaling systems has been demonstrated in a number of systems (Gao et al., 1999). To explain our data, the cross-talk would have to take place in *X*CFTR-, but not in hCFTR-expressing oocytes. To test this possibility, intracellular cAMP was measured under control conditions (in ND96 medium) and after

exposure to either 250 nM PMA or 25 μM forskolin. As seen in Fig. 3, a 20-min exposure to PMA did not increase intracellular cAMP in oocytes injected with water or expressing either hCFTR or *X*CFTR. In contrast, forskolin increased *Xenopus* oocytes intracellular cAMP level by more than 80-fold.

Finally, the difference between hCFTR and *X*CFTR sensitivity to PMA could be in principle explained by the expression system used. One or more cell-specific components in *Xenopus* oocytes could allow *X*CFTR, but not hCFTR, to be directly or indirectly sensitive to PKC-mediated phosphorylation. This question was addressed by experiments in which either *X*CFTR or hCFTR was expressed in COS-1 cells. In four experiments in cells transfected with *X*CFTR cDNA, PMA elicited a conductance of 55 $\pm$ 3% of that produced by 8-Br-cAMP or forskolin.[2] In contrast, in COS-1 cells expressing hCFTR, the increase in conductance elicited by PMA was on average 21 $\pm$ 4%. Taken together, the experiments in *Xenopus* oocytes and COS-1 cells demonstrate that the difference in PKC-mediated activation of human and *Xenopus* orthologues is inherent to the CFTR molecules and not to the host cells.

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[2]In one experiment, there was no response to PMA. If this experiment is included in the calculation, the PMA-stimulated conductance is still significantly increased, i.e., 47 $\pm$ 4% of the 8-Br-cAMP-stimulated current. A negative result was also observed in 1 of 16 experiments in *Xenopus* oocytes expressing *X*CFTR. A possible explanation for the occasional absence of response to PMA is that the cells had low endogenous cAMP levels (see next page).
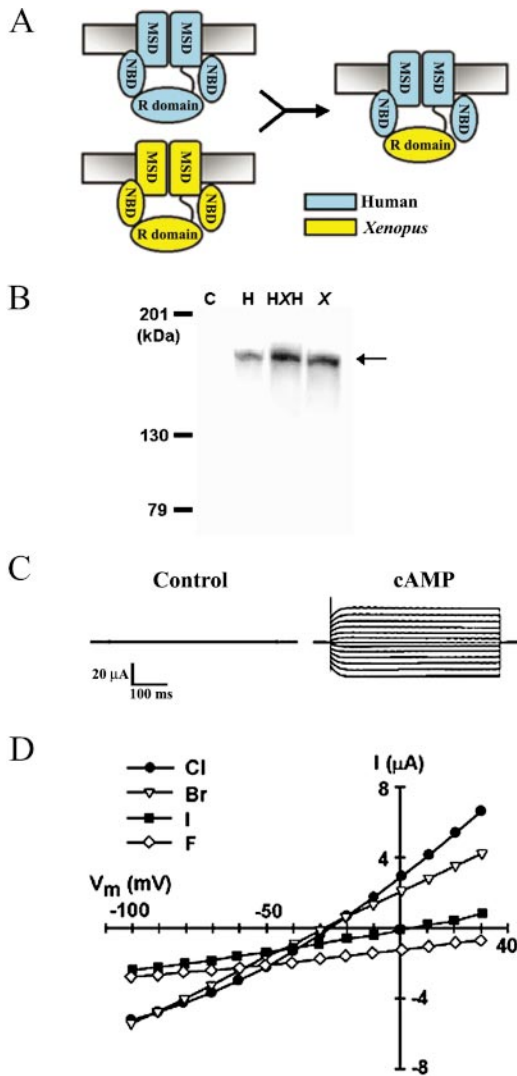
FIGURE 5. Design and expression of the human-*Xenopus* chimera (H*X*H-CFTR). (A) Schematic representation of hCFTR, *X*CFTR, and H*X*H-CFTR, showing the location of the membrane-spanning domain (MSD), nucleotide-binding domain (NBD), and the R domain. (B) Plasma membrane expression of hCFTR, *X*CFTR, and H*X*H-CFTR chimera. Western blots of biotinylated membranes injected with water [C], H*X*H-CFTR [H*X*H], hCFTR [H], or *X*CFTR [X]; there were five oocytes per condition. The arrow denotes the expected size of full-length CFTR (~170 kD). (C) Representative currents from an oocyte expressing H*X*H-CFTR. (D) Halide selectivity sequences of cAMP-activated H*X*H-CFTR. The results show that PKA stimulation elicits a near-linear current with reversal potential near E_{Cl} and halide selectivity identical to that of wild-type hCFTR ($Br^- = Cl^- > I^- > F^-$). See Fig. 1 for additional details.
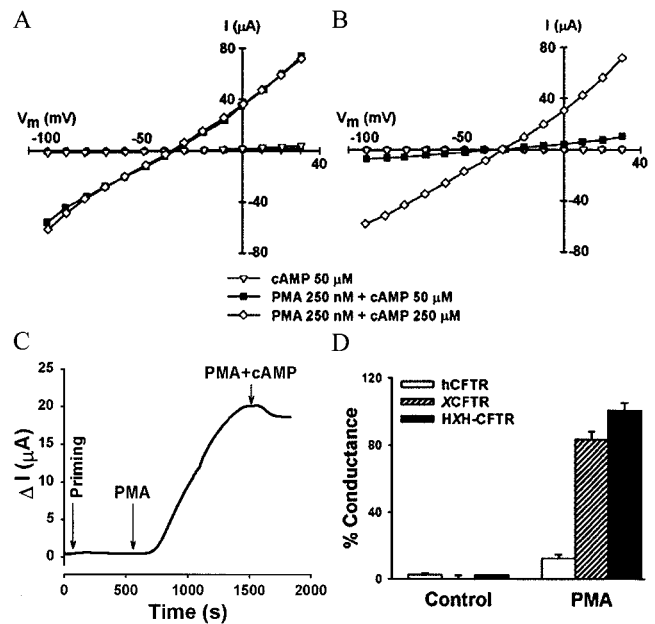

FIGURE 6. Stimulation of H*X*H-CFTR by PKC-mediated phosphorylation. (A) I-V plot from an oocyte expressing H*X*H-CFTR. (B) I-V plot from an oocyte expressing hCFTR. (C) Time course of H*X*H-CFTR activation by PMA. Priming denotes the addition of 50 μM 8-Br-cAMP. (D) Comparison of CFTR activation by PMA in oocytes expressing either wild-type hCFTR or *X*CFTR, or the H*X*H-CFTR chimera. Data were obtained in oocytes primed with 50 μM cAMP (see text), and were normalized to the current elicited by PMA and cAMP cocktail together. Data shown are from 12, 12, and 15 experiments in hCFTR-, *X*CFTR-, and H*X*H-CFTR-expressing oocytes, respectively. The results show that the H*X*H-CFTR chimera is fully sensitive to PMA stimulation. See Fig. 1 for additional details.

The R Domain Accounts for the Differences in the Responses of Human and Xenopus CFTR to PKC Activation

To test for a possible role of the R domain, a chimera was constructed in which residues 607– 811 of hCFTR (Figs. 4 and 5 A) were replaced with the equivalent residues of *X*CFTR. This chimera (H*X*H-CFTR) contains the NH₂- and COOH-terminal transmembrane do-

mains and nucleotide-binding domains from hCFTR. It should exhibit the halide-selectivity sequence of hCFTR, because previous experiments with human/*Xenopus* chimeras revealed that the halide selectivity sequence of hCFTR and *X*CFTR depends on the NH₂-terminal transmembrane domain (Price et al., 1996). In addition, if the R domain from *X*CFTR is the region responsible for the regulation by PKC-mediated phosphorylation, then H*X*H-CFTR should be sensitive to PKC agonists, like *X*CFTR.

H*X*H-CFTR was fully processed and inserted in the plasma membranes of *Xenopus* oocytes as shown by Western blot analysis of plasma membranes (Fig. 5 B). Electrophysiological studies showed no appreciable current in the absence of kinase stimulation (Figs. 5 C and 6 A), as shown above for wild-type hCFTR and *X*CFTR (Figs. 1 and 2). The PKA agonist 8-Br-cAMP reversibly elicited a large, near-linear current, with a reversal potential of approximately −30 mV (Fig. 6, A and C) and halide-selectivity sequence identical to that of wild-type hCFTR (Fig. 5 D).

The response to PMA of oocytes expressing H*X*H-CFTR was similar to those of oocytes expressing either hCFTR or *X*CFTR, in a clearly bimodal distribution,
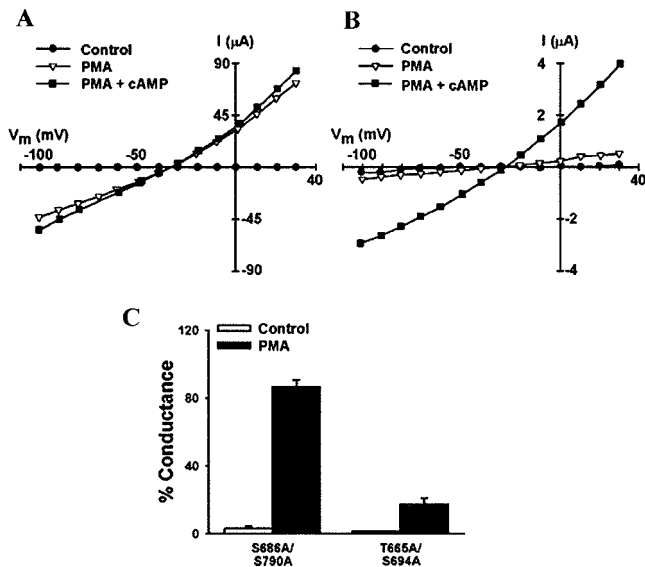
FIGURE 7. Knockout of the unique PKC consensus phosphorylation sites prevents full activation of XCFTR by PMA. (A) Representative I-V relationships from an oocyte expressing the double knockout of conserved PKC consensus phosphorylation sites (S686A/S790A-XCFTR). (B) I-V plot from a cell expressing the double knockout of unique PKC consensus phosphorylation sites (T665A/S694A-XCFTR). (C) Summary of results from experiments identical to those in A ($n = 4$) and B ($n = 6$). The results indicate that at least one of the conserved PKC consensus sites is necessary for full activation by PMA. See Figs. 1 and 2 for additional details.

without intermediate responses. A basal level of PKA-mediated phosphorylation, insufficient to activate XCFTR by itself, is required for the PMA effect (Yamazaki et al., 1999; Button et al., 2000). Since oocyte endogenous cAMP levels from oocyte to oocyte (on average 300 nmol/oocyte) are quite variable, we reasoned that the variability in activation of HXH-CFTR by PMA alone could result from differences in intracellular cAMP. In oocytes with low PKA activity, HXH-CFTR may not be phosphorylated enough to be activated by PMA alone. To "standardize" the level of PKA activity, we exposed the cells to 8-Br-cAMP at a concentration insufficient to activate the current by itself (Fig. 6). We call this "priming." In all 15 experiments, application of PMA to primed oocytes expressing HXH-CFTR elicited full current activation ($102 \pm 8\%$ of the current observed with the subsequent addition of the cAMP cocktail; Fig. 6, A and C), which is not different from the results in oocytes expressing wild-type XCFTR (Fig. 2 C). However, priming did not increase the activation of the wild-type hCFTR by PKC-mediated phosphorylation (Fig. 6, B and C). From these results, we conclude that the R domain is the target for the activation of XCFTR by PKC stimulation, and that the Xenopus R domain is sufficient to confer the sensitivity of CFTR to PKC stimulation.

Conserved Phosphorylation Sites Are Not Involved in the Response to PKC Activation

As shown in Fig. 4, the R domain of XCFTR contains at least seven consensus sites for phosphorylation by PKC. Four of the phosphorylatable residues are also present in hCFTR (Thr[605], Ser[686], Ser[707], and Ser[790]), and two of them (Ser[686] and Ser[790]) are phosphorylated in vitro in hCFTR (Picciotto et al., 1992). The R domain of XCFTR contains two additional sites (Thr[665] and Ser[694]) also present in the *N. maculosus* CFTR homologue, but absent in hCFTR (Fig. 4). Like the *Xenopus* CFTR, the PKC agonist PMA activates the *Necturus* homologue of CFTR (Copello et al., 1993). The role of the "conserved" and "unique" phosphorylation sites in the stimulation of XCFTR by PMA was explored by substituting serine or threonine of the consensus sequences with alanine. In these experiments, priming was carried out as explained above.

Substitution of the two conserved serine residues known to be phosphorylated (Ser[686] and Ser[790]; Picciotto et al., 1992) with alanine residues (S686A/S790A-XCFTR; Fig. 7, A and C) did not affect the activation by PMA. The unstimulated oocytes displayed no significant current, and the subsequent application of 8-Br-cAMP elicited a large, linear, reversible current, with a reversal potential close to E_{Cl} (approximately -30 mV; Fig. 7 A) and a halide-selectivity sequence identical to that of XCFTR ($I^- > Cl^- > Br^- > F^-$; Fig. 1 C). These results indicate that the currents are mediated by CFTR, and agree with the notion that the ion pore is formed by the transmembrane segments (Price et al., 1996). In seven experiments, PMA stimulated the current to $92 \pm 11\%$ of the response to the combination of 8-Br-cAMP and PMA (Fig. 7, A and C). This observation indicates that Ser[686] and Ser[790] are not necessary for the activation of XCFTR by PKC stimulation.

Thr[665] Is Essential for the Response to PKC Activation of XCFTR

To investigate the roles of the unique PKC consensus sites (Thr[665] and Ser[694]), we expressed the mutant lacking both sites (T665A/S694A-XCFTR) in *Xenopus* oocytes. This mutant was also well expressed, did not exhibit basal current, and was sensitive to agonists of PKA-mediated phosphorylation (Fig. 7 B). However, PMA elicited a small current (Fig. 7, B and C) that was not different from that measured in oocytes expressing wild-type hCFTR. The lack of response to PMA could not be overcome by a higher concentration (750 nM, data not shown). This result suggests that at least one of the two unique sites in the R domain of XCFTR is critical for activation by PKC-mediated phosphorylation. Although the degrees of activation of this mutant by PKA and PKC were the same as in the wild-type hCFTR, its halide-selec-
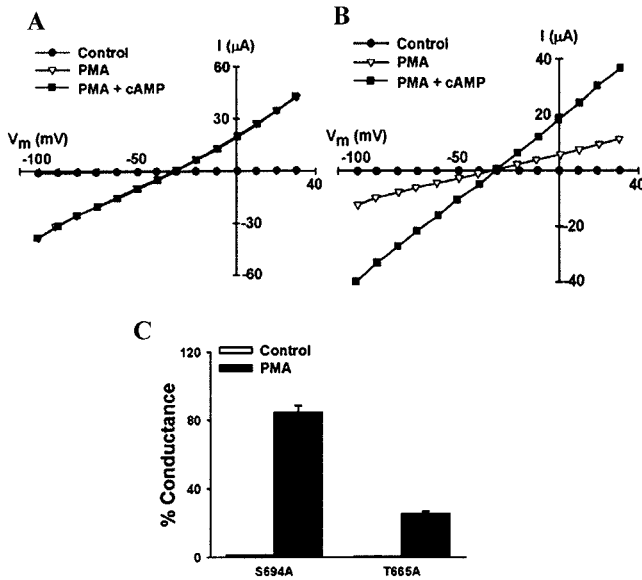
A / **B** panels with I-V plots

FIGURE 8. Thr^{665} is critical for activation of *X*CFTR by PKC-mediated phosphorylation. (A) Representative I-V plot from an oocyte expressing a single knockout of the unique PKC consensus phosphorylation site containing Ser^{694} (S694A-*X*CFTR). (B) I-V plot from an oocyte expressing a single knockout of the unique PKC consensus phosphorylation site containing Thr^{665} (Thr665/A-*X*CFTR). (C) Summary of results from experiments identical to those in A ($n = 6$) and B ($n = 14$). The results denote a critical role of Thr^{665} in the stimulation of *X*CFTR by PMA. See Figs. 1 and 2 for additional details.

FIGURE 9. The mutation Thr^{665} to Ala does not increase cAMP-activated *X*CFTR currents. Time course of the currents after stimulation with the cAMP cocktail in oocytes injected with wild-type *X*CFTR, S686A/S790A-*X*CFTR, or T665A/S694A-*X*CFTR cRNAs. Currents were measured at 30 mV, 20 min after exposure to the cAMP cocktail. The data show that the Thr^{665} to Ala mutation has no significant effect on the level of cAMP-activated currents.

tivity sequence ($I^- > Cl^- > Br^- > F^-$; Fig. 1) was identical to that of the wild-type *X*CFTR, as expected.

To ascertain the roles of the two conserved sites, we mutated Thr^{665} and Ser^{694} separately and tested the effect of PMA in primed oocytes. When S694A-*X*CFTR was stimulated with PMA, there was full current activation (Fig. 8, A and C). In contrast, in oocytes expressing T665A-*X*CFTR, PMA failed to produce full current activation (Fig. 8, B and C). Currents in cRNA-injected oocytes vary from cell to cell. Thus, it is possible that the reduced activation by PMA of *X*CFTR mutants containing the Thr^{665} to Ala mutation is not absolute, but relative to the activation by PKA stimulation (i.e., the mutation increases the activation of CFTR by cAMP). The data in Fig. 9 indicate that this is not the case because the cAMP-activated currents are not different among oocytes expressing wild-type *X*CFTR, S686A/S790A-*X*CFTR, or T665A/S694A-*X*CFTR. These results prove that Thr^{665} is the residue necessary for the activation of CFTR by PKC.

Engineering a PKC Consensus Phosphorylation Site that Includes Thr^{665} Confers PKC-sensitivity to hCFTR

The hCFTR is unique among all known CFTR homologues in that it lacks a PKC consensus site at the location equivalent to residue 665 in *X*CFTR. As shown in
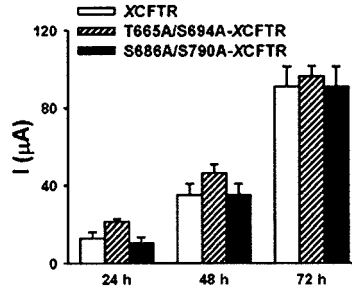
Fig. 4, this is due to the presence of His^{667} instead of arginine or lysine. Since Thr^{665} is present in hCFTR, we decided to determine whether the formation of a PKC consensus phosphorylation site in hCFTR by substituting His with Arg at position 667 is sufficient to confer the *Xenopus* phenotype to hCFTR. The mutant H667R-hCFTR was expressed in *Xenopus* oocytes and tested for activation by PKC stimulation. In oocytes primed with 8-Br-cAMP, as described above, PMA produced a large activation of the conductance, amounting to $68 \pm 6\%$ ($n = 6$) of the current elicited by the combination of PMA and cAMP cocktail (Fig. 10). The PMA-dependent conductance in the presence of priming was significantly greater than that in wild-type hCFTR, although perhaps smaller than the analogous value in H*X*H-CFTR. This demonstrates that the phosphorylation site at Thr^{665} is sufficient to explain CFTR activation by PKC-mediated phosphorylation.

DISCUSSION

The phosphorylation of serines and threonines by cAMP-dependent kinases is a conserved step in the activation of CFTR Cl^- channels from several species (Seibert et al., 1997). It is clear that at least four of the dibasic PKA consensus sites in the R domain are important for activation (Chang et al., 1993; Rich et al., 1993).

PKC stimulation alone produced only a small activation of hCFTR expressed in *Xenopus* oocytes, a result consistent with those previously reported using either heterologous expression systems or native CFTR-expressing cells (Berger et al., 1993; Yamazaki et al., 1999). In contrast, PKC stimulation of *X*CFTR elicited a large Cl^- conductance, similar in magnitude to that activated by PKA agonists. Further, experiments with expression of either hCFTR or *X*CFTR in COS-1 cells yielded similar results to those in *Xenopus* oocytes. Thus, activation of CFTR by PKC is a property of the amphibian CFTR isoforms.

A

- ● Control
- ▽ cAMP 25 μM
- ■ PMA 250 nM + cAMP 25 μM
- ◇ PMA 250 nM + cAMP 250 μM

I (μA)

40

20

V_m (mV)

-100   -50   40

-20

-40

B

120

% Conductance
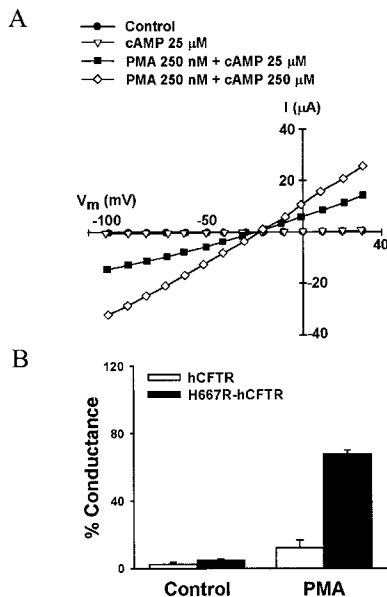
80

40

0

□ hCFTR
■ H667R-hCFTR

Control   PMA

FIGURE 10. PMA produces a large increase in conductance in a mutant hCFTR (H667R-hCFTR) with an engineered PKC consensus phosphorylation sequence that includes Thr[665]. (A) Representative I-V plot from an oocyte expressing H667R-hCFTR. (B) Summary of results from experiments such as those in A. Oocytes were primed with 25 μM 8-Br-cAMP. Data were obtained from six experiments. The results indicate that the engineered PKC consensus site is sufficient to confer a large response to PKC stimulation.

A Single PKC Consensus Phosphorylation Site in the R Domain Is Needed for the Activation of Xenopus CFTR by PKC Stimulation

The chimera in which the R domain of *X*CFTR replaced the R domain of hCFTR exhibited the halide-selectivity sequence of hCFTR, as expected from previous studies indicating that the ion selectivity of CFTR is determined by the NH$_2$-terminal transmembrane spanning domain (Price et al., 1996). In addition, the H*X*H-CFTR chimera was sensitive to PKC stimulation, indicating that the R domain is the target for the activation of *X*CFTR by PKC stimulation, and that the *Xenopus* R domain is sufficient to confer sensitivity of CFTR to PKC stimulation. These experiments also indicate that the interaction between the R domain and other regions of CFTR is preserved in the H*X*H chimera. Although the human and *Xenopus* R domains exhibit only 65% amino acid sequence identity, the *Xenopus* R domain does interact with the nucleotide-binding domains and/or transmembrane domains of hCFTR, eliciting channel activation in response to PKA stimulation.

The mutagenesis experiments presented in this paper indicate that the PKC consensus phosphorylation site including Thr[665] is required for the activation of the channel by PKC stimulation. In hCFTR, Thr[665] is conserved, but the PKC consensus phosphorylation site

is missing. Insertion of this site in the H667R-hCFTR mutant resulted in a large increase in current in response to PKC activation. The experiments on this mutant and the H*X*H chimera were different in some respects (e.g., priming concentration of 8-Br-cAMP and amount of cRNA injected), making a quantitative comparison of the responses to PKC activation difficult. In any event, the results indicate that the PKC consensus phosphorylation site that includes Thr[665] is critical for the response to PMA. If the activation of the H667R-hCFTR mutant is in fact less than that of the H*X*H chimera, then one would conclude also that the phosphorylation of Thr[665], or its functional effect, involves other residues of the R domain of CFTR.

PKC-mediated activation of hCFTR requires endogenous phosphorylation by PKA (Yamazaki et al., 1999). Activation of *X*CFTR by PMA occurred in all oocytes in the absence of priming, whereas H*X*H-CFTR needed priming (Figs. 2 and 4). This difference could be the result of either a lower PKA-mediated phosphorylation of H*X*H-CFTR at basal cAMP levels, or differences in the interaction of the R domain of *X*CFTR with other hCFTR domains. Oocytes expressing a chimera consisting of the R domain and COOH-terminal half of *X*CFTR and the NH$_2$-terminal half of hCFTR did not require priming, whereas a CFTR molecule consisting of the NH$_2$-terminal half and R domain of *X*CFTR and the COOH-terminal half of hCFTR did (Button et al., 2000). Additional studies will be needed to establish the bases of the need for priming of the H*X*H-CFTR.

Our data suggest, but do not prove, that the stimulation of *X*CFTR by PMA is directly mediated by phosphorylation of Thr[665] by PKC. This is the simplest explanation of our results because of the following reasons. First, the inactive phorbol ester 4α-PMA is ineffective, ruling out most nonspecific effects of PMA. Second, PMA treatment does not increase cAMP levels in oocytes, ruling out an indirect effect via PKA activation. Third, cGMP is ineffective in *Necturus* gallbladder epithelial cells (Heming et al., 1994), suggesting no role of the cGMP-dependent kinase in the regulation of amphibian CFTR. Fourth, substitution of Thr[665] in a PKC consensus phosphorylation site of *X*CFTR abolishes the response to PMA. Finally, addition of a PKC consensus phosphorylation site in hCFTR confers sensitivity of this molecule to PMA. Based on these observations, we hypothesize that phosphorylation of Thr[665] by PKC is responsible for the activation of *X*CFTR by PMA. The phosphorylation level of CFTR depends on the rates of phosphorylation and dephosphorylation. Thus, it is conceivable that PMA may inhibit a phosphatase that dephosphorylates Thr[665], and this inhibition solely or in part could account for the increase in phosphorylation of Thr[665]. Resolution of the precise mechanism of *X*CFTR activation will require additional experiments.

The Mechanism of CFTR Activation by Phosphorylation of the R Domain

It is clear that hCFTR is activated by PKA-mediated phosphorylation of residues located mainly in the R domain (Anderson et al., 1991; Tabcharani et al., 1991; Nagel et al., 1992). Phosphorylation of specific residues can modulate CFTR either positively or negatively (Wilkinson et al., 1997), but mutagenesis studies indicate that no single site is responsible for the activation (Cheng et al., 1991; Chang et al., 1993; Rich et al., 1993). Therefore, it is thought that the increase in CFTR channel open probability by PKA activation is a consequence of either a change in the net charge of the R domain (Ostedgaard et al., 2000) or a more complicated phenomenon involving multiple phosphorylation consensus sites (Gadsby and Nairn, 1999; Baldursson et al., 2000). In contrast, our data indicate that a specific residue (Thr^{665}) is necessary for the activation of the *X*CFTR channel by PMA.

It has been assumed that exon 13 of hCFTR (residues 591–830) codes for the R domain (Cheng et al., 1991). However, recent experimental evidence suggests that exon 13 includes three distinct regions. The first region is the last portion of the first nucleotide-binding domain (Chan et al., 2000), based on the homology between CFTR and the ATP-binding subunit of the histidine permease (Hung et al., 1998). The second region is the NH$_2$-terminal region of the R domain, which contains few consensus phosphorylation sites, but includes Thr^{665}, the critical residue for activation of *X*CFTR by PKC stimulation. This region is somewhat structured and phosphorylation by PKA probably changes its secondary structure (Dulhanty and Riordan, 1994). The third region is the COOH-terminal region of the R domain, which contains most PKA and PKC phosphorylation sites. This region is mostly made of random coils, and PKA-mediated phosphorylation has no effect on its secondary structure, which was assessed by circular dichroism spectroscopy (Ostedgaard et al., 2000). Our results are compatible with the notion that activation of CFTR by PKC stimulation involves the more structured NH$_2$-terminal region of the R domain and occurs independently of the net charge. Consistent with this view, a chimera in which part of the COOH-terminal portion of the R domain of hCFTR was replaced with the linker domain of MDR1 was fully activated by PKA (Vankeerberghen et al., 1999). Although the linker region of MDR1 has three sites known to be phosphorylated by PKC, stimulation of this kinase did not increase channel activity significantly (Vankeerberghen et al., 1999). These observations support the conclusion that activation of CFTR by PKC stimulation depends on the specific location of the phosphorylated residue. The demonstration that a single residue is critical for CFTR activation by PKC stimulation provides basic and novel information that will serve for future studies aimed at understanding CFTR regulation at the structural level.

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