Potentiation of effect of PKA stimulation of Xenopus CFTR by activation of PKC: role of NBD2

Yongyue Chen, Brian Button, Guillermo A. Altenberg, and Luis Reuss

Sealy Center for Structural Biology and Department of Neuroscience and Cell Biology, University of Texas Medical Branch, Galveston, Texas 77555-0437

Submitted 23 January 2004; accepted in final form 21 July 2004

Chen, Yongyue, Brian Button, Guillermo A. Altenberg, and Luis Reuss. Potentiation of effect of PKA stimulation of Xenopus CFTR by activation of PKC: role of NBD2. Am J Physiol Cell Physiol 287: C1436–C1444, 2004. First published July 28, 2004; doi:10.1152/ajpcell.00045.2004.—Activity of the human (h) cystic fibrosis transmembrane conductance regulator (CFTR) channel is predominantly regulated by PKA-mediated phosphorylation. In contrast, Xenopus (X)CFTR is more responsive to PKC than PKA stimulation. We investigated the interaction between the two kinases in XCFTR. We expressed XCFTR in Xenopus oocytes and maximally stimulated it with PKA agonists. The magnitude of activation after PKC stimulation was about eightfold that without pretreatment with PKC agonist. hCFTR, expressed in the same system, lacked this response. We name this phenomenon XCFTR-specific PKC potentiation effect. To ascertain its biophysical mechanism, we first tested for XCFTR channel insertion into the plasma membrane by a substituted-cysteine-accessibility method. No insertion was detected during kinase stimulation. Next, we studied single-channel properties and found that the single-channel open probability (Po) with PKA stimulation subsequent to PKC stimulation was 2.8-fold that observed in the absence of PKC pretreatment and that single-channel conductance (γ) was increased by ~22%. To ascertain which XCFTR regions are responsible for the potentiation, we constructed several XCFTR-hCFTR chimeras, expressed them in Xenopus oocytes, and tested them electrophysiologically. Two chimeras [hCFTR NH2-terminal region or regulatory (R) domain in XCFTR] showed a significant decrease in potentiation. In the chimera in which XCFTR nucleotide-binding domain (NBD2) was replaced with the hCFTR sequence there was no potentiation whatsoever. The converse chimera (hCFTR with Xenopus NBD2) did not exhibit potentiation. These results indicate that potentiation by PKC involves a large increase in P0, (with a small change in γ) without CFTR channel insertion into the plasma membrane, that XCFTR NBD2 is necessary but not sufficient for the effect, and that the potentiation effect is likely to involve other CFTR domains.

Cystic fibrosis; chloride channel; protein kinases; ATP binding cassette proteins

The cystic fibrosis transmembrane conductance regulator (CFTR) is a phosphorylation- and ATP hydrolysis-dependent Cl− channel (3, 21, 35, 36, 43). Mutations of the CFTR gene cause cystic fibrosis, an inherited autosomal recessive disease more frequent in Caucasians (44). CFTR is expressed in the apical membrane of secretory epithelia and participates in Cl− and fluid secretion (17, 34). The CFTR molecule contains two repeated motifs; each consists of a membrane-spanning domain followed by a hydrophilic nucleotide-binding domain (NBD). Each membrane-spanning domain has six membrane-spanning helices. A highly charged cytoplasmic regulatory domain, the R domain, links the two halves of the molecule. R-domain phosphorylation by PKA facilitates ATP binding and hydrolysis in the NBDs, which by a complex mechanism causes channel gating (21, 35, 38).

The fact that R-domain phosphorylation is critical for the channel function of CFTR was documented by the following results: 1) CFTR is activated by PKA agonists in cells with native or heterologous expression (28, 36–38, 41); 2) CFTR molecules lacking the R domain display Cl− channel function after exposure to phosphorylated R domain but not when exposed to unphosphorylated R domain (27, 46); 3) there are eight conserved PKA phosphorylation consensus sites in the R domain, and five of these have been shown to be phosphorylated in vivo and in vitro by PKA (12, 32); 4) phosphorylation changes the secondary structure of the R domain in vitro (19); 5) serine-to-alanine substitutions in the PKA phosphorylation consensus sites of the R domain greatly attenuate CFTR activation in response to PKA stimulation (4).

In addition to its activation by PKA-mediated phosphorylation, CFTR could also be activated by other kinases such as PKC (9, 11, 15, 16, 23), PKG (6, 20), and calmodulin kinase (6, 32). However, the molecular mechanism of the activation of CFTR by kinases other than PKA and the interaction between PKA and non-PKA kinases in the activation of CFTR are not fully understood. These issues are important to understand from the points of view of the regulation of CFTR-mediated Cl− transport by epithelial cells and the molecular mechanism of phosphorylation-mediated CFTR Cl− channel gating. Recently, we found that Xenopus laevis (X)CFTR exhibits a severalfold higher response to PKC activation than to PKA activation when expressed in Xenopus oocytes (7). In the epithelial human (h)CFTR isofrom, PKC stimulation potentiates the effect of PKA stimulation (40). In the present study, we confirmed the effect of PKC on the activation of XCFTR by PKA stimulation and then tried to ascertain the biophysical and molecular mechanisms of this effect. We found that PKC potentiates the activation of XCFTR expressed in Xenopus oocytes by PKA stimulation, i.e., the response to PKA stimulation subsequent to PKC stimulation was eightfold greater than that without previous PKC stimulation. This effect was not present in oocytes expressing hCFTR. To identify the biophysical mechanism, we assessed changes of channel number (N), single-channel conductance (γ), and single channel open probability (Po) of oocytes exposed to either PKA agonists or PKA and PKC agonists (potentiation). To identify the XCFTR regions responsible for the potentiation, we con-
structured hCFTR-XCFT chimeras and tested them electrophysiologically. To our surprise, the XCFT NBD2, and not the R domain, appears to be critical for the potentiation effect.

**MATERIALS AND METHODS**

cDNA constructs and mutagenesis. hCFTR (a gift from Dr. Lap-Chee Tsui, The Hospital for Sick Children, Toronto, ON, Canada), and XCFT (a gift from Drs. Margaret Price and Michael Welsh, University of Iowa, Iowa City, IA) were cloned into pOcyt7 as previously described (7). The mutations to generate R334C XCFT and XCFT-hCFTR chimeras (see Fig. 1) were introduced with the QuickChange Multi Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA), with all subcloning done through the plasmid pCR-4Blunt-TOPO with PCR-amplified CFTR DNA fragments. All sequences were directly confirmed by DNA sequencing at the Protein Chemistry Core Laboratory of the University of Texas Medical Branch. The mutagenic primer to generate the R334C mutant was 5’-GGCATTTCATCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTC
interaction of Kinase Effects in Xenopus CFTR

Drugs. To activate PKA, intracellular cAMP was elevated with a cAMP cocktail containing 250 μM 8-bromoadenosine 3′,5′-cyclic monophosphate (8-BrcAMP) and 25 μM forskolin (Sigma-Aldrich). To activate PKC, 250 nM phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) was added to the bath. Stock solutions of these compounds were prepared in water (8-BrcAMP), dimethyl sulfoxide (PMA), or ethanol (forskolin) and diluted to the desired final concentration in ND96 solution immediately before use. [2-(Trimethylamino)ethyl]methanethiosulfonate bromide (MTSET) was dissolved in bath solution to a 1 mM concentration immediately before use from a 1 M stock in water stored in −20°C. β-Mercaptoethanol (β-ME) was dissolved in bath solution to a final 2 mM concentration immediately before use. At the concentrations used, the vehicles had no effects on the CFTR currents (not shown).

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

RESULTS

Response of CFTR to PKA and PKC stimulation. The changes in conductance elicited by stimulation of PKA or PKC are illustrated in Fig. 2. We have observed that the response to PKC stimulation is much larger than that to PKA stimulation in XCFTR (Y. Chen et al., unpublished observations), and the biophysical and molecular mechanisms of this response have been addressed elsewhere (7, 13).

The relative responses to PKA and PKC stimulation in the same oocyte were dependent on the sequence of administration of the kinase agonists. As depicted in Fig. 3, consecutive stimulations of PKA yield similar changes in XCFTR conductance (Fig. 3A). In contrast, after a transient exposure to a PKC agonist (Fig. 3B), the conductance increase elicited by the second PKA stimulation in XCFTR was 8.7 ± 0.8 (n = 8) times the first stimulation. The same experiment in hCFTR (Fig. 3C) yielded no difference between the effects of PKA agonists; the second change in conductance was 0.89 ± 0.08.
(n = 6) relative to the first change (Fig. 3D). This study is focused on the large difference between the effects of PKC stimulation on the responses of hCFTR and XCFTR to the consecutive exposure to cAMP, as summarized in Fig. 3D.

**Potentiation effect does not involve new channel insertion into plasma membrane.** In principle, the increase in CFTR conductance that characterizes the potentiation effect could result from increases in N, P, or γ. First, we addressed the possibility that exocytic insertion of new channels from an intracellular pool is responsible, solely or in part, for the potentiation effect in XCFTR. To this end, we used the substituted-cysteine-accessibility method (1, 2, 24). Arg334, a position thought to be in the extracellular mouth of the CFTR channel and accessible from the extracellular side, was mutated to Cys. Charged, membrane-impermeable thiol reagents react at this site and change the conductance of the CFTR channels (1, 24). In control experiments, the increase in Gm elicited by the thiol reagent MTSET⁺ (1 mM) was 98 ± 5% (n = 6) in activated XCFTR-R334C (Fig. 4, A and B). The effect of MTSET⁺ was consistent but could be fully reversed by the reducing agent β-ME. MTSET⁺ had no effect on wild-type CFTR (data not shown). To test for channel insertion, oocytes expressing XCFTR-R334C were exposed to MTSET⁺ for 10–20 s during the first PKA stimulation and then the agent was washed out from the bath. The oocyte was then stimulated by PKC and PKA sequentially and reexposed to MTSET⁺. The increase in Gm elicited by PKA and PKC stimulation, there was no appreciable change in Gm after PKA stimulation was 7.7 ± 0.5 pS, and Gm elicited by the first cAMP stimulus; Gm, Gm in response to the second cAMP cocktail stimulation.

**Increase in P, o is main mechanism of G, increase by PKC potentiation.** To investigate the possible roles of single-channel P, o or γ on the macroscopic CFTR conductance elicited by the potentiation effect, we used the cell-attached patch-clamp technique. Detailed single XCFTR channel properties in the cell-attached configuration in Xenopus oocytes are described by us elsewhere (13). The gating pattern (Fig. 5A) of XCFTR expressed in Xenopus oocytes resembled that observed in cells with endogenous expression (39). Single-channel slope conductance after PKA stimulation was 7.7 ± 0.5 pS, and γ at the P < 0.025 level and P, o at the P < 0.001 level, indicating that both P, o and γ during PKA stimulation are increased by exposure to PKC agonists. The magnitudes of the changes are very different, however. To-

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**Fig. 3. Transient exposures to PMA potentiate the response to cAMP cocktail of XCFTR, but not of human (h)CFTR.** A: Gm changes elicited by 2 consecutive exposures of XCFTR to cAMP cocktail. B: effect of preexposure to PMA on the activation of XCFTR by cAMP cocktail. Note the much larger response after PMA. C: effect of preexposure to PMA on the activation of hCFTR by cAMP cocktail. The 2 responses to cAMP are about the same. D: in the absence of exposure to PMA (control), 2 consecutive exposures to cAMP cocktail elicited the same conductance changes in either XCFTR- or hCFTR-expressing oocytes. Stimulation with PMA (bars labeled PKC) potentiate the response to cAMP in XCFTR-, but not hCFTR-, expressing oocytes. Gm of 0.14 ± 0.025 level and P, o at the P < 0.001 level, indicating that both P, o and γ during PKA stimulation are increased by exposure to PKC agonists. The magnitudes of the changes are very different, however. To-

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**Fig. 4.** Single-channel conductance changes elicited by cAMP cocktail stimulation in Xenopus oocytes expressing XCFTR or hCFTR. A: Single-channel conductance change elicited by cAMP cocktail stimulation. B: single-channel conductance change elicited by cAMP cocktail stimulation in Xenopus oocytes expressing XCFTR or hCFTR. C: single-channel conductance change elicited by cAMP cocktail stimulation in Xenopus oocytes expressing XCFTR or hCFTR.
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XCFTR chimeras (Fig. 1). The results are illustrated in 

Fig. 6 and summarized in Fig. 7. 

activated CFTR channel by either PKA or PKC (21, 

However, the average increases (γ by 22% and P_o by 180%) should yield an increase in G_m by ~3.5-fold, most of which is attributable to the increase in P_o (see DISCUSSION).

NBD2 of XCFTR is necessary, but not sufficient, for potentiation effect. The fact that potentiation is observed in XCFTR, but not in hCFTR, both expressed in the same system, suggests that differences between these CFTR orthologs, independently of the expression system, account for the different response to kinase stimulation. We tried to identify the regions involved in the potentiation effect by constructing and testing a series of hCFTR-XCFTR chimeras (Fig. 1).

The results are illustrated in Fig. 6 and summarized in Fig. 7. 

The R domain is the phosphorylation domain critical for the activation of the CFTR channel by either PKA or PKC (21, 38). Thus we first tested whether the differences between the R domains of XCFTR and hCFTR are responsible for the fact that the potentiation effect exists in XCFTR but not in hCFTR. We constructed chimeras in which the R domains of hCFTR and XCFTR were exchanged. The conductance elicited by PKA stimulation after exposure to PKC agonist (relative to stimulation of PKA without preexposure) was 4.2 ± 0.5 in chimera A (XCFTR with hCFTR R-domain sequence; P < 0.01, n = 10), as illustrated in Fig. 6A and summarized in Fig. 7. Although the response of this chimera to PKA stimulation after PKC stimulation was smaller than that of XCFTR, the phenomenon was still present. Chimera B (hCFTR with XCFTR R-domain sequence) did not display potentiation (Fig. 7). The potentiation effect persisted in the chimeras in which the NH_2-terminal region (chimera C) or the TM5-NBD1 sequence (chimera D) of XCFTR was replaced by the corresponding sequence from hCFTR (Fig. 7). These results indicate that differences in the sequences of the XCFTR and hCFTR TM5-NBD1 do not explain the potentiation effect and that sequences in the R domain and the NH_2-terminal domain are not necessary for, but significantly decrease the magnitude of, the potentiation effect.

In contrast with the above results, replacing the COOH-terminal region of XCFTR with the corresponding sequences from hCFTR in chimera E eliminated the PKC potentiation effect (Fig. 7), i.e., the conductance response to PKA agonists after PKC stimulation was the same as that before PKC stimulation. To narrow down the responsible region, we studied chimeras in which only the NBD2 (chimera F) or the COOH-terminal region distal to NBD2 (chimera G) was replaced with the corresponding sequence from hCFTR, respectively. In chimera F (Figs. 6B and 7) the potentiation disappeared, whereas in chimera G the potentiation (ratio of conductances with PKA activation after and before PKC stimulation = 4.7 ± 1.3; n = 4) persisted (Fig. 7). These results indicate that the NBD2 sequence is necessary for the kinase potentiation effect of XCFTR. To explore whether the COOH-terminal XCFTR sequence including NBD2 and the COOH-terminal region is sufficient to confer the PKC potentiation effect, we constructed a chimera (H) in which this XCFTR sequence replaced the corresponding hCFTR sequence. This chimera did not display the PKC potentiation effect (Fig. 7). These results indicate that NBD2 is necessary, but not sufficient, for the PKC potentiation effect observed in the XCFTR. Together, these results suggest that the molecular bases for the potentiation effect in XCFTR are complex (see DISCUSSION).
DISCUSSION

The central result in this study is that in XCFTR the activation of PKA subsequent to PKC stimulation elicits a response larger than that to PKA stimulation without pretreatment with PKC agonist. The PKC potentiation effect studied here is not observed in hCFTR when both CFTR orthologs are expressed in Xenopus oocytes, suggesting that this phenomenon is CFTR molecule specific, but at present we cannot rule out the possibility that the potentiation effect involves one or more factors of the host cell in addition to the CFTR molecule. In other words, the Xenopus oocyte may express a protein that is involved in the PKC potentiation effect and interacts with XCFTR but not with hCFTR. In addition, to our knowledge, this effect has not been investigated in cells with native expression of XCFTR.

The PKC potentiation in XCFTR is different from the PKC activation (7) and the PKC permissive effect (Ref. 23 and below). Our previous results (7) demonstrated that PKC activation in XCFTR depends on a single PKC phosphorylation consensus site (T665LRR) in the R domain. A PKC consensus site at this location exists in CFTR from all species so far sequenced (from dogfish to chimpanzee) except human and zebra fish (TLHR). We found that the activation by PKC stimulation was abolished in the mutant T665A XCFTR and transferred to the hCFTR mutant TLHR → TLRR (7). The chimera in which the XCFTR R domain was replaced with the corresponding hCFTR sequence (chimera B) lacks the PKC activation site; nevertheless, it exhibits potentiation, indicating that these two processes have different molecular bases. A third phenomenon is the requirement for a certain basal level of PKC activity for the activation of CFTR by PKA stimulation.

Fig. 5. Single-channel records reveal that cAMP after transient exposure to PMA increases both single-channel open probability ($P_o$) and single-channel conductance ($\gamma$) relative to the values observed with cAMP in the absence of PMA pretreatment. Cell-attached patches in XCFTR-expressing oocytes are shown. A, a: bursting single-channel activity elicited ~5 min after exposure to cAMP cocktail at pipette potential ($V_p$) = −40 mV. c, Closed state; o, open state. b: Similar single-channel activity in response to cAMP after PMA stimulation. $I-V$ curves from single-channel records obtained by stimulation with cAMP after PMA stimulation; $i$ = single-channel current; $n = 5$ experiments, each identified with a different symbol. C: summary of single-channel responses to cAMP cocktail stimulation without (Pre) or with (Post) exposure to PMA. The $\gamma$ increased by 22% (left, $n = 5$) and the $P_o$ increased by ~180% (right, $n = 6$). Both changes were statistically significant.

Fig. 6. The potentiation of the response to cAMP by PKC stimulation is abolished in chimera F (XCFTR with NBD2 hCFTR sequence). A: time course of $G_m$ in response to sequential exposure to cAMP cocktail, PMA, and cAMP cocktail stimulation in an oocyte expressing chimera B (XCFTR with hCFTR R-domain sequence). Similar results were obtained in oocyte expression chimeras in which the XCFTR NBD2 sequence was not replaced by the equivalent hCFTR sequence. B: similar experiment in an oocyte expressing chimera F. The potentiation was not present. Schematic representations of the chimeras are shown (see Fig. 1). Note the different scales of A and B.
named the PKC permissive effect by Hanrahan’s group (23). This effect, present in both XFCTR and hCFTR, depends on several PKC phosphorylation consensus sites in the R domain and NBD1 (10, 13). Hence, the three modes by which PKC stimulation affects CFTR function can be distinguished because of the ortholog specificity (hCFTR vs. XFCTR) or because of the different amino acid residues involved.

Concerning the biophysical mechanism of potentiation, we tested whether the insertion of new channels plays a role. Channel insertion has been assessed from changes in plasma membrane electrical capacitance in parallel with increases in conductance, which are interpreted to result from vesicle fusion (26, 42, 45). A problem with this method is that accurate measurements of membrane capacitance are difficult, in particular when \( G_{m} \) is changing, as is the case with the stimulation of CFTR by kinase agonists (12). In addition, the possibility exists that the membrane vesicles fusing with the membrane do not contain the channels. In our hands, there were no consistent correlations between the apparent capacitance and conductance changes during kinase stimulation (data not shown). Membrane protein labeling and detection by immunofluorescence or Western blot analysis has also been used to address this question. A problem with this approach is that the labeling process is slow, taking tens of minutes to hours, and therefore makes it difficult to assess acute effects of kinase stimulation. In addition, this methodology does not allow for identification of functional copies of the protein under study. For these reasons, we used the substituted-cysteine-accessibility method (1, 2, 24) to assess whether or not the potentiation phenomenon involves CFTR channel insertion. The results with the substituted-cysteine-accessibility method indicate that insertion of new channels does not play a significant role in the potentiation effect. These results extend the observations of Dawson’s group (24), who showed a lack of insertion of new channels when hCFTR-expressing oocytes were treated with PKA agonists. Confirming their observations, we also obtained a negative result with PKA stimulation (data not shown). We conclude that the proposal of channel insertion based on increases in membrane capacitance observed when oocytes expressing hCFTR are exposed to PKA agonists (24, 42, 45) is incorrect. Either the assessment of the capacitance is in error (12) or vesicles fuse with the plasma membrane but do not contain activatable CFTR molecules. An important point is that exocytic insertion of CFTR channels may vary among cell types and/or among orthologs (25). Thus the results of Dawson’s group and ours, obtained in Xenopus oocytes, cannot be generalized to other systems.

Both single-channel \( \gamma \) and \( P_o \) are increased in response to PKA stimulation after PKC stimulation. The change in \( P_o \) was quite large, indicating that this is the main mechanism of increase of macroscopic conductance; the change in \( \gamma \) was small, but significant, and we also observed it after stimulation of XFCTR with PKC agonist alone (13). There was a significant difference between the relative changes in whole cell conductance and single-channel properties. However, these numbers may not be directly comparable because of the faster superfusion of the oocytes in the whole cell experiments and difficulties in determining the time of maximal stimulation of on-cell CFTR channels. Regulation of ion channels by PKC-mediated phosphorylation is not generally expected to involve changes in single-channel \( \gamma \), but it has been observed in a few instances (18, 29, 30).

The R-domain sequence differences between XFCTR and hCFTR are not responsible for the lack of kinase potentiation effect in hCFTR. This is a surprising result, given the dominant role of the R domain in the regulation of CFTR by phosphorylation (9, 31, 32). Interestingly, Chang et al. (9) noted that hCFTR could still be activated by PKA stimulation in a mutant in which all PKA consensus phosphorylation sites were knocked out in the R domain. Also, both hCFTR and XFCTR could be partially activated by PKA or PKC stimulation when Ser/Thr residues in all PKC phosphorylation consensus sites in the R domain were mutated to Ala (13). Possible explanations for these results are phosphorylation of cryptic sites outside the R domain, indirect phosphorylation of CFTR by another kinase activated by PKA or PKC, or a protein kinase-sensitive accessory protein that would interact with CFTR. All these possibilities could in principle apply to the kinase potentiation effect on XFCTR.

Even if the effect of kinase stimulation on CFTR requires phosphorylation of one or more unknown accessory proteins, the accessory protein(s) must interact with CFTR molecules to change CFTR channel function. Thus the different PKC potentiation effects in hCFTR and XFCTR must stem from the difference in the structure of these two CFTR homologs. Elimination of the PKC potentiation effect of XFCTR in chimeras in which NBD2 was replaced by the corresponding

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Received for publication June 29, 2004. Accepted for publication September 29, 2004.

Fig. 7. Summary of results from the hCFTR-XFCTR chimeras. A schematic representation of chimeras A–H (see Fig. 1) is displayed at bottom. The data shown are \( G_{ax}/G_{ax} \) (i.e., response to PKA activation after PKC agonist divided by the response to PKA activation before PKC agonist). Dashed lines show the mean values for XFCTR and hCFTR. The data in this figure were statistically compared with those from XFCTR (8.65 ± 0.42; Fig. 3D) or with those from hCFTR (0.98 ± 0.09; Fig. 3D). A, XFCTR with R domain of hCFTR (4.2 ± 0.5, \( n = 10; P < 0.001 \) vs. XFCTR); B, hCFTR with R domain of XFCTR (1.1 ± 0.3, \( n = 8; P < 0.001 \) vs. hCFTR); C, XFCTR with NH$_2$-terminal region of hCFTR (2.7 ± 0.3, \( n = 7; P < 0.001 \) vs. XFCTR); D, XFCTR with sequence from transmembrane helix (TM)5 to the R domain replaced with the corresponding hCFTR sequence (7.9 ± 2.9, \( n = 4; P < 0.001 \) vs. hCFTR); E, XFCTR with NBD2 and COOH-terminal region of hCFTR (1.0 ± 0.1, \( n = 7; P < 0.001 \) vs. XFCTR); F, XFCTR with NBD2 of hCFTR (1.4 ± 0.5, \( n = 10; P < 0.001 \) vs. hCFTR); G, XFCTR with COOH-terminal region of hCFTR (4.7 ± 1.3, \( n = 4; P < 0.001 \) vs. XFCTR); H, hCFTR with XFCTR sequence from NBD2 to the COOH-terminal end (1.1 ± 0.1, \( n = 5; NS \) vs. XFCTR).
hCFTR sequences indicates that the difference between the NBD2s of XCFTR and hCFTR is critical for the PKC potentiation effect observed in XCFTR. The fact that replacement of the hCFTR sequence beyond the beginning of NBD2 with the corresponding XCFTR sequence did not confer the PKC potentiation effect indicates that the NBD2 difference is not sufficient for this effect. It is possible that other domains are involved; these domains would have a positive effect in XCFTR and/or a negative effect in hCFTR, always in association with XCFTR NBD2. The significant decrease of the potentiation effect in chimeras A and C (compared with XCFTR) suggests that the R domain and the NH2-terminal domain may also be involved. In contrast, the differences of the NBD1s, TM5 and 6, and the COOH-terminal region of XCFTR are not necessary for the PKC potentiation effect. Additional experiments will be required to identify the specific sequence in NBD2 that is responsible for the PKC potentiation effect and the roles of other CFTR domains.

The molecular basis for the critical role of NBD2 in mediating the PKC potentiation effect remains unknown. One possibility is a difference in NBD2 phosphorylation between XCFTR and hCFTR. There are five PKA or PKC phosphorylation consensus sites in XCFTR NBD2 (2 of them are also present in hCFTR), but there is currently no evidence for phosphorylation of these sites. In contrast, the critical sites for ATP binding and hydrolysis in NBD2 are conserved in these two molecules. Another possibility is phosphorylation of accessory protein(s) that may interact with NBD2. Already-known CFTR accessory proteins such as CAP70 and syntaxin 1A are unlikely candidates because they do not interact with the NBD2 of hCFTR (22, 44).

In summary, we found that the response to PKA stimulation increases severalfold by preexposure of XCFTR to PKC agonist. The biophysical bases for the potentiated macroscopic response to PKA stimulation are a large increase in single-channel Po and a modest increase in single-channel y with no detectable change in number of channels in the plasma membrane. The differences between the NBD2s of hCFTR and XCFTR are critical for the XCFTR-specific PKC potentiation effect, and the NH2-terminal domain and R domain are not essential but may participate in the effect. Future efforts to dissect the molecular mechanism of the potentiation effect may help our understanding of the regulation of CFTR by phosphorylation.

ACKNOWLEDGMENTS

We thank Dr. Owen P. Hamill for advice with the Xenopus oocyte single-channel experiments and Drs. Michael J. Welsh and Steven A. Weinman for helpful discussions. We also thank Lynette Durant for secretarial help.

REFERENCES

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