Curcumin Opens Cystic Fibrosis Transmembrane Conductance Regulator Channels by a Novel Mechanism That Requires neither ATP Binding nor Dimerization of the Nucleotide-binding Domains*

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Cystic fibrosis transmembrane conductance regulator (CFTR) chloride channels are essential mediators of salt transport across epithelia. Channel opening normally requires ATP binding to both nucleotide-binding domains (NBDs), probable dimerization of the two NBDs, and phosphorylation of the R domain. How phosphorylation controls channel gating is unknown. Loss-of-function mutations in the CFTR gene cause cystic fibrosis; thus, there is considerable interest in compounds that improve mutant CFTR function. Here we investigated the mechanism by which CFTR is activated by curcumin, a natural compound found in turmeric. Curcumin opened CFTR channels by a novel mechanism that required neither ATP nor the second nucleotide-binding domain (NBD2). Consequently, this compound potently activated CF mutant channels that are defective for the normal ATP-dependent mode of gating (e.g. G551D and W1282X), including channels that lack NBD2. The stimulation of NBD2 deletion mutants by curcumin was strongly inhibited by ATP binding to NBD1, which implicates NBD1 as a plausible activation site. Curcumin activation became irreversible during prolonged exposure to this compound following which persistently activated channels gated dynamically in the absence of any agonist. Although CFTR activation by curcumin required neither ATP binding nor heterodimerization of the two NBDs, it was strongly dependent on prior channel phosphorylation by protein kinase A. Curcumin is a useful functional probe of CFTR gating that opens mutant channels by circumventing the normal requirements for ATP binding and NBD heterodimerization. The phosphorylation dependence of curcumin activation indicates that the R domain can modulate channel opening without affecting ATP binding to the NBDs or their heterodimerization.

The CFTR chloride channel regulates salt and water transport across epithelial tissues (1). Mutations that reduce the synthesis or functional activity of this channel cause cystic fibrosis (CF),2 the most common lethal genetic disorder among Caucasians (2). CFTR is a member of the ATP-binding cassette (ABC) transporter superfamily (3) based on its general domain structure that includes two transmembrane domains (which form the pore) and two nucleotide-binding domains (NBD1 and NBD2, which bind and hydrolyze ATP). CFTR also has a large centrally localized regulatory domain (R domain) that is a special feature of this ABC transporter. The R domain has many sites for phosphorylation by cyclic nucleotide-dependent protein kinases, which are the main physiologic activators of this channel (4). CFTR channel opening typically requires both kinase-mediated phosphorylation of the R domain and ATP binding to both NBDs (reviewed in Refs. 5 and 6). Regarding the role of ATP in promoting channel opening, the available evidence supports a model in which two ATP molecules bind in pockets at an interface between the two NBDs (i.e. at the interface of an NBD1-NBD2 dimer) (7, 8). This model is based in part on structural and functional information that has been obtained for other ABC transporters (notably bacterial transporters (9–11)). Nucleotide-dependent dimerization of the NBDs has been proposed to drive conformational rearrangements of the translocation pathway (e.g. by a “tweezers-like” mechanism (11)) for ABC transporters such as the bacterial maltose transporter (11).

How phosphorylation of the R domain controls CFTR channel opening is less clear. Phosphorylation appears to have little effect on nucleotide interactions with the NBDs as assayed by photolabeling the NBDs with azido-ATP analogs (12, 13). Accordingly, it has been proposed that phosphorylation controls a gating step downstream of ATP binding (13). However, this view point is not universally accepted. Vais et al. (14) have proposed that ATP binding to the NBDs is enhanced by phosphorylation of the R domain based on the results of a mutational analysis of the R domain.

The focus of this study is on the mechanism by which curcumin, a dietary compound found in turmeric, activates wild type

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2 The abbreviations used are: CF, cystic fibrosis; CFTR, CF transmembrane conductance regulator; NBD, nucleotide-binding domain; ABC, ATP-binding cassette; WT, wild type; PKA, protein kinase A; TES, 2-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)amino)ethanesulfonic acid; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; ER, endoplasmic reticulum; AMP-PNP, adenosine 5'-[β,γ-iminotriphosphate].
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and mutant CFTR channels. Curcumin was reported originally by Egan et al. (15) to enhance the synthesis and cell surface delivery of the most common CF mutant channel, ∆F508-CFTR, a processing mutant that normally is exported from the endoplasmic reticulum with very low efficiency (16). The extent to which curcumin enhances ∆F508-CFTR processing is controversial, i.e. this biochemical effect could not be reproduced in several other laboratories (17, 18). However, some of the functional effects of curcumin that were reported by Egan et al. (15) might have been due to more direct activation of surface resident channels by this compound. In this regard, we (19) and Berger et al. (20) observed that curcumin substantially enhances the activities of wild type and ∆F508-CFTR channels in excised membrane patches.

Here we show that curcumin promotes CFTR channel activity by a novel mechanism that is independent of ATP binding and dimerization of the two NBDs. This compound strongly activates mutant CFTR channels that are defective for ATP binding and/or NBD heterodimerization including channels that lack all of NBD2. CFTR constructs that are robustly activated by curcumin include two of the more common CF mutants, G551D (mutation in NBD1 (21)) and W1282X (nonsense mutation deleting most of NBD2 (22)). The mode of curcumin activation is interesting in several ways including a strong dependence on prior phosphorylation of the R domain by protein kinase A. The requirement for phosphorylation but not ATP binding or NBD2 provides good functional evidence that phosphorylation of the R domain can regulate CFTR channel opening without affecting nucleotide binding or the dimerization of the two NBDs. Our results indicate that curcumin is a useful functional probe of CFTR gating, which strongly activates mutant channels that otherwise cannot be opened by the normal ATP-dependent mode of gating.

MATERIALS AND METHODS

DNA Constructs, Cell Culture, and Transfections—Human wild type (WT) CFTR and the tested mutants were subcloned into the pCDNA3 mammalian expression vector (Invitrogen) unless otherwise indicated. Point mutants and deletion constructs (e.g. ∆1198-CFTR) were generated by PCR mutagenesis using appropriate mutagenic oligonucleotides and verified by DNA sequencing of the entire PCR product. The ∆R/S660A-CFTR mutant was provided by M. Welsh (University of Iowa) (23), subcloned into the pCDNA3 vector, and truncated by PCR mutagenesis to make ∆1198/∆R/S660A-CFTR. HEK-293T cells were transiently transfected with WT or mutant CFTR cDNA using the Lipofectamine transfection kit following the manufacturer’s recommendations (Invitrogen). HEK-293T cells were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and 1 mM penicillin-streptomycin. HeLa cells stably transfected with W1282X-CFTR were provided by J. P. Clancy at the University of Alabama at Birmingham. The cells were grown on plastic coverslips for patch clamp recording and were used 1–3 days after transfection.

Patch Clamp Analysis and Curcumin—Macroscopic and single channel currents were recorded in the excised, inside-out configuration. Patch pipettes were pulled from Corning 8161 glass to tip resistances of 1–2 mΩ (macroscopic recordings) or 9–12 mΩ (unitary current recording). CFTR channels were activated following patch excision by exposure of the cytoplasmic face of the patch to catalytic subunit of protein kinase A (PKA; 110 units/ml; Promega) and MgATP (1.5 mM). CFTR currents were recorded in symmetrical solution containing 140 mM N-methyl-d-glucamine-Cl, 3 mM MgCl2, 1 mM EGTA, and 10 mM TES (pH 7.3). Macroscopic currents were evoked using a ramp protocol from +80 to −80 mV with a 10-s time period, unless otherwise indicated. The patches were held at ± 40 or 60 mV for unitary current recordings. All of the patch clamp experiments were performed at 21–23 °C. Signals from macroscopic and single channel recordings were filtered at 20 and 200 Hz, respectively. Data acquisition and analysis were performed using pCLAMP8 and pCLAMP9 software (Axon Instruments). The averaged data are presented as the means ± S.E. Statistical comparisons were made by performing unpaired t tests unless otherwise indicated.

The curcumin preparation used for most patch clamp experiments was purchased from Sigma (catalog number C7727; >94% curcuminoid content with >80% curcumin; the rest primarily desmethoxycurcumin). Identical results were obtained using a different preparation from Fluka (also purchased through Sigma; catalog number 28260; >95% curcuminoid content).

Cell Surface Biotinylation, Immunoprecipitations, and Immunoblotting—CFTR cDNAs were transfected in HEK 293T cells seeded in 100-mm dishes according to the Lipofectamine 2000 protocol (Invitrogen). Cell surface proteins were biotinylated using EZ-Link® Sulfo-NHS-SS-Biotin (Pierce) 48 h after transfection. Briefly, the cells were rinsed three times with PBS containing 1 mM MgCl2 plus 0.1 mM CaCl2, pH 7.4, and rinsed once with PBS, pH 8.0. The cells were then incubated 30 min at 4 °C with EZ-Link® Sulfo-NHS-SS-Biotin (1 mg/ml) in PBS (pH 8.0) or with PBS only (control experiment). After the biotinylation step the excess biotin was neutralized by incubating cells for 10 min at 4 °C with 1% bovine serum albumin in PBS. Biotinylated cells were lysed in 1% Triton X-100 in PBS supplemented with protease inhibitors (complete, EDTA-free; Roche Applied Science) (1 ml/dish). The cells were scrapped in lysis buffer and kept on ice for 1 h to achieve cell lysis. The lysate was cleared by centrifugation (14,000 rpm for 15 min at 4 °C), and the total protein content of the supernatant was determined (MicroBCA protein assay kit; Pierce).

Part of each sample (8.5 mg of total proteins) was incubated with streptavidin beads (streptavidin-agarose; Novagen) and part (850 μg of total proteins) with a monoclonal antibody directed against the CFTR amino-terminal tail (monoclonal antibody 3482; Chemicon) cross-linked to A/G-agarose beads (Protein A/G Plus-agarose; Santa Cruz Biotechnology). Streptavidin pulldowns and immunoprecipitations were carried out overnight at 4 °C. The beads were then rinsed three times with 1% Triton X-100 in PBS, and proteins were eluted by a 30-min incubation at 37 °C with reducing Laemmli buffer (50 mM dithiothreitol, 1.2% β-mercaptoethanol). After a quick centrifugation (1 min, 3500 rpm), proteins in the supernatants were resolved by SDS-PAGE analysis on a 4–15% acrylamide gel (Criterion gel; Bio-Rad) and then transferred to polyvinylidene difluoride membrane (Immobilon-P transfer membranes;
Chemiluminescent Substrate (Pierce) and then exposed using
Following extensive washing in TBS plus 0.2% Tween 20, each
1:10,000 in the same solution used for the primary antibody.


cation with a goat anti-mouse IgG horseradish peroxidase-conju-

ate antibody (ImmunoPure® antibody; Pierce) diluted at
1:10,000 in the same solution used for the primary antibody.
Following extensive washing in TBS plus 0.2% Tween 20, each
blot was incubated 5 min with the SuperSignal West Pico
Chemiluminescent Substrate (Pierce) and then exposed using
HXR film (Hawkins X-Ray Supply).

RESULTS

ATP-independent Activation of G551D-CFTR and Wild Type
CFTR Channels by Curcumin—In a previous study we observed
that the G551D regulation mutant is resistant to activation by a
class of CFTR agonist that strongly activates AF508-CFTR and
wild type channels (5-nitro-2-(3-phenylpropylamino)benzoic
acid (NPPB) analogs; Ref. 19). G551D-CFTR channels normally
exhibit very low single channel open probabilities (Po) because
this mutation disrupts the ABC signature sequence in NBD1,
which lines the ATP-binding pocket at the NBD dimer inter-
face in other ABC transporters (9–11). Fig. 1A shows that
G551D-CFTR channels are strongly stimulated by curcumin.
Depicted is a typical macroscopic current record for a macro-
patch that was excised from a transfected HEK-293T cell. The
cytosolic face of the patch was bathed in a solution that con-
tained normally saturating concentrations of MgATP (1.5 mM)
and PKA catalytic subunit (110 units/ml; see “Materials and
Methods” and Fig. 1 legend for details). PKA inhibitory peptide
was added at the first arrow to prevent continued phosphoryl-
atation of the channels (and to rule out an effect of the compound
on channel phosphorylation). As noted previously, the neutral
NPPB derivative (NPPB-AM) only weakly activates G551D-
CFTR channels at a dose that maximally activates the wild type
channel (10 μM; Ref. 19). Conversely, curcumin exhibited a
strong and dose-dependent activation of the G551D-CFTR
currents (see also single channel data in Fig. 3). The activation
by curcumin could be largely reversed by washing the curcumin
from the bath if the time of exposure was shorter than 5 min
(see below for discussion of persistent effects of curcumin).
The gradual deactivation kinetics were presumably because of slow
“off rates” for curcumin; for comparison, washing ATP from
the bath rapidly deactivated wild type channels (Fig. 1C). Identical
results were obtained with two different curcumin prepara-
tions (see “Materials and Methods”).

Because the G551D-CFTR mutant is strongly disrupted for
the normal ATP-dependent mode of channel gating, we tested
whether curcumin also could activate this mutant in the
absence of bath ATP. Fig. 1B shows that curcumin strongly
stimulates G551D-CFTR currents after removing bath ATP
and by adding hexokinase/glucose to enzymatically eliminate
residual ATP. (In pilot experiments we observed that this dose
of hexokinase/glucose completely inhibited WT-CFTR cur-
rents in the presence of 1.5 mM MgATP within 30 s). We also
tested the effects of curcumin on wild type channels in the
absence of bath ATP (Fig. 1C). Here the activation by curcumin
was apparently more modest than that observed for the NBD1
mutant, but nonetheless, a reproducible activation by curcumin
was observed in the absence of ATP. The WT-CFTR currents
that were activated by curcumin in the absence of bath ATP
approached 10–15% of the maximal currents achieved in the
presence of saturating concentrations of PKA and ATP. Thus,
both wild type channels and G551D-CFTR channels can be
activated by curcumin under these conditions.

The preceding results indicate that curcumin can activate
wild type or mutant channels under conditions in which the
normal ATP-dependent mode of gating is disrupted. To explore
this point more fully we determined whether wild type
channels that had been oxidized by a mixture of glutathione and
diamide (strong thiol oxidizer) also could be activated by cur-
cumin. Previously we determined that this mixture inactivates
CFTR channels by oxidizing (apparently by glutathionylation) a
cysteine near the ABC signature sequence in NBD2 (Cys-1344
(25)). This modification disrupts channel gating, possibly by
inhibiting ATP binding to NBD2 or the heterodimerization of
the two NBDs. Fig. 1D shows that channels that have been ox-
idized in this way are strongly and reversibly stimulated by cur-
cumin. The return of the current to near base-line levels follow-
ing the removal of curcumin by bath perfusion rules out an
antioxidant effect of this compound to reverse the covalent
modification at NBD2 by diame/GSH. For comparison, the
subsequent addition of dithiothreitol also activated the current,
but this effect could not be reversed by washing the reducing
agent from the bath.

Curcumin Activates CFTR Channels That Lack NBD2—The
finding that curcumin promotes the opening of G551D-CFTR
channels and wild type channels in the absence of ATP raised
the possibility that this compound acts at a step downstream of
ATP binding and possibly NBD dimerization. To explore this
point further, we determined whether curcumin also activates
CFTR channels that lack all or part of NBD2. To our initial
surprise we found that CFTR channels that lack the entire
NBD2 as well as the COOH-terminal tail (∆1198-CFTR)
express at high levels in HEK-293T cells where they localize to
the cell surface as efficiently as wild type or G551D-CFTR chan-
nels (see schematic of constructs in Fig. 2A and surface biotiny-
lation results in Fig. 2B). (Most CFTR deletion constructs are
processing mutants that are exported inefficiently from the ER,
including NBD1 deletion constructs (16).)3 Not surprisingly,
∆1198-CFTR channels exhibit very low (but detectable) activity
in excised membrane patches irrespective of the presence or
absence of ATP (see also single channel records in Fig. 3). Like
for G551D-CFTR channels, however, the currents mediated by
∆1198-CFTR are markedly stimulated by curcumin (Fig. 2C).
The EC50 for ∆1198-CFTR activation by curcumin was ∼20
μM, as determined in a series of titration experiments. (Fig. 2E).
This stimulatory effect of curcumin was specific in that

3 W. Wang, K. Bernard, G. Li, and K. L. Kirk, unpublished observations.
CFTR channels were negligibly activated by equivalent doses of two other compounds that activate wild type and ΔF508-CFTR channels, i.e. NPPB-AM (see also Fig. 1) and genistein (26), or by a “half-curcumin” derivative, 4-(4-hydroxy-3-methoxyphenyl)-3-buten-2-one (results not shown). The activation of Δ1198-CFTR channels by curcumin did not require bath ATP; in fact, ATP substantially reduced the curcumin response for this construct (see Fig. 4 and W1282X-CFTR below).

Fig. 2C provides a good example of another interesting feature of the stimulatory effect of curcumin; namely, the irreversible activation that develops during longer exposures (>5 min) to this compound. The magnitude of the persistently activated currents that remained following washout of 30 μM curcumin (25 min of exposure) was 52 ± 3% (n = 5) of the peak current that was measured prior to perfusing the compound from the bath. This feature was not unique to the activation of Δ1198-CFTR currents, i.e. persistently activated currents were observed for every CFTR construct that was tested including G551D and wild type CFTR (e.g. Fig. 1A). Conceivably, this persistent effect of curcumin is related to its reported ability to covalently react with nucleophilic amino acids in proteins (Refs. 27–29 and “Discussion”).
FIGURE 2. Curcumin activates CFTR channels that lack NBD2. A, schematic of WT-CFTR and deletion constructs that were tested for curcumin activation. B, cell surface expression of different CFTR constructs in HEK cells determined by biotinylation (top panel) and total CFTR protein expression determined by immunoprecipitation (bottom panel; see “Materials and Methods” for details). C, strong activation of Δ1198-CFTR currents by curcumin. Note that curcumin activation was readily reversed by bath perfusion following brief curcumin exposure but became largely irreversible following longer exposure to curcumin. These persistent currents remained stable in the absence of any bath agonists (also see Fig. 3). D, curcumin also stimulates K95E/Δ1198-CFTR currents, which show strong outward rectification. Inset, I-V curves for control (curve 1), after adding curcumin (curve 2), after re-adding curcumin with no ATP (curve 3), and after curcumin washout (curve 4). E, curcumin titration for Δ1198-CFTR channels in the absence of bath ATP. Inset, mean titration data fit to a single Michaelis-Menten function (EC₅₀ = 20.8 ± 5.4 μM, n = 4). F, curcumin also activates W1282X-CFTR channels. All of the records are representative of at least four experiments except D (n = 2).
Because the Δ1198-CFTR construct is new to the CFTR field, we validated that the currents that are mediated by this construct are authentic CFTR currents in two ways: (i) by performing unitary currents recordings, which confirmed that this NBD2 deletion construct exhibits the appropriate single channel conductance (6–8 pS) and linear I-V behavior expected for CFTR channels (Fig. 3) (5, 6) and (ii) by introducing a point mutation (K95E) in this deletion construct that had been shown by Lindsell (30) to induce strong outward rectification of the currents mediated by the full-length channel. Fig. 2D shows that the currents mediated by K95E/Δ1198-CFTR exhibit strong outward rectification, as expected, and that these rectifying currents are robustly activated by curcumin.

We next determined whether W1282X-CFTR channels also can be activated by curcumin. W1282X is a fairly common nonsense mutation in certain CF populations including Ashkenazi Jews, for whom it is the most frequent CF mutation (22). This nonsense mutation truncates CFTR in NBD2, resulting in the deletion of motifs that are essential for ATP binding and presumably NBD dimerization (i.e. Walker B and ABC signature sequences). W1282X-CFTR channels are targeted to the cell surface, albeit less efficiently than Δ1198-CFTR channels (see biotinylation results in Fig. 2B). Like Δ1198-CFTR channels, W1282X-CFTR channels exhibit low (but detectable activity) in excised patches either in the absence or presence of bath ATP (Fig. 2F). W1282X-CFTR channel currents were also robustly stimulated by curcumin under either condition (Fig. 2F), although the effect of curcumin is more pronounced in the absence of bath ATP (see also Fig. 4).

Gating Properties of Curcumin-activated Channels; Curcumin Increases the Opening Rates of G551D-CFTR Channels and the NBD2 Deletion Mutants—To explore how curcumin influences the gating properties of these mutant constructs, we tested its effects on G551D-CFTR, Δ1198-CFTR, and W1282X-CFTR channels in excised micropatches containing small numbers of channels (less than eight). Fig. 3 shows that curcumin increased the single channel open probabilities (P_o) of these constructs primarily by increasing the rates of channel opening. Two doses of curcumin were used for the micropatch analysis: a submaximal concentration of 5 μM and the nearly saturating dose of 30 μM (see titration curve in Fig. 2E). The lower concentration was tested because unitary currents still could be resolved clearly following activation at this submaximal dose. Individual records for G551D-CFTR, Δ1198-CFTR, and W1282X-CFTR channels are shown in Fig. 3 (A, B, and D). Mean data for the stimulation of Δ1198-CFTR channels by 5 μM curcumin are summarized in Fig. 3C. Not surprisingly, the most obvious effect of curcumin was to increase the opening rates of G551D-CFTR and the NBD2 deletion constructs, which otherwise open at extremely low rates.

We also examined the gating behavior of channels that were persistently activated by long term exposure to curcumin (30 μM for 20 min followed by washout; Fig. 3E). Our interest here was to determine whether channels that have been irreversibly activated by curcumin continue to gate dynamically (i.e. continuously open and close) or, alternatively, if these channels become “locked open.” In this regard, highly phosphorylated wild type channels can be locked open by non-hydrolyzable ATP analogs such as AMP-PNP (31, 32). Fig. 3E shows that channels that have been irreversibly activated by curcumin gate dynamically rather than becoming locked open. This dynamic gating behavior persisted for as long as 45 min following the removal of curcumin (and ATP) by bath perfusion. (Note that the bath was perfused continuously for several minutes with curcumin-free solution (∼20 times the bath volume) to ensure efficient removal of the agonist). Thus, persistently active channels continue to close and reopen in the absence of curcumin unbinding and rebinding, which is distinct from the apparently tight link between ATP hydrolysis/unbinding and channel closing in the wild type channel.

Curcumin Activation of Δ1198-CFTR Channels Is Inhibited by ATP Binding to NBD1—Fig. 4 shows another striking feature of the effect of curcumin on the NBD2 deletion constructs (both Δ1198-CFTR and W1282X-CFTR), namely strong inhibition of this activating effect by bath ATP. Fig. 4A shows that the inhibition by ATP can be observed either by adding curcumin first in the presence of bath ATP and then again following removal of the ATP or alternatively by adding ATP after initially adding curcumin. The curcumin-activated Δ1198-CFTR currents were reduced 3–5-fold by the presence of 1.5 mM ATP in the bath (see mean data in Fig. 4E) with a somewhat greater inhibition observed at ATP concentrations greater than 10 mM (Fig. 4, A and B). The time course of the inhibition of the curcumin-activated current by bath ATP was relatively slow (t_1/2 of 1–3 min), similar to the time course for current recovery after removing curcumin from the bath (Fig. 1A). The similar time courses raise the possibility that curcumin must first unbind from the channel before ATP can bind and inhibit subsequent activation by this compound. Consistent with this interpretation, ATP had no effect on channels that were persistently (irreversibly) activated by long term exposure (> 20 min.) to curcumin (Fig. 4B). The inhibition of the curcumin-activated currents by bath ATP could be overcome partially by further increasing the curcumin concentration (Fig. 4C), which raises the possibility that these agonists compete for binding to the channel.

The only known ATP-binding site in this deletion construct is in NBD1, conferred by the Walker A and B motifs in this domain. Fig. 4 (D and E) shows that the inhibitory effect of bath ATP on the curcumin response was eliminated by introducing a mutation in the Walker A sequence (A462F) in NBD1. This mutation was shown previously by Berger et al. (33) to strongly inhibit ATP binding to NBD1. The lack of effect of ATP on the curcumin activation of the A462F/Δ1198-CFTR construct

**FIGURE 3. Curcumin activates CFTR mutants primarily by increasing channel opening rates.** Unitary currents were recorded at ±60 mV across micropatches obtained using small tip pipettes (see “Materials and Methods”). A, G551D-CFTR. B, Δ1198-CFTR. C, effect of curcumin on mean NP, and mean opening rate per patch for Δ1198-CFTR channels. The conditions are the same as those in B (protein kinase inhibitor (PKI); 1.5 mM ATP; ±5 μM curcumin; n = 4). D, W1282X-CFTR; −60 mV E, representative patch showing dynamics of opening and closing of Δ1198-CFTR channels during the reversible and persistent phases of curcumin activation. Left, whole record; right, selected segments at ±60 mV. All of the records are representative of at least three experiments except D and E (n = 2 each).
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A

Curcumin (30 μM) → ATP (1.5 mM) → ATP (11.5 mM)

B

Curcumin (30 μM) → ATP (10 mM)

C

Curcumin (30 μM) → ATP (1.5 mM) → ATP (11.5 mM)

D

Curcumin (30 μM) → ATP (1.5 mM)

E

Fold increase of current

Δ1198

A462F/Δ1198

ATP + ATP −
rules out a trivial effect of ATP to chelate the curcumin in the bath; instead, ATP must bind to CFTR (presumably to NBD1) to inhibit the curcumin activation of the NBD2 deletion construct. It should be noted that ATP did not obviously blunt the curcumin activation of channels that possess NBD2 (e.g. G551D-CFTR and wild type channels; results not shown). The results obtained for the latter constructs are harder to interpret because of the existence of multiple ATP-binding sites and cooperative interactions between the NBDs in full-length channels. In this regard, it should be pointed out that the wild type channel may bind ATP more stably at NBD1 than constructs that cannot form NBD1-NBD2 heterodimers. This might explain why wild type channels are not as strongly stimulated by curcumin in the absence of bath ATP; nucleotide may remain tightly bound to NBD1 in wild type CFTR even after removing ATP from the bath (12, 13).

Curcumin Activation Requires Channel Phosphorylation—The fact that curcumin activates CFTR channels that lack NBD2 (or minus bath ATP) provided us with the opportunity to determine whether channel opening still is controlled by phosphorylation in the absence of nucleotide binding or NBD heterodimerization. Fig. 5 shows that the activation of Δ1198-CFTR channel activation by curcumin is highly phosphorylation-dependent. A, curcumin strongly activates Δ1198-CFTR currents only after phosphorylation by PKA. B, λ-phosphatase (APP), a Mn⁷⁺-dependent protein phosphatase, rapidly deactivates WT-CFTR currents. C, λ-phosphatase partially reverses the curcumin-induced Δ1198-CFTR currents when added during the initial phase of curcumin activation. D, curcumin activates Δ1198/ΔR-S660A-CFTR channels in the absence of ATP and PKA. All of the records are representative of at least three experiments.

FIGURE 4. ATP inhibits Δ1198-CFTR channel activation by curcumin. A, bath ATP inhibits Δ1198-CFTR channel activation by curcumin either when present before curcumin addition (1.5 mM ATP) or when added during the initial, reversible phase of curcumin activation. B, 10 mM ATP markedly limits initial Δ1198-CFTR channel activation by curcumin but has no effect on the persistent currents that develop during prolonged exposure to curcumin. C, ATP inhibition of curcumin-activated Δ1198-CFTR currents can be partially reversed by increasing the bath curcumin concentration. D, bath ATP does not inhibit A462F/Δ1198-CFTR channels. E, mean data comparing the effects of bath ATP (1.5 mM) on Δ1198-CFTR (n = 16) and A462F/Δ1198-CFTR (n = 10) activation by 30 μM curcumin. The asterisks denote p < 0.001 for comparison between plus and minus ATP for Δ1198-CFTR (unpaired t test). Note that the absolute currents mediated by A462F/Δ1198-CFTR are lower because the A462F mutation partially disrupts ER processing and cell surface localization (33). All of the records are representative of at least three experiments.
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CFTR channels by curcumin is strongly dependent on channel phosphorylation by PKA. This requirement was observed in two protocols: (i) by adding curcumin to excised patches first in the absence of PKA and then again after adding PKA (Fig. 5A) and (ii) by adding a strong phosphatase (λ-phosphatase) (34) at early time points following curcumin activation (Fig. 5, B and C). A similar PKA dependence of the curcumin effect was observed for G551D-CFTR, W1282X-CFTR, and wild type channels (results not shown). Interestingly, whereas λ-phosphatase could partially reverse the activation by curcumin when added soon after the compound (Fig. 5C), this phosphatase had no effect on persistently active channels following long term curcumin exposure (results not shown). The latter result indicates that persistently active channels become resistant to dephosphorylation by the phosphatase or, alternatively, that channels are no longer phosphorylation-dependent under these conditions. We also tested the effect of curcumin on channels that lack a large portion of the R domain in addition to NBD2 (Δ1198/ΔR/S660A), because previous work has shown that ΔR/S660A-CFTR channels exhibit constitutive activity in the absence of added kinase (23). Fig. 5E shows that curcumin activates this R domain deletion construct without the need for PKA. The results of Fig. 5 provide strong evidence that PKA phosphorylation (probably of sites within the R domain) can stimulate CFTR channel opening without affecting ATP binding or the heterodimerization of the two NBDs.

DISCUSSION

Our data indicate that curcumin strongly activates mutant CFTR channels that normally have very low activities because of defects in ATP binding and/or NBD heterodimerization (e.g. G551D-CFTR and W1282X-CFTR channels). This compound acts independently of nucleotide binding or NBD dimerization to increase the probabilities that individual channels will open. That the stimulatory effect of curcumin does not require heterodimerization of the two NBDs is best exemplified by its potent activation of channels that lack all (Δ1198-CFTR) or part (W1282X-CFTR) of NBD2. The latter constructs (especially Δ1198-CFTR) have proven useful for investigating this unconventional, ATP-independent mode of CFTR channel activation. Δ1198-CFTR channels exhibit modest (but detectable) activity in the absence of ATP or curcumin (Fig. 3), but this activity is markedly increased by curcumin. Cui et al. (35) recently reported that a generally similar NBD2 deletion mutant (1248X) also exhibits detectable channel activity, although they did not address the effects of ATP (or curcumin) on this activity. We should emphasize that our results do not speak against the currently held view that NBD dimerization is a key step in the normal ATP-dependent mode of CFTR channel opening (8, 36). Our findings do indicate that CFTR channels can be induced to open by an alternative mechanism that bypasses this normal requirement for ATP binding and NBD heterodimerization.

Curcumin Activation Is Phosphorylation-dependent—Curcumin activates the NBD2 deletion constructs or G551D-CFTR channels in the absence of active PKA in the bath or following deletion of most of the R domain. Thus, its effects are not due to enhanced phosphorylation of the R domain. However, the stimulation by curcumin is nonetheless strongly dependent on prior phosphorylation of the channel by PKA, even though neither ATP nor NBD2 are required for this activation. The latter observation provides good evidence that phosphorylation of the R domain can enhance channel opening without promoting ATP binding or NBD dimerization. This argument is consistent with the reportedly modest effects of channel phosphorylation on the photo-labeling of the CFTR NBDs by azido-ATP analogs (12, 13). Apparently the R domain can control a gating step that is downstream of (or in parallel with) nucleotide binding and NBD heterodimerization. At first glance this would appear to conflict with recent evidence from Mense et al. (36) that PKA promotes cross-linking of the two NBDs in split CFTR channels. However, phosphorylation of the R domain may control multiple steps in CFTR channel gating including a step that is independent of NBD dimerization.

Does Curcumin Directly Interact with the CFTR Channel?—Currently we do not know whether curcumin activates CFTR channels by a direct binding mechanism. This lipophilic compound might indirectly affect CFTR gating by influencing bilayer thickness or membrane stiffness, as reported for other compounds and ion channels by Andersen and co-workers (37–39). However, four results seem more consistent with a direct binding model: (i) the activation of CFTR channels by curcumin saturates at increasing concentrations (Fig. 2); (ii) the effect of curcumin appears to be somewhat specific: three other test compounds could not mimic the strong activating effect of curcumin on Δ1198-CFTR channels including genistein, which was reported previously to indirectly stimulate the opening of gramicidin channels and CFTR channels by affecting bilayer mechanics (39); (iii) the curcumin effect is reversible at short exposure times but becomes progressively irreversible during prolonged exposure to this compound, which seems most easily explained by covalent cross-linking to residues at or near a curcumin-binding site; and (iv) bath ATP inhibits activation of the NBD2 deletion constructs by this compound. Rigorous proof of a direct binding mechanism will require the development of biochemical assays of curcumin binding and the identification of a functionally relevant binding site for this putative CFTR agonist.

The persistent activation of CFTR channels by curcumin that develops over time is an intriguing feature that may prove useful for identifying a curcumin-binding site on the CFTR polypeptide. Curcumin is known to covalently react with nucleophilic amino acids (e.g. cysteines and lysines) in other target proteins (27–29). The time-dependent transition from reversible to irreversible activation by this compound is possibly due to such a covalent interaction between curcumin and a reactive amino acid within or near its putative binding site. Identifying such a reactive amino acid would provide an important clue as to the location and nature of the putative curcumin-binding site.

Channel Closing Does Not Require Curcumin Dissociation—Once CFTR channels became irreversibly activated by curcumin, they remained active for many minutes (>45 min) in the apparent absence of any agonist (curcumin) or activator (ATP or PKA) in the bath. To our knowledge this is the first example of CFTR channel activity that persists for many minutes in the
absence of an agonist or activator. Wild type channels can be nearly locked open by poorly hydrolyzable ATP analogs such as AMP-PNP under certain conditions (high PKA, 22 °C), but these channels deactivate following the removal of the ATP analog with a time course that presumably reflects the dissociation of the analog (31, 32). Interestingly, channels that are irreversibly activated by prolonged exposure to curcumin continue to gate dynamically, i.e. they continue to open and close in the absence of the compound. This result implies that curcumin increases the probability that CFTR channels will open but that subsequent channel closure does not require curcumin dissociation. This behavior is apparently distinct from that of the poorly hydrolyzable ATP analogs for which channel closing is closely linked to nucleotide dissociation (probably from NBD2 (13)).

**NBD1 Is a Plausible Interaction Site for Curcumin**—ATP strongly inhibits the activation of channels that lack NBD2 (Δ1198-CFTR and W1282X-CFTR) by curcumin. The only known ATP-binding site in these deletion constructs is within NBD1, which is lined with residues from the Walker A and B motifs and the Q loop (40). Introducing a mutation in the Walker A motif that was shown previously to disrupt ATP binding to NBD1 (A462F (33)) severely blunted the inhibitory effect of ATP on Δ1198-CFTR channel activation by curcumin. This implies that ATP must bind to its “conventional” binding site in NBD1 to blunt the curcumin response. (The CFTR NBDs have been reported to possess a second “unconventional” enzymatic activity (adenylate kinase activity) that may also regulate channel gating (41). If so, this activity likely would require a second nucleotide-binding site within each NBD). The observation that ATP binding to NBD1 inhibits the curcumin response provides a simple explanation for the relatively poorer stimulation of wild type CFTR channels by curcumin in the absence of bath ATP. Several groups have provided biochemical evidence that ATP tightly binds to NBD1 in wild type CFTR for many minutes following ATP washout (12, 13). Thus, even though we performed our patch clamp experiments in the absence of bath ATP, it is possible that ATP remained bound to NBD1 for the majority of wild type channels during our experiments. Tight ATP binding to NBD1 is less likely for mutants that are disrupted for ATP binding and/or the dimerization of the two NBDs (e.g. G551D, W1282X, and Δ1198-CFTR), given that dimerization would be expected to stabilize ATP binding to NBD1 (9–11).

Conceivably, ATP inhibits the binding of curcumin to the NBD2 deletion constructs by competing for overlapping binding sites within NBD1. The fact that ATP does not inhibit the irreversibly activated currents that persist following curcumin washout (Fig. 4B) is consistent with an inhibitory effect of ATP on curcumin binding, although not necessarily by direct competition for a common binding site. Interestingly, the activation of G551D-CFTR channels by curcumin is not blunted by ATP (results not shown). Presumably this difference is due to the presence of the second NBD in the G551D mutant that also modulates channel gating and that may inhibit the curcumin response in the absence of ATP (e.g. by reducing curcumin access to its binding site). It is also possible that curcumin binds to a second site within NBD2 or the carboxyl-terminal tail to enhance the ATP-dependent gating of full-length CFTR channels. In sum, the inhibitory effect of ATP on the activation of the NBD2 deletion constructs by curcumin points to NBD1 as one plausible interaction site. To be fair, however, we cannot rule out other possible sites of interaction. For example, this lipophilic compound might bind to a site within the membrane-spanning domains, a site whose accessibility is potentially regulated by ATP binding to NBD1.

**Curcumin as a CF Therapeutic**—Is curcumin worth considering as a natural treatment option for CF patients? Egan et al. (15) originally reported that oral curcumin increased the survival of ΔF508-CFTR mice. These investigators argued that the positive effect of curcumin was due to enhanced maturation of the ΔF508-CFTR protein, which otherwise is exported inefficiently from the ER. To what extent curcumin promotes the maturation of the ΔF508-CFTR polypeptide is controversial (17, 18). Because the block in ER export of this mutant is incomplete, it is possible that some of the beneficial effects of oral curcumin that were reported by Egan et al. (15) were due to more direct effects of this compound on surface resident ΔF508-CFTR channels. If so, then curcumin (or more potent analogs) might have value for treating CF patients with mutations that primarily disrupt the normal ATP-dependent mode of channel regulation (e.g. G551D).

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