Covalent Modification of the Regulatory Domain Irreversibly Stimulates Cystic Fibrosis Transmembrane Conductance Regulator*

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The cystic fibrosis transmembrane conductance regulator (CFTR) Cl⁻ channel is regulated by three cytosolic domains, the regulatory domain (R domain) and two nucleotide binding domains. To learn more about how the cytosolic domains regulate channel activity, we used chemical modification to probe their structure. When we applied the sulfhydryl-modifying reagent N-ethylmaleimide (NEM) and other N-substituted maleimides to the cytosolic domains, we found that they rapidly and irreversibly stimulated channel activity. CFTR contains 14 intracellular cysteine residues that might be targets for NEM modification. We identified one, Cys⁸³₂, that was essential for the response. Cys⁸³₂ is located in the R domain. Single channel studies showed that NEM stimulated CFTR by increasing the duration of bursts of activity and by shortening the closed interval between bursts. At the single channel level, CFTR in which Cys⁸³₂ was mutated to alanine behaved identically to wild-type CFTR, except that it failed to respond to NEM. Additional studies showed that NEM modification increased the potency of ATP-mediated stimulation. Previous work has shown that modification of the R domain by phosphorylation, which introduces negative charge, or replacement of multiple serines by negatively charged aspartates stimulates the channel. Our current data show that covalent modification of the R domain with a neutral, hydrophobic adduct at a site that is not phosphorylated can also stimulate CFTR. This finding suggests that an alteration in the conformation of the R domain may be a key feature that regulates channel activity.

The cystic fibrosis transmembrane conductance regulator (CFTR) is a regulated epithelial Cl⁻ channel (for review see Refs. 1 and 2). CFTR is composed of two membrane-spanning domains (MSD 1 and 2), which form the Cl⁻ conducting pore, and three cytosolic domains, including the regulatory (R) domain and two nucleotide binding domains (NBD 1 and 2). Fig. 1 shows a schematic representation. Phosphorylation of the R domain by cAMP-dependent protein kinase (PKA) is a prerequisite for channel activity. ATP binding and hydrolysis at each of the two nucleotide binding domains then mediate the opening and the closing of the Cl⁻ conducting pore.

Our current understanding of CFTR regulation comes primarily from studies of how function is affected by nucleotides and nucleotide analogs and by site-directed mutations. The effect of nonhydrolyzable nucleotide analogs (e.g. AMP-PNP, pyrophosphate, orthovanadate, and BeF) on function has revealed the contribution of nucleotide hydrolysis to channel activity (3–9). Studies of CFTR bearing site-directed mutations have identified the structural basis for nucleotide- and phosphorylation-mediated regulation (9–16). To date, however, we still have a limited understanding of R domain and nucleotide binding domain structures.

Chemical modification provides another means of probing protein structure. When modification of a protein has a functional consequence, identification of the site of modification can provide clues regarding structure-function relationships. Cysteine residues are useful targets for chemical modification. Their uniquely reactive sulfhydryl side chains can be selectively and covalently modified by a number of different sulfhydryl-reactive reagents. As an example, Akabas and co-workers used sulfhydryl-reactive reagents to identify residues in the pore of CFTR (17, 18). N-Ethylmaleimide (NEM) is a sulfhydryl-reactive reagent that has been frequently used as a tool to disrupt protein structure and function through cysteine modification. For example, Kaback and co-workers used NEM extensively to probe the lactose permease structure (19, 20).

CFTR contains 18 cysteines, 14 of which are located within intracellular domains (Fig. 1). With this in mind, our first goal was to determine if NEM added to the cytoplasmic surface alters CFTR function. Our second goal was to identify the cysteine(s) responsible for any effect by mutating them to alanines.

**EXPERIMENTAL PROCEDURES**

*Site-directed Mutagenesis and Transfection—CFTR mutants were constructed in the pTM1-CFTR4 plasmid by the method of Kunkel (21). Mutations were verified by restriction digest at silently introduced sites by sequencing around the site of mutation and by in vitro transcription and translation. Wild-type and mutant CFTRs were transiently expressed in HeLa cells using the vaccinia virus/T7 bacteriophage hybrid expression system as described previously (11). Cells were studied routinely 4–24 h after transfection depending on the level of expression desired.*

*Patch Clamp Technique—Methods used for excised, inside-out patch clamp recording were as described previously (22, 23). Voltages were referenced to the extracellular side of the membrane. Macropatch and single channel data were collected at −40 and −80 mV, respectively, and at 34–36 °C.*

In excised, inside-out patches of membrane, CFTR was activated by exciting into a bath solution containing 1 mM ATP (disodium salt, Sigma) and the catalytic subunit of PKA (Promega Corp., Madison, WI).
at a concentration of 75 mM. Prior to use, PKA was suspended in a phosphate-free buffer containing 10 mM potassium aspartate, 10 mM EDTA, 1 mg/ml bovine serum albumin, and 52 mM β-mercaptoethanol, pH 6.7. For excised inside-out patch experiments, the pipette (extracellular) solution contained (in mM): 140 N-methyl-D-glucamine, 100 aspartic acid, 35.5 HCl, 5 CaCl₂, 2 MgCl₂, 10 TES, pH 7.3, with HCl. The bath (intracellular) solution contained (in mM): 140 N-methyl-D-glucamine, 135.5 HCl, 3 MgCl₂, 10 TES, 4 Cs(OH)/1/2 EGTA, pH 7.3, with HCl (ICa²⁺,in = < 10⁻⁸ M).

Data Analysis—Single channel current amplitudes were determined from all points histograms. Single channel conductances were derived from the slope of a line fit by a linear least squares regression to single channel I-V relationships at negative potentials where the I-V relationships were linear. Excised macropatch data were filtered at 1 kHz using a variable 8-pole Bessel filter (Frequency Devices Inc., Haverhill, MA) and digitized at 2 kHz. For single channel analysis, replayed data were filtered at 1 kHz, digitized at 5 kHz, and digitally filtered at 500 Hz. Events lists for single channel analysis were created using a half-height transition protocol; transitions less than 1 ms in duration were excluded from analysis. All analyses were done using the pClamp 6.0 software package (Axon Instruments Inc., Foster City, CA).

Open and closed time constants were derived from the fit of a one- or two-component exponential using the maximum likelihood method. Burst analysis was performed as described previously using a tₐ (the time that delineates interburst from intraburst closures) of 20 ms derived from single channel closed time histograms (16). Similar results were obtained with a value of 10 or 30 ms for both wild-type CFTR and CFTR-C832A (+PKA). Regions of data from multichannel patches where there were no superimposed openings were used for burst analysis for the CFTR-C832A mutant following PKA washout, as described previously (16). Results are presented as the means ± S.E. For n observations. Statistical significance was determined as indicated in the figure legends. p values < 0.05 were considered statistically significant.

Chemicals—Maleimide reagents were dissolved in ethanol just prior to use. N-Propyl and N-butylmaleimide were obtained from Fluka Chemie (Switzerland). All other chemicals were obtained from Sigma.

RESULTS

NEM Irreversibly Stimulates CFTR in Excised Macropatches—To learn whether NEM can modify CFTR and alter its function, we applied NEM to the cytoplasmic surface of CFTR Cl⁻ channels that had been phosphorylated by PKA and ATP. Fig. 2A shows an example from an excised, inside-out macropatch. We found that NEM (100 μM) rapidly stimulated CFTR Cl⁻ channel activity. The effect was irreversible. When ATP was removed following stimulation, current returned to base line, indicating that CFTR still required ATP for channel activity. Fig. 2B shows the data quantitatively. Because NEM stimulated CFTR in the absence of PKA, its effects were unlikely due to inhibition of a membrane-associated phosphatase.

Addition of the vehicle (ethanol) alone had no stimulatory effect on CFTR. As previously reported (3), we routinely observed a small amount of “run-down” in channel activity with solution changes (e.g. see Wash in Fig. 2B) in both NEM-modified and unmodified patches; the cause of this is not known. When we added a 10-fold higher concentration of NEM (1 mM) to patches, there was a rapid and transient stimulation followed by a slow (~10 min) and irreversible inhibition (n = 3; data not shown). This response is likely due to the initial covalent modification of an accessible site(s) that stimulates activity followed by modification of sites less accessible to NEM that inhibit activity.

Effect of N-Substitution on Maleimide-mediated Stimulation of CFTR—To understand better the structural requirements on the maleimide for Cl⁻ channel stimulation, we examined the effect of several N-substituted maleimides. We found the following rank order for N-alkyl maleimide-mediated stimulation: N-ethyl ≈ N-propyl > N-butyl ≡ unsubstituted (Fig. 3, N-H). The fact that all of these analogs stimulated CFTR suggests that N-substitution with a range of aliphatics can enhance activity and that the site(s) of modification is accessible to reagents as large as N-butylmaleimide.

NEM Stimulates in Part through R Domain Modification—To identify the cysteine(s) through which NEM was mediating its stimulatory effect, we examined the NEM response of CFTR variants in which individual or groups of cysteines were mutated to alanine(s) or deleted (F1450X deletes Cys1458).

None of these mutations entirely eliminated the stimulatory effect of NEM, suggesting that modification of more than one site was responsible for the stimulatory effect (Fig. 4). However one mutant, CFTR-C832A, showed a marked decrease in its response to NEM (Fig. 4).
Covalent Modification of the R Domain of CFTR

Cys\(^{832}\) lies in the C-terminal region of the R domain, a domain important in phosphorylation-dependent regulation of CFTR. As an additional test of the site specificity of NEM stimulation, we studied the CFTR\(_{\Delta 708-835}\) variant, in which a portion of the C terminus has been deleted (F1450X). Results are from excised, inside-out macropatches. All experiments were done in the presence of 0.3 mM ATP with membrane voltage clamped at \(-40\) mV. Data are the means \(\pm\) S.E. of current expressed as a percentage of basal current following application of 100 \(\mu\)M NEM to the cytosolic surface. The number within each bar indicates the \(n\) value. An asterisk indicates significant difference determined using an unpaired, two-tailed Student’s \(t\) test.

Because NEM, like phosphorylation, covalently modifies the R domain, we did two types of experiments to investigate further the relationship between modification by phosphorylation and by NEM. First, we exposed CFTR-containing patches to NEM in the presence of PKA. Our initial studies (Fig. 2) were done following removal of PKA. Removal of PKA results in a rapid decrease in channel activity (see below) possibly due to the action of membrane-associated phosphatases on CFTR (25). We thought that if NEM were mimicking the effect of phosphorylation, we might not observe any further stimulation in the presence of PKA when CFTR was presumably fully phosphorylated. Fig. 5 (A and B) shows that NEM stimulated CFTR even in the presence of PKA. In this experiment, however, we may be observing two simultaneous effects: NEM stimulation of CFTR superimposed on NEM inhibition of PKA. As a test of this possibility, we added PKA to an unphosphorylated patch in the presence of 1 mM NEM and found that current was stimulated (data not shown), suggesting that PKA remains functional under the conditions used. Nevertheless, because subtle alterations in PKA activity may have occurred, caution is warranted in interpreting these data quantitatively.

Second, we examined the ability of NEM to activate CFTR in the presence of ATP prior to phosphorylation by PKA. Unlike PKA, NEM modification did not elicit channel activity (Fig. 5C), indicating that it could not entirely replace the effect of channel phosphorylation. Interestingly, a second application of NEM following phosphorylation failed to elicit any stimulation (Fig. 5C). This result indicates that NEM modification had occurred prior to phosphorylation and that PKA could still activate CFTR despite the modification. Together, these data show that NEM stimulates CFTR through covalent modification of its R domain. NEM modification, however, is not identical to the effects of phosphorylation. In addition, the data suggest that the site of NEM modification is accessible both before and after the channel is phosphorylated.

Effect of NEM on Single Channel Properties—We undertook single channel studies to understand better how CFTR activity

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**Fig. 3.** Effect of \(N\)-substitution on maleimide-mediated stimulation of CFTR. Data were from excised macropatches studied in the presence of 0.3 mM ATP. 100 \(\mu\)M maleimide was added in each case. Membrane voltage was clamped at \(-40\) mV. Data are the means \(\pm\) S.E. \(n = 12, 6, 7, 6\) for N-ethyl, N-propyl, N-butyl, and unsubstituted \((\text{N-H})\), respectively. Inset shows the chemical structure of an \(N\)-substituted maleimide. An asterisk indicates significant difference determined using a one-way ANOVA with a post-hoc Newman-Keuls test.

**Fig. 4.** Effect of NEM on CFTR containing cysteine to alanine mutations, on CFTR\(_{\Delta 708-835}\), and on CFTR in which a portion of the C terminus has been deleted (F1450X). Results are from excised, inside-out macropatches. All experiments were done in the presence of 0.3 mM ATP with membrane voltage clamped at \(-40\) mV. Data are the means \(\pm\) S.E. of current expressed as a percentage of basal current following application of 100 \(\mu\)M NEM to the cytosolic surface. The number within each bar indicates the \(n\) value. An asterisk indicates significant difference relative to response of wild-type CFTR determined using a one-way ANOVA with a post-hoc Dunnett’s test.

**Fig. 5.** Effect of the presence of PKA on NEM-mediated stimulation. A, time course of the effect of NEM in the presence of PKA. B, effect of 1 mM NEM added in the presence of 75 mM PKA and 0.3 mM ATP (\(n = 4\)). C, effect of NEM addition before and after phosphorylation by PKA. Current records in A and C were collected as in Fig. 2. PKA buffer introduces \(-0.5\) mM \(\beta\)-mercaptoethanol into the bath solution; a higher concentration of NEM (1 mM) was used in A and B to offset the effect of \(\beta\)-mercaptoethanol, which inactivates NEM. The bars indicate interventions made to the cytoplasmic surface. An asterisk indicates significant difference determined using an unpaired, two-tailed Student’s \(t\) test.
is modulated by NEM. Fig. 6A shows representative single channel tracings of wild-type CFTR. Each tracing is from the same patch of membrane. The top trace was obtained in the presence of ATP and PKA; the middle trace shows current following PKA removal, in the presence of ATP alone; and the bottom trace shows current in the presence of ATP and NEM. Table I shows that when PKA was removed the open state probability (P₀) decreased due to a decrease in the length of bursts of activity; none of the other time constants were changed significantly. The addition of 200 μM NEM increased P₀ because the duration of bursts of activity increased and the length of the time the channel spent in the interburst closed state decreased (τ₁; Table I). Fig. 7 shows an example of the effect of NEM on the distribution of closed times. Because the distributions of bursts were not fit by simple exponential functions, mean values are reported (Table I). The open time (τ₀), fast-closed time (τ₁), and single channel conductance were not affected by NEM.

We also examined the effect of NEM on the single channel properties of CFTR-C832A. We did this for two reasons. First, if the CFTR-C832A mutation produced a channel with a very high P₀ (e.g. nearing a value of 1.0), then it might appear to be resistant to NEM because P₀ was already near maximal. Second, we wished to learn whether the Cys to Ala mutation altered channel function.

The behavior of CFTR-C832A was very similar to that of wild-type protein (Fig. 6B). In the presence of PKA and ATP, its conductance, P₀, burst duration, and gating time constants (τ₀, τ₁, and τ₂) were not statistically different from wild-type CFTR (Table I). Like wild-type CFTR, P₀ and burst duration decreased when PKA was removed from CFTR-C832A. However, in contrast to wild-type channels, burst duration, P₀, and τ₀ were unchanged by 200 μM NEM (p values equal to 0.65, 0.62, and 0.30, respectively; Table I). These data indicate that CFTR-C832A is functionally and structurally similar to wild-type CFTR; however, the failure to respond to NEM identifies Cys832 as an important site of modification.

Figure 6. Single channel traces of wild-type CFTR (A) and CFTR-C832A (B) studied in excised, inside-out membrane patches. Each 5-s tracing was acquired in the presence of 1 mM ATP and, where indicated, 200 μM NEM or 75 nM PKA. Membrane potential was clamped at −80 mV. Dashed lines indicate closed state, and downward deflections indicate channel openings. We used 1 mM ATP for these studies rather than 0.3 mM ATP to facilitate comparisons with previous studies.

Table I: Single channel data for wild-type CFTR and CFTR-C832A

| Condition     | n | ATP + PKA | ATP | ATP + NEM |
|---------------|---|-----------|-----|-----------|
| Wild-type CFTR|   |           |     |           |
| P₀ (pS)      | 11 | 0.51 ± 0.02 | 0.38 ± 0.02 | 0.57 ± 0.02 |
| T₀ (ms)      | 8  | ND        | 9.6 ± 0.4 | 10 ± 0.6  |
| T₁ (ms)      | 5  | 184 ± 16  | 149 ± 8  | 237 ± 17  |
| T₂ (ms)      | 5  | 68 ± 7    | 58 ± 7   | 56 ± 7    |
| T₃ (ms)      | 5  | 2.16 ± 0.09 | 2.37 ± 0.11 | 2.39 ± 0.15 |
| T₄ (ms)      | 5  | 141 ± 25  | 152 ± 12 | 118 ± 8   |
| CFTR-C832A   |   |           |     |           |
| P₀ (pS)      | 7–9 | 0.55 ± 0.03 | 0.39 ± 0.02 | 0.42 ± 0.04 |
| T₀ (ms)      | 6–8 | ND        | 10.2 ± 0.3 | 10.3 ± 0.2 |
| T₁ (ms)      | 3–5 | 194 ± 19  | 130 ± 10 | 133 ± 10  |
| T₂ (ms)      | 3–5 | 62 ± 3    | 51 ± 5   | 50 ± 4    |
| T₃ (ms)      | 3–5 | 2.07 ± 0.13 | 2.63 ± 0.37 | 2.18 ± 0.28 |
| T₄ (ms)      | 3  | 164 ± 46  | 206 ± 75 | 185 ± 81  |

a p < 0.05 relative to CFTR in the presence of PKA and ATP.
b p < 0.05 relative to CFTR in the presence of ATP only.

DISCUSSION

We found that the sulphydryl-reactive reagent NEM rapidly and irreversibly stimulated CFTR channel activity through covalent modification of Cys832 in the C-terminal region of the R domain. These data provide new insights into the mechanism by which the R domain regulates CFTR Cl⁻ channel activity.

Previous work has shown that modification of the R domain by phosphorylation (10, 12–14, 24, 26, 27), which introduces negative charge, or by replacement of multiple serines with negatively charged aspartates (14) stimulates the channel. One interpretation of those findings was that the net negative charge or charge density of the R domain activated the channel, perhaps by an electrostatic mechanism. Our current data do not exclude an important role for charge in stimulation through the R domain. However, they show that a modification of the R domain with a neutral, bulky, hydrophobic cysteine adduct rather than a charged phosphoserine could also stimulate activity. This result suggests that an alteration in conformation of the R domain might be a key feature that regulates the channel. Consistent with this, Dulhanty and Riordan (28) showed that phosphorylation of a peptide encoding much of the
phosphorylation sites (Ser660–Ser813) can also affect function. Moreover, channel stimulation was not limited to modification of phosphorylation sites. The R domain contains nine consensus dibasic sites for phosphorylation ((R/K)(R/K))X(S/T)). Five of those sites (Ser660, Ser700, Ser737, Ser795, and Ser813) are phosphorylated in vivo, and a cystic phosphorylation site (Ser753) has been identified (10, 12, 13, 15). Studies in which individual serines or groups of those serines were mutated to alanine have shown that no one site is required for activity, that mutation of some of the sites reduces channel activity, and that mutation of all the sites yielded a channel that was still functional after phosphorylation, albeit at a significantly reduced level (10, 13–15). Our data indicate that modification of a site other than a consensus phosphorylation site and a site that is outside (at least in the primary structure) the region of the R domain that contains the phosphorylation sites (Ser660–Ser813) can also affect function. Moreover, channel stimulation was not limited to modification with a single molecular structure; several different N-substituted maleimides (N-ethyl ≥ N-propyl > N-butyl ≥ unsubstituted) stimulated activity. The phosphorylation studies and our NEM data indicate that covalent modification of many different sites in the R domain can affect function.

The effect of NEM and phosphorylation have some similarities. Both treatments involve covalent modification of the R domain. Both modifications stimulate channel activity. Moreover, both interventions decrease the EC50 for ATP-dependent regulation. However, the two modifications differ in that phosphorylation can activate the channel on its own, whereas the effect of NEM could only be observed when channels were phosphorylated. Perhaps maleimide modification may have enhanced the effect of phosphorylation or perhaps modification of sites other than those in the C-terminal end of the R domain may be required to activate CFTR.

Our data make some predictions about the environment in which Cys832 resides. The results indicate that Cys832 is both accessible to and reactive with N-ethylmaleimide. Furthermore, its accessibility and reactivity were not significantly altered by the phosphorylation state of the R domain. Stimulation by NEM was complete in less than 2 min, which includes delays due to the time that solutions were changing. When pipetted directly onto the patch, the effect was complete within seconds. In contrast, NEM modification of a cysteine introduced into a transmembrane sequence of the lactose permease required greater than 10 min for completion (29). A sulfhydryl-NEM reaction will occur preferentially in an aqueous environment due to solvation of the reactive thiolate species (30, 31). Though NEM is lipophilic, the rapidity of its effect suggests that Cys832 is located on the surface of the R domain possibly in an aqueous environment.

Based on our data and previous results we speculate that in the R domain the structural requirements for stimulation of activity may not be rigorously defined. That is, precise modification by phosphorylation may not be critical for regulation. This interpretation agrees well with the observation that there is substantial sequence variability in the R domain of CFTR from different species (32, 33); in residues 679–798 interspecies sequence identity is only 23%, despite the fact that this region contains the majority of phosphorylation sites. It is also consistent with the observation that cystic fibrosis-associated mutations are uncommon in the C-terminal two-thirds of the R domain.

Mutations in the gene encoding CFTR cause cystic fibrosis (34). Our work may have some implications for cystic fibrosis, particularly in patients who bear mutations that produce a
protein that is correctly processed to the cell membrane. Though the lack of protein specificity of maleimides prohibits their therapeutic use, their stimulatory effect begins to clarify the structural and functional role of the R domain. Increased understanding of these issues may lead to pharmacologic and/or other rationale approaches by which channel activity can be augmented through modification of R domain structure.

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