Functional dissection of the R domain of cystic fibrosis transmembrane conductance regulator

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Abstract Exogenously expressed unphosphorylated sub-domains of the R domain block CFTR Cl^- channels in the planar lipid bilayer, though the block differs from block with full length R domain. Full length R domain peptide (aa 588–855) blocks CFTR Cl^- channels quickly, completely and permanently [1]. Two sub-domains, RD1RD2 (aa 588–805) and RD2TM (aa 672–855), also inhibit CFTR Cl^- channels, but the block takes longer to effect and is not complete. Shorter sequences, RD1 (aa 588–746) and RD2 (aa 672–805), fail to effect any block. These data suggest that either the amino-terminal or carboxy-terminal portions of the R domain protein or its stabilized secondary structure are critical to functional regulation.

Key words: Cystic fibrosis; CFTR; R domain; Channel regulation

1. Introduction

CFTR is unique in the ABC transporter family in that along with two membrane spanning domains and two nucleotide binding domains, CFTR has a large intracellular domain (R domain) that is involved in regulating its chloride channel activity [1–3]. ATP binding and hydrolysis at one or both of the nucleotide binding folds must occur [4–6] and sites within the R domain must be phosphorylated to activate the channel [2,7]. The R domain has an inhibitory role, since deletion of the R domain (amino acids 708–835) relieves the requirement for phosphorylation in order to open channels [8]. This inhibition may be the result of the R domain changing charge [8] or confirmation [9,10] or both. CFTR proteins mutated to remove all or part of the R domain have channel activity, therefore the R domain has not been implicated in pore formation or ion conduction [11–13]. Exogenous R domain can block CFTR channels in vivo [14] and in vitro [1]. 9HTEo$^-$ cells that have been transfected with an expression vector containing the R domain (amino acids 589–855) have reduced basal Cl^- transport and are not stimulated by cAMP agonists in SPQ experiments, whereas, cells transfected by the corresponding empty vector have brisk basal transport of Cl^- and exhibit cAMP stimulated Cl^- current [14]. The R domain block is accomplished in a phosphorylation dependent manner [1]. In planar lipid bilayer experiments, exogenous unphosphorylated R domain (amino acids 589–855) inhibits the activity of single channels of CFTR but, once phosphorylated, the exogenous R domain no longer inhibits channel activity [1]. However, exogenous phosphorylated R domain has a stimulatory effect on channel activity of CFTR with much of the R domain deleted (CFTRΔR 708–835) [13–15]. Thus, the R domain has complex interactions in CFTR which are inhibitory in the unphosphorylated state but stimulatory when phosphorylated.

We took a reconstructive approach to determine whether a sub-domain of the R domain is responsible for its inhibitory activity. The R domain was divided into regions based on the multiple domain model proposed by Dulhanty and Riordan [9]. These investigators postulated, based on homology, that the R domain has three distinct regions. When R domain regions from 10 species were aligned, the first and third region of the R domain showed more than 64% homology at the amino acid level while the second region showed only 23% homology. They speculated that the second region had less constraint due to its lack of function or the versatility of its functional domain. However, the putative phosphorylation sites, which mostly reside within this second domain, are highly conserved between species ($>90\%$). In the present study peptides prepared based on the regions described by Dulhanty and Riordan [9], were added to already open CFTR channels in a bilayer system to ascertain the portion of the R domain that exhibited the blocking ability.

2. Materials and methods

2.1. Definition and in vitro expression of R domain sub-domains

The R domain (from glutamate 588 to valine 855) was divided into three separate sub-domains (Fig. 1). The first, RD1, spanned amino acids 588 through 746. The second sub-domain, RD2, spanned amino acids 672 through 805 and shared 75 amino acids with RD1. The third sub-domain, RTM, included leucine 806 through valine 855. DNA templates were constructed by polymerase chain reaction (PCR) to amplify sequences for the sub-domains. The 5′ primers contained a Kozak sequence (gccaccatgg) and added an initial methionine to the proteins made, which reduced context dependent leaky scanning of the mRNA by the eukaryotic ribosomes [16]. All DNA template PCR products were cloned into pCRII or pCR2.1 using the TA cloning kit (Invitrogen). The sub-domains were then subcloned into pcDNA3 (Invitrogen) using PCR added *Bam*HI and *Pst*I sites (5′ and 3′, respectively). pcDNA3 has the advantage over pCRII or pCR2.1 of a polyadenylation signal and transcription termination sequence from

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Abbreviations: CFTR, cystic fibrosis transmembrane conductance regulator; wt, wild-type; PCR, polymerase chain reaction; R domain, regulatory domain; PKA, protein kinase A; PKI, protein kinase inhibitor; PKC, protein kinase C; ABC, ATP binding cassette; RDP, R domain protein; PAGE, polyacrylamide gel electrophoresis; pS, picosiemen; fA, femtoamperes; SPQ, 6-methoxy-*N*-(3-sulfopropyl) quinolinium; DPC, diphenylcarboxylate; aa, amino acids; NEM, *N*-ethylmaleimide

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Table 1
RD1RD2 block of CFTR channels

	Control	+PKI	+RD1RD2 before block	+RD1RD2 after block
1	56.18 ± 5.16	39.69 ± 7.15	n.a.	21.04 ± 1.31
2	40.94 ± 3.90	40.55 ± 4.63	26.33 ± 1.45 < 19 min	2.07 ± 1.41 > 19 min
3	72.52 ± 4.13	70.63 ± 4.48	61.66 ± 3.40 < 6 min	9.89 ± 0.820 > 6 min
4	62.78 ± 6.98	77.89 ± 2.79	67.27 ± 3.29 < 12 min	0.313 ± 0.089 > 12 min
5	31.50 ± 6.18	28.20 ± 2.61	n.a.	11.77 ± 1.42

All entries are percent mean open probability (P_o) ± standard error. Data from five separate experiments (labeled in column 1) were presented. Following incorporation of CFTR channels into the bilayer, P_o were measured over a period of 2.5 to 7.5 min (control). Then 50 μg of PKI were added to the *cis* solution (+PKI), followed by the addition of RD1RD2 sub-domain (+RD1RD2). The onset of RD1RD2 effect varied from < 1 min to 19 min. Thus, data were divided into before block and after block.

the bovine growth hormone gene. These enhanced translation of the protein products of the sub-domains. All DNA templates were sequenced for base integrity and the protein products, all of which contained the epitope sequence for mAb13-1 (aa 729–736, Genzyme), were immunoprecipitated using this antibody and phosphorylated using protein kinase A (Fig. 1). The RD1 construct was extended from the domain defined by Dulhanty and Riordan [9] on the 3′ end so that the translation product included the epitope sequence for mAb 13-1 (mouse monoclonal anti-human CFTR exon 13, Genzyme).

The *Bam*HI-*Pst*I orientation allowed for sense transcription of the R domain DNA templates from a T7 promoter. The R sub-domain proteins (RD1, RD2, RD1RD2 and RD2TM) were synthesized in vitro using the TnT-coupled reticulocyte lysate system (Promega). SDS-PAGE was performed on the translation products with electro-elution of the gel onto polyvinylidene difluoride membrane (PVDF). The PVDF membranes were blocked with PBS-T (50 mM sodium phosphate, pH 7.4, 150 mM NaCl, 0.05% Tween-20) and 1% bovine serum albumin and then incubated with antibody against the R domain (mAb13-1). A secondary peroxidase conjugated affinity purified goat antibody to mouse IgG was added to allow visualization of the RDP-antibody complex using the ECL system (Amersham). For every in vitro translation, production of R sub-domain proteins was verified by either incubating an aliquot of the translation mix with [^{35}S]methionine and subsequent SDS-PAGE and autoradiography to identify the labeled band of appropriate molecular weight or by immunoprecipitation and phosphorylation of an aliquot of unlabeled translation product with antibody against the R domain of CFTR to identify the R sub-domain proteins.

2.2. Phosphorylation inhibition

Previous work has shown that the interaction of exogenous full length RDP on open CFTR channels is phosphorylation dependent [1]. Protein kinase A is required in order for channels to open, but when exogenous R domain is phosphorylated, it no longer blocks Cl^- channel activity. All sub-domains used contain at least two of the major sites know to be phosphorylated in vivo. It is conceivable that the actions of the sub-domains would also be phosphorylation dependent. To stop phosphorylation once the CFTR channel has opened, a specific inhibitor of PKA, protein kinase inhibitor (PKI, Sigma) was added to the *cis* chamber. A ratio of PKI/PKA of 50 ng PKI/1 unit PKA completely blocked phosphorylation of RDP in *cis* chamber buffer (data not shown). Experiments with R domain fragments were performed with ratios of 1 μg/unit or higher to insure a complete block of phosphorylation.

2.3. Addition of sub-domains to captured CFTR channels

Once a CFTR channel was captured in the planar lipid bilayer, baseline data were collected for at least 2.5 min. Then 5 μl of PKI at a concentration of 10 μg/μl was added to the *cis* chamber and stirred for at least 15 s to ensure mixing. Data were again collected for at least 2.5 min before addition of sub-domain proteins. Then 20–50 μl of rabbit reticulocyte lysate containing sub-domain proteins (at a concentration of 5–20 nM) were added to the *cis* chamber and stirred for at least 15 s to ensure mixing. Data were then collected for 8–20 min, depending on the lifetime of the bilayer. All experiments ended with either breakage of the bilayer or addition of DPC to the trans solution, to insure identity of the CFTR channel. In order to be confident that the sub-domains did not inhibit chloride current we only considered experiments with bilayers in which a single, or at most, two wild-type CFTR channel(s) was (were) captured, since we found that inhibition by intact R domain was more reliably demonstrated in these preparations than in those containing multiple channels. All experiments were conducted under conditions in which a concentration of peptide could be achieved that was at least three-fold higher (five-fold higher for RD1 and RD2) than the concentration of R domain protein required to inhibit channel openings, and in which CFTR channel activity could be followed for at least 8 min following peptide addition, since we observed that block from the smaller peptides sometimes takes longer than block from the intact R domain protein.

For expression of CFTR in HEK 293 cells, microsomal vesicle collection, immunoprecipitation and planar lipid bilayer techniques see Ma et al. [1].

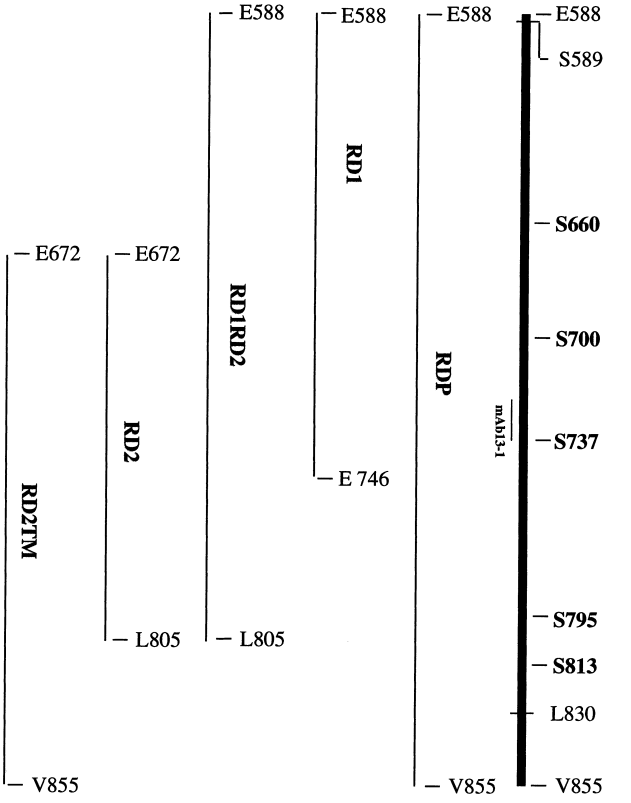

Fig. 1. R domain and sub-domains. Amino acid numbering taken from published CFTR sequence [3] with in vivo phosphorylated serines shown. Antibody binding site (m13-1) is underlined (aa 729–736). Exon 13 boundaries (from S589 through L830) are shown for reference.

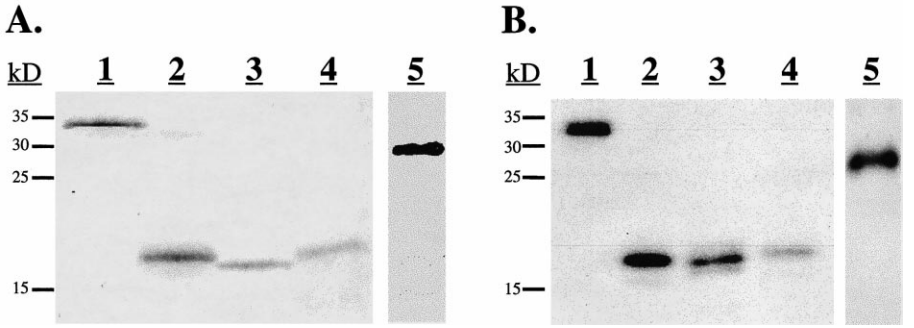

Fig. 2. Expression of R domain sub-domain in reticulocyte lysate. Panel A: [^{35}S]methionine labeled proteins expressed in rabbit reticulocyte lysates: Lane 1, protein marker (Amersham); lane 2, RDP full length; lane 3, RD1RD2; lane 4, RD2; lane 5, RD2TM; lane 6, RD1. Panel B: Samples from panel A that were immunoprecipitated with R domain antibody (m13-1, Genzyme) and phosphorylated with [^{32}P]-γ-ATP by PKA. Lane 1, protein marker; lane 2, RDP full length; lane 3, RD1RD2; lane 4, RD2; lane 5, RD2TM; lane 6, RD1.

3. Results

3.1. Identification and expression of sub-domains

Dulhanty and Riordan [17] suggested, based on homology, that the R domain may be composed of several regions. The first region spans amino acids 588–672, and was completely contained within our proteins RD1 and RD1RD2. The second region spans amino acids 679–798 and was contained within our proteins RD2, RD1RD2 and RD2TM. The third region, thought to be a tether to the seventh membrane spanning helix, spans amino acids 806–859 and was tested using the RD2TM protein. These are illustrated in Fig. 1.

As shown in Fig. 2, DNA templates were transcribed and translated into proteins (panel A) which could be immunoprecipitated with the R domain antibody and were phosphorylated by PKA (panel B). Labeling of translated proteins with [^{35}S]methionine showed that these proteins were the only detected translation products. SDS-PAGE analysis of the proteins' mobility versus standards (Fig. 2) indicated that the proteins migrated within 10% of their predicted size. The DNA templates were also sequenced to insure base integrity.

3.2. Effects of R domain sub-domains on CFTR channel

Previous studies [1,15] have shown that exogenous full length RDP interacts with CFTR in both an inhibitory and excitatory role. To determine the effect of the sub-domains on CFTR, these proteins were added to the *cis* chamber of the planar lipid bilayer in which a CFTR channel had been captured, in concentrations up to 40-fold greater than that of the full length RDP which produced inhibition of CFTR Cl^- channels (15 pM) [1]. Concentrations were determined based on incorporation of [^{35}S]methionine in the protein products purified by SDS-PAGE and analyzed in a scintillation counter (see Fig. 1) and ranged between 126 pM and 715 pM in the *cis* chamber for RD1 and RD2. The effects of addition of each of the sub-domains on CFTR Cl^- channels are shown in Tables 1–4. Figs. 3–6 show representative traces (panel A) from the diary plot shown in panel B. Although channel activity is stochastic with episode open probabilities varying widely, the mean open probability over time within an experiment yields a useful measure of channel activity. The addition of sub-domains RD1 and RD2 separately showed no effect on channel activity ($n = 5$, 5; respectively). In the experiments with RD1 and RD2, mean open probability after protein addition never fell below 50% of the pre-addition value. In some experiments ($n = 4$) the mean open probability actually increased, in one case, by more than 100%. In contrast, both RD1RD2 and RD2TM showed an inhibitory effect when added to the *cis* chamber ($n = 5$, 3; respectively). In no experiment with RD1RD2 and RD2TM did the mean open probability increase and, over all experiments, the mean open probability was significantly reduced ($P \leq 0.002$; Mann-Whitney Rank Sum test). The inhibitory effect of the added sub-domain proteins is not as rapid as the inhibitory effect of the full length R domain. As previously reported, block of the Cl^- channel activity of CFTR by the full length R domain occurred within 1 min of protein addition [1]. Both RD1RD2 and RD2TM may take as long as 12 min to block (Tables 1 and 4). Previous experiments have shown that this blocking effect is specific to the R domain proteins, since addition of expressed proteins from Bore Mosaic virus and luciferase in rabbit reticulocyte lysates failed to block channel activity even after protracted incubation [1].

Table 2
Lack of effect of RD1 on CFTR channels

	Control	+PKI	+RD1
1	23.08 ± 1.95	24.88 ± 2.10	23.77 ± 1.21
2	33.48 ± 3.76	25.45 ± 1.79	26.80 ± 1.00
3	11.12 ± 1.61	6.04 ± 0.729	9.55 ± 0.550
4	69.53 ± 3.31	51.26 ± 6.89	41.72 ± 3.83
5	17.20 ± 2.93	16.05 ± 3.00	34.71 ± 2.14

The experimental procedures are identical to those listed in Table 1. Data from the five individual experiments show that RD1 had no effect on the CFTR channel.

Table 3
Lack of effect of RD2 on CFTR channels

	Control	+PKI	+RD2
1	40.19 ± 5.38	31.44 ± 3.05	38.74 ± 1.19
2	66.34 ± 3.74	60.51 ± 7.10	39.33 ± 1.95
3	26.74 ± 15.84	54.19 ± 8.45	54.08 ± 6.01
4	56.66 ± 1.94	39.23 ± 2.33	34.19 ± 1.65
5	46.94 ± 5.81	35.26 ± 2.76	24.59 ± 1.64

See Table 1 for legend. Five data show that RD2 had no effect on the CFTR channel. For representative single channel records, see Fig. 5.

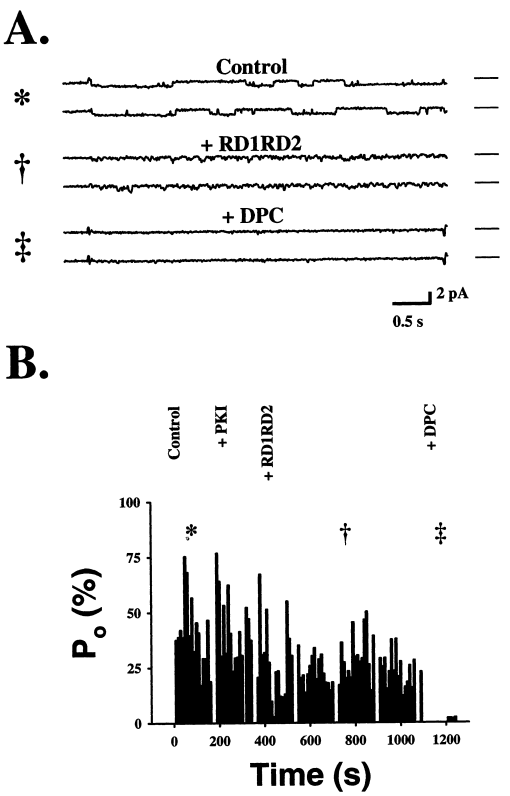

Fig. 3. Exogenous RD1RD2 blocks CFTR channel. Panel A: Representative traces of CFTR channels in the planar lipid bilayer. Conditions indicated above traces, as described in Section 2. Panel B: Diary plot with conditions indicated above (control, +PKI, etc.). Each bar is the mean of 16 episodes, two of which are displayed in panel A. Traces from panel A are taken from the diary plot at times indicated by symbols above plot (*, †, ‡).

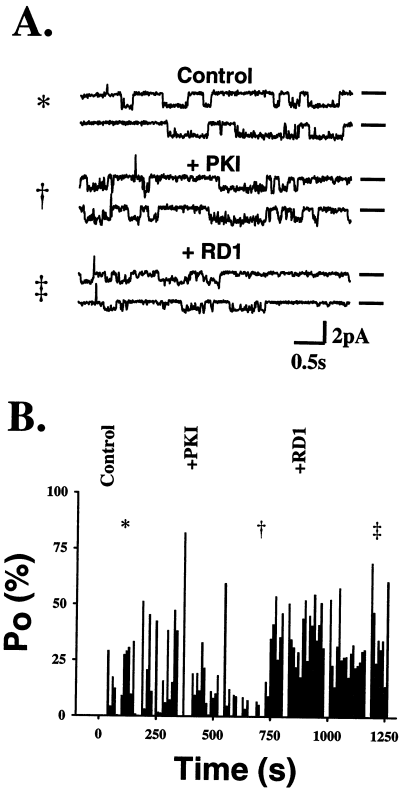

Fig. 4. RD1 has no effect on CFTR channel. Panel A: Consecutive traces of Cl^- currents of CFTR channels in the planar lipid membranes. Conditions of each set of two traces are indicated above the traces (control, +PKI, etc.). Each condition is described in Section 2. Panel B: Diary plot of single channel recording. Changing conditions are listed above (control, +PKI, etc.). Single episode recordings in panel A are taken from the diary plot at times indicated (*, †, ‡).

4. Discussion

Unphosphorylated exogenous R domain protein (aa 588–855) inhibits CFTR channel openings when applied to the intracellular side of the channel captured in the planar lipid bilayer. This inhibition is observed at concentrations of R domain as low as 15 pM and occurs within 1–2 min of addition of the exogenous R domain protein [1]. However, inhibitory activity is lost as the exogenous R domain protein becomes phosphorylated, and no inhibition is observed for CFTRΔR (708–835) channels, which have normal channel properties but do not require PKA to open [15]. These data, taken together, suggested that the inhibitory properties of the R domain protein are conformation dependent or charge dependent and probably result not from a 'plug' interacting with the channel pore, but from interaction with the nucleotide binding domains to prevent channel openings. In order to discover which portions of the large exogenous R domain protein were responsible for the inhibition, we expressed portions of the R domain, based on the domain model of Dulhanty and Riordan [9], and tested their activity in the planar lipid bilayer system.

Using criteria presented previously, we found that two of the R domain peptides, spanning amino acids 588–805 and amino acids 672–855, could inhibit the CFTR channel. These two peptides have in common amino acids 672–805, designated RD2 in the Dulhanty and Riordan model, which contains three of the five phosphorylation sites used in vivo. Apart from these sites, RD2 is not highly conserved among species [9]. However, this RD2 domain, expressed separately, failed to block even when applied at high concentrations for extended periods of time. At least two explanations are pos-

Table 4
RD2TM block of CFTR channels

	Control	+PKI	+RD2TM before block	+RD2TM after block
1	24.76 ± 3.49	23.09 ± 2.96	44.05 ± 2.78 < 11 min	11.26 ± 0.835 > 11 min
2	41.76 ± 8.45	85.58 ± 4.37	50.15 ± 3.90 < 2 min	0.102 ± 0.038 > 2 min
3	15.85 ± 4.73	16.95 ± 3.72	n.a.	8.71 ± 2.58 > 0 min

Data from three separate experiments are listed. See Table 1 for legend. See also Fig. 6 for representative single channel records and diary plot.

sible for these observations. First, the active region may reside within RD2 but require the additional extended sequence either at the N-terminus (RD1-RD2) or C-terminus (RD2-RTM) to stabilize the inhibitory conformation. Alternatively, there may be two sites in the R domain capable of inhibiting channel openings. One of these sites may reside in the RTM region or the RD2-RTM juncture, requiring at least some amino acids from the RTM region for full activity, and the other must require the secondary structure conferred by the extended sequence RD1-RD2, since neither of the overlapping pieces of this protein, expressed separately (RD1 or RD2), could effect inhibition under these conditions. The observations of Adams and Ma [18] that a small peptide from the RTM region can inhibit channel openings favors the second explanation. Support for this hypothesis also comes from experiments using an R domain piece containing amino acids 645 through 834 [13]. This protein, missing the last four amino acids of the Adams and Ma peptide, shows no inhibition of chloride currents in the unphosphorylated state. Since these four amino acids are found in two peptides that block (RD2TM and the Adams and Ma peptide), and are missing from the non-blocking protein, they may be important in the blocking effect.

The block effected by RD1RD2 and RD2TM differs in important ways from the block observed with the full length R domain protein. The block takes longer to effect, requires higher concentrations of added protein, and appears to leave flickery channel openings, whereas the R domain protein block occurred within 1–2 min and was virtually complete

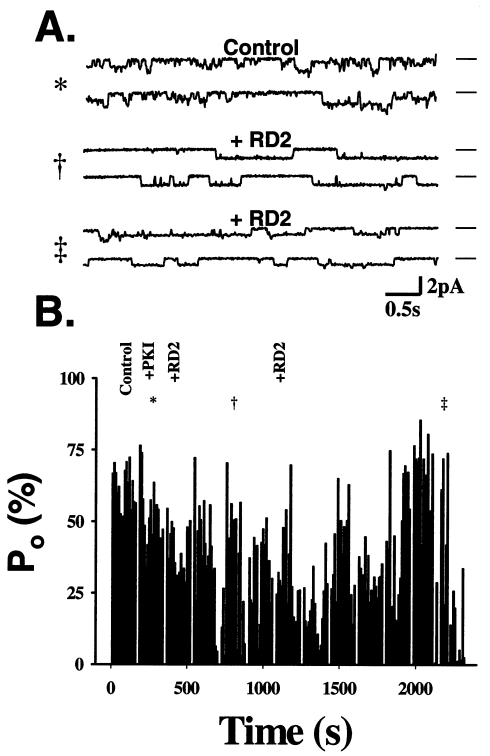

Fig. 5. RD2 has no effect on CFTR channel. Panel A: Downward deflections in current traces indicate channel openings. Each set of two episodes is taken from the diary plot in panel B at the times indicated by the symbols (*, †, ‡). Conditions indicated above traces (control, +PKI, etc.), are described in Section 2. Panel B: Diary plot of channel open probability with 16 episodes averaged for each bar.

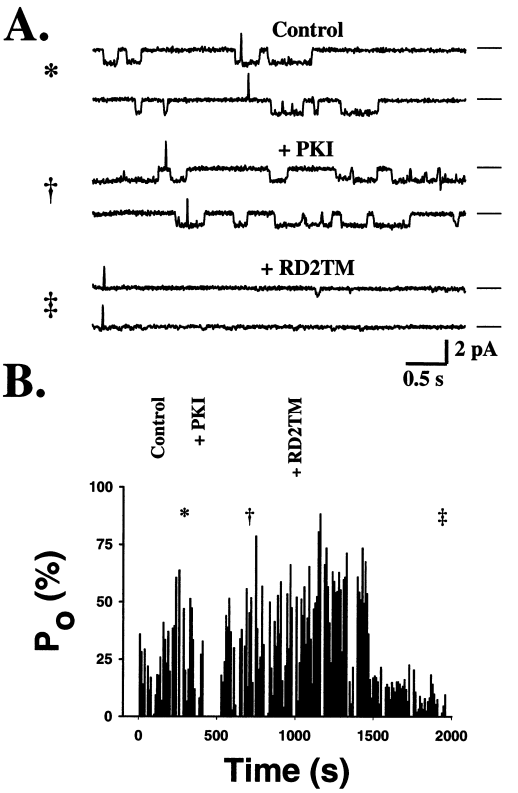

Fig. 6. Exogenous RD2TM blocks CFTR channel. Panel A: Representative traces of CFTR channels in the planar lipid membranes. Consecutive traces were measured at a test potential of −80 mV. Conditions indicated above traces, as described in Section 2. Panel B: Diary plot with conditions indicated above (control, +PKI, etc.). Each bar is the mean of 16 episodes, two of each condition are displayed in panel A. Traces from panel A are taken from the diary plot at times indicated by symbols above plot (*, †, ‡).

[1]. These observations may be explained if the longer chain length of the R domain protein stabilizes the inhibitory conformation, whereas the inhibitory conformation is less stable in the shorter peptides. This would lower the effective concentration of the inhibitory form of the protein in the shorter peptides, and also allow more rapidly reversible binding (giving rise to the flickery block). Alternatively, or in addition, portions of the full length R domain protein may bind to CFTR in a non-inhibitory fashion, improving the availability of the inhibitory portion to its favored site of inhibition. The flickery block does not arise simply from the addition of the mixture of proteins in the reticulocyte lysate preparation, for it does not occur with translated luciferase protein or Bore Mosaic virus proteins which span a spectrum of sizes [1].

The inhibitory function of the unphosphorylated R domain on CFTR chloride channel activity represents one important component of the function of this region of CFTR unique among ABC transporters. Together with the activation function of phosphorylated R domain [15], it contributes to the precise and likely carefully graded regulation of chloride transport by phosphorylation in vivo. The present data raise the possibility that more than one inhibitory site may exist in the R domain, and suggest that the activity of these site(s) may be highly dependent on the conformation assumed by the R domain.

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