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## Direct effects of 9-anthracene compounds on cystic fibrosis transmembrane conductance regulator gating

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### Abstract

Anthracene-9-carboxylic acid (9-AC) has been reported to show both potentiation and inhibitory effects on guinea-pig cardiac cAMP-activated chloride channels via two different binding sites, and inhibition of  $Mg^{2+}$ -sensitive protein phosphatases has been proposed for the mechanism of 9-AC potentiation effect. In this study, we examined the effects of 9-AC on wild-type and mutant human cystic fibrosis transmembrane conductance regulator (CFTR) chloride channels expressed in NIH3T3 or CHO cells. 9-AC inhibits whole-cell CFTR current in a voltage-dependent manner, whereas the potentiation effect is not affected by membrane potentials. Anthracene-9-methanol, an electro-neutral 9-AC analog, fails to block CFTR, but shows a nearly identical potentiation effect, corroborating the idea that two chemically distinct sites are responsible, respectively, for potentiation and inhibitory actions of 9-AC. 9-AC also enhances the activity of  $\Delta R$ -CFTR, a constitutively active CFTR mutant whose R-domain is removed. In excised inside-out patches, 9-AC increases  $P_o$  by prolonging the mean burst durations and shortening the interburst durations. We therefore conclude that two different 9-AC binding sites for potentiation and inhibitory effects on CFTR channels are located outside of the R-domain. We also speculate that 9-AC potentiates CFTR activity by directly affecting CFTR gating.

### Keywords

Chloride channel; Cystic fibrosis; Patch clamp; Single channel

### Introduction

Anthracene-9-carboxylic acid (9-AC), an aromatic compound, has been widely used as an anion probe to study various chloride channels such as cardiac cAMP-activated chloride currents in guinea pig ventricular myocytes [33], swelling-activated chloride currents in canine ventricular myocytes [6], CLC-1 chloride channel expressed in *Xenopus* oocytes [11], and  $Ca^{2+}$ -activated chloride currents in mouse kidney inner medullary collecting duct cells [25]. Most of these studies show that 9-AC blocks the anion permeation pathway. However, Zhou et al. [33] reported dual effects of 9-AC on the cardiac isoform of cystic fibrosis transmembrane

conductance regulator (CFTR). In addition to a voltage-dependent block of whole-cell cardiac CFTR currents, 9-AC voltage-independently increases the cAMP-dependent chloride conductance. They concluded that 9-AC blocks cardiac CFTR  $\text{Cl}^-$  conductance from the extracellular side but enhances the conductance from the intracellular side by inhibiting a  $\text{Mg}^{2+}$ -sensitive protein phosphatase [33,35].

CFTR, a member of the ABC (ATP binding cassettes) superfamily [27], is a chloride channel that is regulated by protein kinase A (PKA)-dependent phosphorylation of the regulatory (R)-domain, and gated by ATP binding/hydrolysis at its nucleotides binding domains [14]. Thus, inhibition of cellular protein phosphatases can increase the steady-state phosphorylation level of CFTR and thereby increases the activity of the channel [19,31]. However, many well-characterized CFTR activators, including isoflavonoids [21], benzimidazolone compounds [2,16,23], and benzoquinolinium compounds [3,15] seem to potentiate CFTR activity by directly binding to the channel protein [4,10,13,29,30].

Although 9-AC inhibits  $\text{Mg}^{2+}$ -sensitive protein phosphatase isolated from cardiac myocytes [35], whether this is the sole mechanism for the potentiation effect of 9-AC on CFTR remains unclear. Considering the fact that many structurally divergent compounds can affect CFTR gating independently of phosphorylation/dephosphorylation, we set out to test the hypothesis that 9-AC directly modulates CFTR gating. In this paper, we first confirmed the dual effects of 9-AC on CFTR using human epithelial CFTR exogenously expressed in NIH3T3 and CHO cells and then focused on the mechanisms of CFTR potentiation by 9-AC mainly using single-channel recording techniques.

## Materials and methods

### Cell culture

NIH3T3 cells stably expressing wild-type CFTR (NIH3T3/CFTR) or K1250A-CFTR mutant channels were grown as previously described [32] at 37°C and 5%  $\text{CO}_2$  in DMEM supplemented with 10% FBS. CHO cells were maintained as described in Powe et al. [24]. For whole-cell experiments, cell suspensions were prepared with trypsinization (0.25% trypsin and 1 mM EGTA in PBS). For excised inside-out experiments, cells were grown on small glass chips in a 35-mm Petri dish 1–2 days prior to use.

### Construction of $\Delta\text{R}$ -CFTR mutant

The plasmids, pGEMHE-1–633 and pGEMHE-837–1480 [8], were gifts from Dr. David Gadsby's laboratory (Rockefeller University, New York, N.Y., USA). We obtained the pBudCE4.1 CFTR1–633 by subcloning the 2-kb *Pst*I-*Xho*I fragment from pGEMHE1–633 subcloned into *Pst*I and *Sal*I sites of pBudCE4.1 expression vector (Invitrogen, Carlsbad, Calif., USA). The 1.9-kb *Kpn*I-*Xho*I fragment from pGEMHE837–1480 was then ligated to *Kpn*I and *Xho*I sites of pBudCE4.1 CFTR1–633. This final construct, pBudCE4.1  $\Delta\text{R}$ -CFTR, allows, in a single plasmid, the expression of the N-terminal half of CFTR (amino acids 1–633) under CMV promoter and the expression of the C-terminal half of CFTR (amino acids 837–1480) under human elongation factor 1 $\alpha$  (EF1 $\alpha$ ) promoter. All constructs were confirmed by automated sequencing (DNA Core, University of Missouri, Columbia, Mo., USA).

### Transient expression of $\Delta\text{R}$ -CFTR

To transiently express  $\Delta\text{R}$ -CFTR, CHO cells were grown in 35-mm tissue culture dishes with or without glass chips 1 day prior to transfection. The plasmid pBudCE4.1 split  $\Delta\text{R}$ -CFTR was co-transfected with pEGFP-C3 (Clontech, Palo Alto, Calif., USA) encoding green fluorescent protein using SuperFect transfection reagent (Qiagen, Valencia, Calif., USA) according to the manufacturer's protocols. The  $P_o$  of the  $\Delta\text{R}$ -CFTR expressed in CHO cells is consistently

higher than that reported by Csanády et al. [8]. Perhaps different expression systems could account for this discrepancy.

### Patch-clamp experiments

Pipette electrodes were made from Corning 7056 glass capillaries (Warner Instruments, Hammed, Conn., USA). The pipette resistance was  $\sim 3 \text{ M}\Omega$  in the bath solution. For whole-cell experiments, the membrane potential was held with an EPC9 or 10 amplifier (HEKA, Lambrecht/Pfalz, Germany) at 0 mV, following break-in with suction. Repetitive ramp voltages ( $\pm 100 \text{ mV}$ , 2 s in duration, every 6 or 10 s) were generated with Pulse software (HEKA) to create  $I$ - $V$  relationships. Currents traces were filtered at 1 kHz with a built-in four-pole Bessel filter and then digitized at 2 kHz. The currents were recorded at room temperature ( $\sim 25^\circ\text{C}$ ). The pipette solution contained (in mM): 10 EGTA, 121 TEA-Cl, 10 MgATP, 2  $\text{MgCl}_2$ , 5.5 glucose, and 10 HEPES (pH 7.4 with CsOH). The bath solution contained (in mM): 119 NaCl, 2  $\text{MgCl}_2$ , 1  $\text{CaCl}_2$ , 5 glucose, and 5 HEPES (pH 7.4 with NaOH). Sucrose (20 mM) was added to the bath solution to prevent activation of swelling-induced currents.

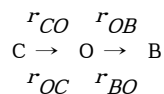
Single-channel CFTR currents were recorded at room temperature ( $\sim 25^\circ\text{C}$ ) with an EPC10 patch-clamp amplifier. Data were filtered at 100 Hz with an eight-pole Bessel filter (Warner Instruments) and captured onto a hard disk at 500 Hz. To obtain recordings with a high signal/noise ratio, we discarded patches with a seal resistance  $< 20 \text{ G}\Omega$ . The pipette solution contained (in mM): 140 *N*-methyl-D-glucamine chloride (NMDG-Cl), 2  $\text{MgCl}_2$ , 5  $\text{CaCl}_2$ , and 5 HEPES (pH 7.4 with NMDG). After the membrane patch was excised into an inside-out mode, the pipette was continuously perfused ( $\sim 0.8 \text{ ml/minute}$ ) with a solution containing (in mM): 150 NMDG-Cl, 2  $\text{MgCl}_2$ , and 5 Trizma base (pH 7.4 with NMDG). PKA and/or 1 mM Mg-ATP were applied to activate wild-type CFTR or  $\Delta\text{R}$ -CFTR activity (see Results).

### Reagents

Forskolin, purchased from Alexis (Lausen, Switzerland), was stored as 20 mM stock in DMSO at  $4^\circ\text{C}$ . Anthracene-9-carboxylic acid was purchased from Aldrich (Milwaukee, Wis., USA) and stored as 1 M stock in DMSO at  $-20^\circ\text{C}$ . 9-Anthracene-methanol (9-AM) was also purchased from Aldrich and stored as 100 mM stock in DMSO at  $-20^\circ\text{C}$ . Mg-ATP was purchased from Sigma (St Louis, Mo., USA) and stored as 250 mM stock in  $\text{H}_2\text{O}$  at  $-20^\circ\text{C}$ .

### Data analysis

Whole-cell and part of the single-channel analysis was done with Igor software (Wavemetrics, Lake Oswego, Ore., USA). For single-channel kinetic analysis, we used the program suite developed by Csanády [7]. Briefly, current traces were baseline corrected, idealized and fitted to a three-state model:



where O and C are open and closed states, respectively; B is a blocked state induced by an intrinsic blocker [34]; and  $r_{CO}$ ,  $r_{OC}$ ,  $r_{OB}$ , and  $r_{BO}$  are corresponding rate constants. Mean interburst, burst durations, and channel open probability ( $P_o$ ) were calculated as  $\tau_{ib} = 1/r_{CO}$ ,  $\tau_b = (1/r_{OC})(1 + r_{OB}/r_{BO})$ ,  $P_o = 1/(1 + r_{OC}/r_{CO} + r_{OB}/r_{BO})$ , respectively. All values are presented as mean  $\pm$  SEM. Student's *t*-test was performed with Sigmaplot (SPSS Science, Chicago, Ill., USA).  $P < 0.05$  was considered significant.

## Results

### Effects of 9-AC on whole-cell CFTR channel currents

The effects of 9-AC on wild-type CFTR were examined first with the whole-cell patch-clamp technique. Figure 1A shows a continuous whole-cell current trace from an NIH3T3 cell stably expressing wild-type CFTR. The basal conductance in the absence of forskolin is minimal (a) and the application of forskolin elicits a conductance with a linear  $I$ - $V$  relationship (b–a). 9-AC, added in the presence of forskolin, increases the whole-cell CFTR currents at positive membrane potentials, but inhibits inward currents at a pipette potential of  $-100$  mV (c–a). These dual effects of 9-AC are very similar to those reported previously on cardiac CFTR  $\text{Cl}^-$  channels [33].

The whole-cell CFTR currents in the presence of different concentrations of 9-AC at a positive and negative pipette potentials  $+100$  mV and  $-100$  mV were normalized to the current level with  $10$   $\mu\text{M}$  forskolin and plotted against 9-AC concentrations (open symbols in Fig. 1B). The enhancement effects were underestimated because of the inhibitory effect. Since K1250A-CFTR channels exhibit a long-lived locked-open state ( $P_o > 0.85$  [17,24,34], cf. [5,26]), we reason that 9-AC may show a negligible potentiation effect on this mutant and thus the inhibitory effect can be isolated and quantified. Figure 1C shows the whole-cell current trace from an NIH3T3 cell stably expressing K1250A-CFTR. Indeed, 9-AC only exerts a voltage-dependent inhibitory effect on this mutant. Figure 1D shows the dose-response relationships of this inhibitory effect of 9-AC on K1250A-CFTR at three different membrane potentials ( $-60$ ,  $-80$ , and  $-100$  mV). A fit to the Hill equation yielded  $K_i$  values of  $2.55$  mM,  $1.70$  mM, and  $1.26$  mM, and a Hill coefficient of  $0.98$ ,  $0.98$ , and  $0.95$  (at  $-60$ ,  $-80$ , and  $-100$  mV, respectively).

Once the inhibitory effect of 9-AC was quantified, we used the resulting parameters to correct the underestimated enhancement effects. Compensated values for potentiation at  $+100$  mV and  $-100$  mV nearly overlap (closed symbols in Fig. 1B), indicating that, unlike the inhibitory effect, the potentiation effect is voltage-independent. It is also interesting to note that the  $K_d$  for the potentiation effect (compensated  $K_d$ :  $291$   $\mu\text{M}$  at  $100$  mV) is lower than those for the inhibitory effect, suggesting the presence of two distinct binding sites. We now focus on exploring the mechanisms of potentiation.

### Effects of 9-AM on wild-type CFTR

The voltage-dependent inhibition by 9-AC suggests that the negative charge moiety is important for binding of 9-AC to the inhibitory site. If so, elimination of the negative charge of 9-AC may abolish its inhibitory effects. Indeed, 9-AM, which has an electrically neutral side chain, no longer inhibits the CFTR currents (Fig. 2A). However, the potentiation effect is still observed (Fig. 2B). Maximally dissolvable concentration of 9-AM ( $100$   $\mu\text{M}$ ) enhanced CFTR currents by  $1.25 \pm 0.07$  fold ( $n=4$ ), a value very close to the potentiation by  $100$   $\mu\text{M}$  9-AC (see dose-response relationships in Fig. 1B). These results indicate that the negative charge is not required for the potentiation effect and the potentiation site is chemically distinct from the inhibitory site.

### Effects of 9-AC on constitutively active CFTR mutant

It has been reported that several lipophilic CFTR activators such as genistein and MPB compounds potentiate CFTR currents by phosphorylation-independent mechanisms [10,29]. The R-domain is the main regulatory domain for CFTR channel activity via phosphorylation/dephosphorylation process [14]. We next tested 9-AC on  $\Delta\text{R}$ -CFTR, a CFTR construct whose regulatory domain (amino acids 634–836) was completely removed, and therefore is constitutively active [8]. Figure 2C shows significant basal  $\text{Cl}^-$  conductance from a CHO cell

transiently expressing  $\Delta R$ -CFTR. This conductance is not affected by the addition of forskolin or CPT-cAMP (data not shown). However, 9-AC exerts a similar potentiation effect as that on wild-type CFTR as seen with the outwardly rectified  $I$ - $V$  curve in the presence of 9-AC (Fig. 2D).

### Effects of 9-AC on CFTR gating in excised inside-out patches

Next we examined effects of 9-AC on CFTR gating in excised inside-out patches. Once wild-type CFTR currents were activated by exogenous PKA (25 U/ml) and ATP (1 mM), the channel activity is well maintained in the presence of ATP alone, e.g., [32]. Subsequent application of 9-AC altered the CFTR gating both at positive (+50 mV, Fig. 3A) and negative membrane potential (−50 mV, Fig. 3B). In the presence of 9-AC, the open channel noise is higher and the single-channel current amplitudes are smaller at −50 mV. This inhibition, typical of a fast flickering channel block, is voltage dependent (Fig. 3C). Figure 3D shows that the fold increases of  $NP_o$  at +50 mV and −50 mV are very similar, again demonstrating the voltage-independent nature of the potentiation site. Thus, these results firmly establish that 9-AC acts on CFTR by interacting with two different binding sites.

### Effects of 9-AC on single-channel kinetics of $\Delta R$ -CFTR

To further quantify the effects of 9-AC on CFTR gating, we recorded single-channel current of  $\Delta R$ -CFTR in excised inside-out patches.  $\Delta R$ -CFTR was chosen because it is technically easier to obtain patches containing few channels (maximal current steps  $\leq 2$ ) and this channel is resistant to run-down by membrane-associated protein phosphatases. The current was recorded at +50 mV to ensure a high signal/noise ratio, an essential requirement for rigorous kinetic analysis. Figure 4A shows a continuous current trace of  $\Delta R$ -CFTR in an excised inside-out patch. The addition of 1 mM 9-AC slightly decreases the single-channel amplitude (also see Fig. 4B) but significantly increases the  $P_o$  of the CFTR channel. As summarized in Fig. 4C, 9-AC (1 mM) increased  $P_o$  by prolonging the mean burst durations ( $\tau_b$ ) and shortening the mean interburst durations ( $\tau_{ib}$ ) significantly.

## Discussion

A  $Cl^-$  channel blocker, 9-AC, has been previously reported to have a voltage-independent potentiation effect as well as a voltage-dependent inhibitory effect on guinea pig cardiac CFTR channels using whole-cell recordings [33]. In this study, we focused on the mechanism of the potentiation effect of 9-AC on human CFTR channels exogenously expressed in NIH3T3 or CHO cells mainly using single-channel recordings. We largely confirmed the results previously reported by Zhou et al. [33], but also obtained several original and some different results from those in their reports.

### Are protein phosphatases involved in 9-AC potentiation?

PKA-dependent phosphorylation of consensus serine/threonine residues in the R-domain is a prerequisite to activate CFTR [14]. Therefore, inhibiting protein phosphatases is a potential mechanism for enhancing CFTR activity. Several different types of phosphatases have been implicated in dephosphorylating CFTR. Luo et al. [22] showed that phosphatase 2C can deactivate CFTR in excised patches. Coimmunoprecipitation experiments suggest that phosphatase 2C and CFTR form a stable complex to facilitate regulation of the channel [36]. Travis et al. [28] indicated that phosphatase 2C reduced CFTR activity in epithelia by increasing inactivation rate. Our previous studies showed that okadaic acid and calyculin A, both inhibitors of phosphatase 1 and 2A, enhance cAMP-dependent whole-cell CFTR currents in guinea pig ventricular myocytes or cell-attached CFTR expressed in insect Hi-5 cells [19, 31]. Inhibition of protein phosphatase may also account for the pharmacological effects of other CFTR activators, such as deltamethrin [12] and bromotetramisole [23].



Zhou et al. [35] showed that the magnitude of cAMP-activated cardiac whole-cell CFTR conductance is inversely proportional to intracellular free  $[Mg^{2+}]$ . Since the potentiation effect of low  $[Mg^{2+}]$  mimics that of 9-AC, they propose that 9-AC enhances CFTR activity by inhibiting  $Mg^{2+}$ -sensitive protein phosphatase(s). By measuring phosphatase activity in the presence of various  $[Mg^{2+}]$ , they also showed that 9-AC inhibits phosphatase activity in a  $Mg^{2+}$ -dependent manner.

In this paper, we have demonstrated that 9-AC can affect ATP-dependent gating of wild-type CFTR in excised inside-out membrane patches. Similar potentiation of CFTR gating by 9-AC was also observed for  $\Delta R$ -CFTR, a construct whose R-domain is completely removed. Although removal of the R-domain eliminates most of the consensus sites for PKA- and PKC-dependent phosphorylation, CFTR protein is known to possess potential phosphorylation sites outside the R-domain [10,14]. Therefore, our  $\Delta R$ -CFTR experiments can not completely rule out the possibility of involvement of phosphorylation/dephosphorylation in 9-AC action in whole-cell recordings. However, it is worth noting that  $\Delta R$ -CFTR expressed in our systems using CHO cells does not respond to cAMP agonists in whole-cell and cell-attached modes, whereas it has been reported that  $\Delta R$ -CFTR expressed in oocytes can still be enhanced by cAMP stimulation [8]. Exogenous PKA also fails to increase ATP-dependent  $\Delta R$ -CFTR activity in excised inside-out patches (T. Ai et al., unpublished observations). Moreover the magnitude of  $P_o$  increase by 9-AC for wild-type and  $\Delta R$ -CFTR activity in excised inside-out patches is very similar to those obtained in the whole-cell configuration. Thus, while we cannot rule out the possibility of additional involvement of protein phosphatases in 9-AC potentiation, the simplest explanation for these results is that 9-AC, like numerous other CFTR activators characterized previously (e.g., isoflavonoids, benzimidazolone compounds), modulates CFTR gating independently of phosphorylation/dephosphorylation.

Our proposition that 9-AC affects CFTR seems to contradict the conclusion reached by Zhou et al. [33]. It should be noted, however, that the experiments were performed on CFTRs expressed in different systems. We obtained a  $K_d$  value of  $\sim 290 \mu M$  for the potentiation effect which was more than tenfold higher than that reported by Zhou et al. ( $\sim 13 \mu M$ ) [33]. This suggests that the mechanisms underlying 9-AC-induced potentiation in guinea pig cardiac myocytes might be different from what we report here.

### How does 9-AC modify CFTR gating?

Zhou et al. [33] previously reported the existence of two different effects of 9-AC: one is an extracellular inhibitory effect with a low affinity and another is an intracellular potentiation effect with a high affinity. Our data also clearly demonstrated that 9-AC acts on two different binding sites for its potentiation and inhibitory effects. This conclusion is supported by several observations: (1) the apparent affinity for the potentiation effect is higher than that of the inhibitory effect; (2) while the inhibition is voltage-dependent, the potentiation is not; and (3) removing the negative charge in 9-AC completely abolishes the block but does not affect the potentiation effect.

It is also noteworthy that 9-AC blocks CFTR channels from the cytoplasmic side of the membrane in our experiments (Fig. 3), whereas Zhou et al. [33] did not observe an inhibitory effect upon cytoplasmic application of 9-AC and concluded that 9-AC blocks CFTR only from the extracellular side. The 5.3% and 31.4% decreases of the single-channel amplitude at +50 and  $-50$  mV, respectively (Fig. 3C), are consistent with a cytoplasmic-accessible binding site  $\sim 50\%$  across the field. This is perhaps the same binding site thoroughly characterized for numerous organic anion blockers (reviewed by Hwang and Sheppard [18]).

The exact molecular nature of the binding site for potentiation effect is unclear. It is, however, interesting to note that all the CFTR gating modifiers (including genistein and capsaicin ) we

have characterized so far show very similar actions on the single-channel kinetics. They all increase the  $P_o$  by shortening the closed time and prolonging the open time [1,2,20,29]. These studies also indicate that the binding site is not located in the R-domain or at the interface between the R-domain and other domains. As proposed previously [4,29], the binding site might be in the nucleotide binding domains for their obvious roles in CFTR gating. The structural simplicity of anthracene compounds makes them useful tools for future explanation of the molecular basis of gating modification.

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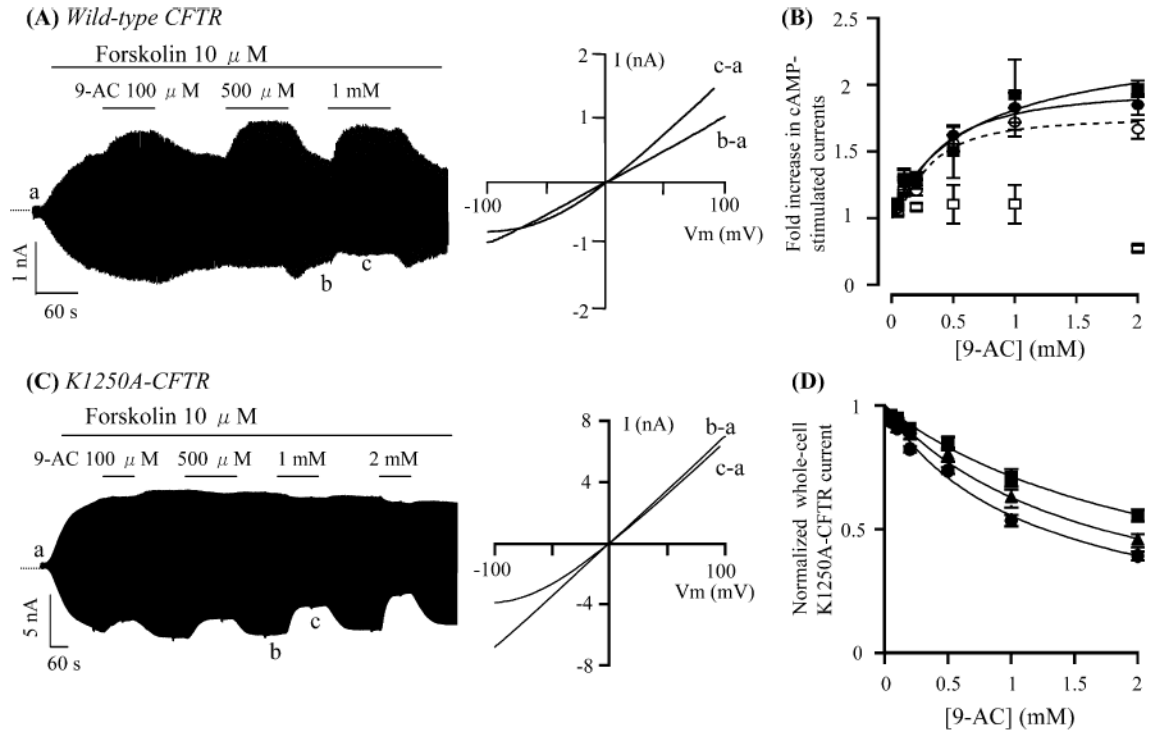
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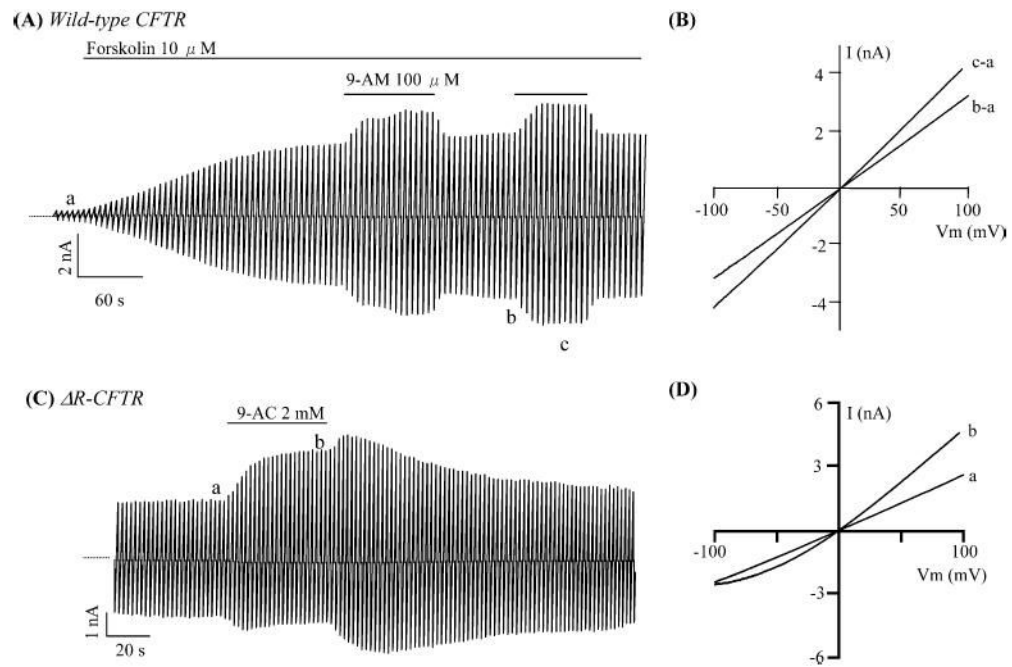


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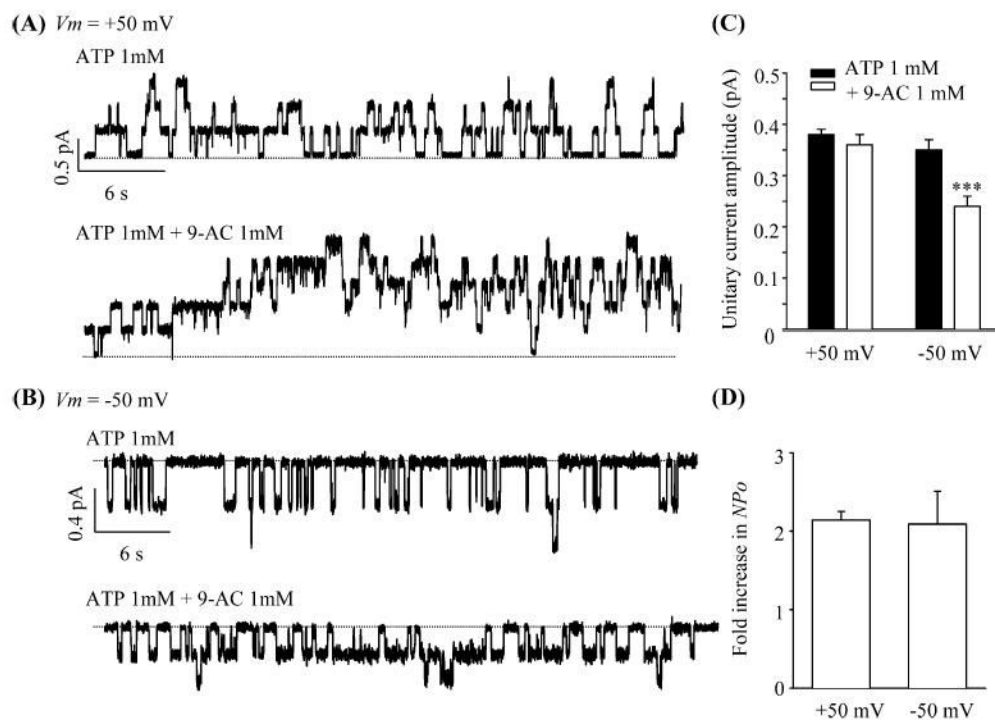


**Fig. 1A–D.**

Effects of anthracene-9-carboxylic acid (9-AC) on whole-cell cystic fibrosis transmembrane conductance regulator (CFTR) currents. **A** A whole-cell wild-type CFTR current trace in the presence of 10  $\mu$ M forskolin and various concentrations of 9-AC. *I-V* relationships shows net CFTR currents in different conditions as marked in the *left panel*. **B** Dose-response relationships of 9-AC potentiation effects. Net potentiation effects in wild-type CFTR were calculated by correcting for the inhibitory effects obtained from recordings of 9-AC on K1250A-CFTR currents.  $y_{comp} = y_{raw}/(1 - x/100)$ , where  $y_{comp}$ , is the compensated value;  $y_{raw}$ , the measured fold increase;  $x$ , the percentage inhibition of whole-cell K1250A-CFTR currents with 1 mM 9-AC at a pipette potential ( $V_p$ ) of +100 mV or -100 mV. *Open circles*:  $y_{raw}$  for 1 mM 9-AC at +100 mV; *filled circles*:  $y_{comp}$  at +100 mV; *open squares*:  $y_{raw}$  at -100 mV; *filled squares*:  $y_{comp}$  at -100 mV. **C** A whole-cell K1250A-CFTR current trace. *I-V* relationships show net K1250A-CFTR currents in the presence or absence of 9-AC. **D** Dose-response relationships of the inhibitory effect on K1250A-CFTR currents at three different voltages. *Filled squares*: -60 mV; *filled triangles*: -80 mV; *filled circles*: -100 mV. All values are represented by mean  $\pm$  SEM ( $n=3$ ). Lines were fitted using the Hill equation:  $y=1+(y_{max} - 1)/[1+(K_d/x)^n]$  or  $y=1/[1+(x/K_i)^n]$ , where  $y$  is normalized current;  $y_{max}$  the maximal response;  $x$  the concentration of 9-AC;  $K_d$  the half-maximal concentration of potentiation;  $K_i$  the half-maximal concentration of inhibition; and  $n$  is the Hill coefficient

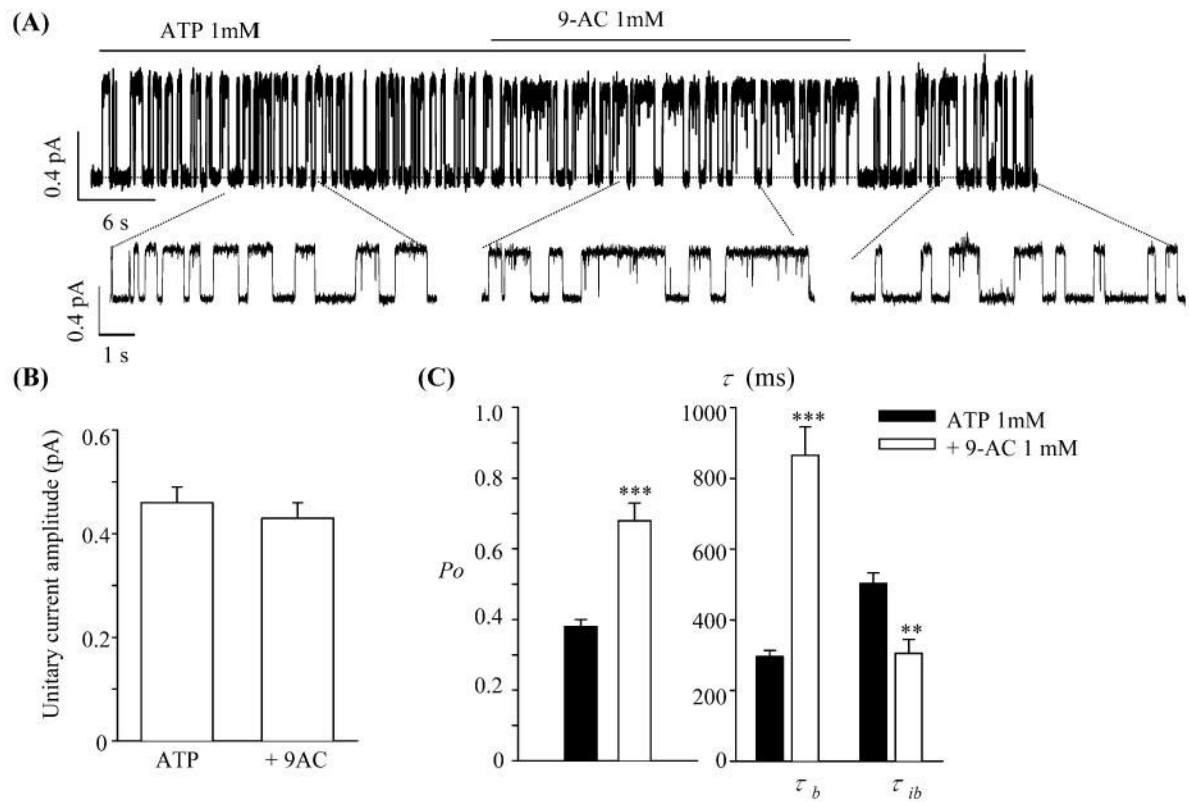


**Fig. 2A–D.** Effects of 9-anthracene compounds on CFTR activity. **A** Effects of 9-anthracene methanol (9-AM) on whole-cell cAMP-stimulated wild-type CFTR currents ( $n=4$ ). **B**  $I$ - $V$  relationships in different conditions as marked in **A**. **C** Effects of 9-AC on whole-cell  $\Delta R$ -CFTR currents ( $n=5$ ). Notice that basal currents were observed in the absence of cAMP agonists. **D**  $I$ - $V$  relationships in different conditions as indicated in **C**



**Fig. 3A–D.**

Direct effects of 9-AC on CFTR gating. Single-channel traces of wild-type CFTR in excised inside-out patches in the presence or absence of 9-AC at two different membrane potentials: +50 mV (**A**) and -50 mV (**B**). **C** Summary of unitary current amplitudes. **D** Fold increase in  $NP_0$  at two different potentials.  $NP_0$  was calculated by dividing the steady-state mean currents with unitary current amplitudes. All values are mean $\pm$ SEM ( $n=5$ ). \*\*\* $P<0.001$  versus ATP 1 mM



**Fig. 4A-C.**

Effects of 9-AC on single-channel kinetics of  $\Delta R$ -CFTR. **A** A single-channel  $\Delta R$ -CFTR current trace in an excised inside-out patch ( $V_m=50$  mV). **B** Summary of unitary current amplitudes in the presence or absence of 9-AC. **C** Summary of single-channel kinetics parameters. All values are mean $\pm$ SEM ( $n=6$ ). \*\* $P<0.01$ , \*\*\* $P<0.001$  versus ATP 1 mM