

## Phosphate Stimulates CFTR Cl<sup>-</sup> Channels

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**ABSTRACT** Cystic fibrosis transmembrane conductance regulator (CFTR) Cl<sup>-</sup> channels appear to be regulated by hydrolysis of ATP and are inhibited by a product of hydrolysis, ADP. We assessed the effect of the other product of hydrolysis, inorganic phosphate (P<sub>i</sub>), on CFTR Cl<sup>-</sup> channel activity using the excised inside-out configuration of the patch-clamp technique. Millimolar concentrations of P<sub>i</sub> caused a dose-dependent stimulation of CFTR Cl<sup>-</sup> channel activity. Single-channel analysis demonstrated that the increase in macroscopic current was due to an increase in single-channel open-state probability ( $p_o$ ) and not single-channel conductance. Kinetic modeling of the effect of P<sub>i</sub> using a linear three-state model indicated that the effect on  $p_o$  was predominantly the result of an increase in the rate at which the channel passed from the long closed state to the bursting state. P<sub>i</sub> also potentiated activity of channels studied in the presence of 10 mM ATP and stimulated Cl<sup>-</sup> currents in CFTR mutants lacking much of the R domain. Binding studies with a photoactivatable ATP analog indicated that P<sub>i</sub> decreased the amount of bound nucleotide. These results suggest that P<sub>i</sub> increased CFTR Cl<sup>-</sup> channel activity by stimulating a rate-limiting step in channel opening that may occur by an interaction of P<sub>i</sub> at one or both nucleotide-binding domains.

### INTRODUCTION

The cystic fibrosis transmembrane conductance regulator (CFTR) is a Cl<sup>-</sup> channel with novel structure and regulation (for reviews see Welsh et al., 1992; and Riordan, 1993). Amino acid sequence analysis suggests that CFTR is composed of five domains. There are two membrane-spanning domains, each consisting of six proposed transmembrane segments. The membrane-spanning domains are thought to contribute to formation of the Cl<sup>-</sup> channel pore (Anderson et al., 1991b; Sheppard et al., 1993; Tabcharani et al., 1993). There are also two cytoplasmic nucleotide binding domains (NBDs) that contain Walker A and B sequences for interaction with nucleotides (Walker et al., 1982). The fifth domain is a unique cytoplasmic domain, called the R domain, which has numerous consensus sequences for phosphorylation by cAMP-dependent protein kinase (PKA) and protein kinase C.

Functional studies have shown that at least two conditions must be fulfilled for the CFTR Cl<sup>-</sup> channel to open. First, the R domain must be phosphorylated (usually by PKA) (Tabcharani et al., 1991; Berger et al., 1991; Cheng et al., 1991; Chang et al., 1993). Previous studies suggest that phosphorylation may relieve tonic inhibition of channel activity (Rich et al., 1991, 1993). Second, nucleoside triphosphates such as ATP must be present on the cytosolic surface to open

the channel. Functional (Anderson et al., 1991a; Anderson and Welsh, 1992) and biochemical (Thomas et al., 1991, 1992; Hartman et al., 1992; Travis et al., 1993; Ko et al., 1994) studies have shown that ATP interacts directly with the NBDs. As the concentration of intracellular ATP increases, channel activity, measured as either macroscopic Cl<sup>-</sup> current or the single-channel open-state probability ( $p_o$ ) increases. It has been proposed that channel activity may require hydrolysis of ATP because activity is greatly reduced in the absence of Mg<sup>2+</sup> and because hydrolyzable nucleoside triphosphates are required for channel opening. However, whether hydrolysis actually occurs and what role it may play in channel activity is unclear.

In previous studies we showed that ADP inhibits CFTR Cl<sup>-</sup> channels (Anderson and Welsh, 1992). Therefore, we considered the possibility that P<sub>i</sub>, the other product of ATP hydrolysis, might also inhibit CFTR. Product inhibition of ATP-dependent reactions by intracellular P<sub>i</sub> has been observed in other biological systems. For example, in skeletal muscle, accumulation of intracellular P<sub>i</sub> may be among the most important metabolic changes responsible for a decrease in contractile force (Ruegg et al., 1971; Dawson et al., 1978; Nosek et al., 1987). To test the hypothesis that P<sub>i</sub> might inhibit CFTR, we assessed the effect of P<sub>i</sub> on PKA-phosphorylated CFTR Cl<sup>-</sup> channels using the excised, inside-out configuration of the patch-clamp technique.

Contrary to our expectations, we found that P<sub>i</sub> stimulated CFTR channel activity. Single-channel analysis demonstrated that P<sub>i</sub> stimulated CFTR channel activity by increasing  $p_o$  and not by permeating the channel. Kinetic modeling demonstrated that P<sub>i</sub> stimulated CFTR channel activity predominantly by increasing the rate at which the channel enters the bursting state. These and other functional studies plus biochemical studies showing that P<sub>i</sub> competes with ATP binding to CFTR suggest that P<sub>i</sub> exerts its stimulatory influence through interaction at the NBDs.

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**Abbreviations used:** CFTR, cystic fibrosis transmembrane conductance regulator; NBD, nucleotide binding domain; P<sub>i</sub>, inorganic phosphate;  $p_o$ , single-channel open-state probability; Cl<sup>-</sup>, chloride; 8-N<sub>3</sub>ATP, 8-azidoadenosine 5'-triphosphate; PKA, catalytic subunit of cAMP-dependent protein kinase.

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## MATERIALS AND METHODS

### Chemicals and solutions

ATP (adenosine 5'-triphosphate, disodium salt), sodium phosphate ( $\text{Na}_2\text{PO}_4$ , P<sub>i</sub>), 8-N<sub>3</sub>ATP, and all other reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted. Sodium sulfate ( $\text{Na}_2\text{SO}_4$ ) was from EM Science (Gibbstown, NJ). Catalytic subunit of PKA was obtained from Promega Corp. (Madison, WI). [ $\alpha$ -<sup>32</sup>P]8-N<sub>3</sub>ATP was from ICN Radiochemicals (Irvine, CA).

For experiments with excised inside-out membrane patches, the pipette (extracellular) solution contained (in mM): 140 NMDG (*N*-methyl-D-glucamine), 100 aspartic acid, 35.5 HCl, 5  $\text{CaCl}_2$ , 2  $\text{MgCl}_2$ , 10 HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), pH 7.3 with 1 N NaOH. The bath (intracellular) solution contained (in mM): 140 NMDG, 135.5 HCl, 3  $\text{MgCl}_2$ , 10 HEPES, 4 Cs, and 1 EGTA, pH 7.3 with 1 N HCl ( $[\text{Ca}^{2+}]_{\text{free}} < 10^{-8}$  M). For whole-cell experiments, pipette (intracellular) solution contained (in mM): 120 NMDG, 115 aspartic acid, 3  $\text{MgCl}_2$ , 4 Cs, 1 EGTA, and 1 mM  $\text{Na}_2\text{ATP}$ , pH 7.3 with 1 N HCl ( $[\text{Ca}^{2+}]_{\text{free}} < 10^{-8}$  M). Bath (extracellular) solution contained (in mM): 140 NaCl, 10 HEPES, 1.2  $\text{MgSO}_4$ , 1.2  $\text{CaCl}_2$ , and 30 mM sucrose, pH 7.3 with 1 N NaOH. Stock solutions of sodium phosphate (P<sub>i</sub>) and sodium sulfate were 200 mM in bath solution without CsEGTA, pH 7.3, and diluted in bath solution to give desired final concentration.

### Cells and transfection procedure

We used three different cell types expressing wild-type and mutant CFTR: stably transfected National Institutes of Health 3T3 fibroblasts and C127 mouse mammary epithelia cells, or transiently transfected HeLa cells as previously described (Anderson and Welsh, 1992). Transient transfection of HeLa cells with the vaccinia virus/bacteriophage T7 hybrid expression system was as previously described (Karkaria et al., 1990; Carson and Welsh, 1993). Briefly, cells were infected with two recombinant viruses (10–20 MOI each), one containing the bacteriophage T7 RNA polymerase driven by a viral promoter and a second recombinant virus containing wild-type or mutant CFTR cDNA driven by a T7 promoter. HeLa cells were plated at  $\sim 5 \times 10^4$  cells/cm<sup>2</sup> on collagen-coated glass coverslips 24 h before infection and were studied 18 h after infection. Similar results were obtained with all three cell types, and the data are combined.

### Patch-clamp technique

Methods for excised, inside-out patch-clamp recording are similar to those previously described (Hamill et al., 1981; Anderson et al., 1991a; Carson and Welsh, 1993). An Axopatch 200 amplifier (Axon Instruments, Inc., Foster City, CA) was used for voltage clamping and current amplification. A microcomputer (Dell 333D, Dell Computer Corp., Austin, TX) and the pClamp software package (Axon Instruments, Inc.) were used for data acquisition and analysis. Patch pipettes were fabricated as previously described (Carson and Welsh, 1993). Patch pipettes of 2–15 M $\Omega$  were used, and seals of  $> 5$  G $\Omega$  were routinely obtained. Data were recorded on videotape after pulse-code modulation using a PCM-2 A/D VCR adapter (Medical Systems Corp., Greenvale, NY). Voltages are referenced to the extracellular aspect of the membrane. Whole-cell and excised macropatch experiments were performed at a holding potential of  $-40$  mV; single-channel data were recorded at a holding potential of  $-80$  mV. Experiments were conducted at 34–36°C using a temperature-controlled microscope stage (Brook Industries, Lake Villa, IL).

Whole-cell and excised macropatch replayed records were filtered at 1 kHz using an eight-pole Bessel filter (Frequency Devices Inc., Haverhill, MA) and digitized at 2 kHz. Each time course data point represents the average current from 1 s with one data point collected every 5 s. Average currents for an intervention were determined as the average of the last 12 data points during the intervention. To compensate for any channel rundown during an experiment, specific interventions were bracketed with current measurements made with similar concentrations of ATP but without the test compound; the intervention current was then compared with the average of

pre- and postintervention currents. Single-channel replayed records were filtered at 1 kHz, digitized at 5 kHz, and digitally filtered at 500 Hz. Idealized records were created using a half-height transition protocol; transitions less than 1 ms in duration were not included in the analysis. For the purpose of illustration, time-course figures are inverted so that an upward deflection represents an inward current, data points during solution perfusion were not included in some figures, and single-channel traces were digitally filtered at 200 Hz.

Burst analysis was performed using a  $t_c$  (the time that separates interburst closures from intraburst closures) of 20 ms. This value was derived from analysis of wild-type CFTR closed-time histograms derived from excised inside-out membrane patches containing a single channel studied in the presence of 1 mM ATP plus PKA. When plotted with a logarithmic  $x$ -axis, the two closed states appear as distinct populations and the time constants differ by greater than two orders of magnitude (average fast closed time constant,  $\tau_{\text{cp}} = 1.79 \pm 0.22$  ms, average slow closed time constant,  $\tau_{\text{cs}} = 187 \pm 13$  ms,  $n = 6$ , data not shown). The burst delimiter,  $t_c$ , was chosen as the nadir between the two populations of closures. The large difference between time constants suggests that misclassification errors made when defining bursts should be small (Colquhoun and Sakmann, 1985). A value of 20 ms was also derived using the method of Sigurdson et al. (1987). Closures longer than 20 ms were considered to define gaps between bursts, while closures shorter than this time were considered gaps within bursts.

### Maximum likelihood analysis and kinetic modeling

Maximum likelihood analysis and kinetic analysis were performed as described (Winter et al., 1994). Briefly, unfiltered single-channel data were digitized on a microcomputer (Apple Macintosh, Apple Computer, Inc., Cupertino, CA) equipped with a multifunctional data acquisition board (NB-MIO-16) and LabVIEW 2 software (National Instruments, Austin, TX) at 5 kHz after filtering with an eight-pole Bessel filter (Frequency Devices Inc., Haverhill, MA) at a corner frequency of 1 kHz with subsequent digital filtering at 500 Hz. Our previous work has shown that a linear three-state model ( $C_1 \leftrightarrow C_2 \leftrightarrow O$ ) best describes the kinetics of CFTR channel activity (Winter et al., 1994). The Maple 5 symbolic algebra program (Waterloo Maple Software, Waterloo, Canada) was used to derive the open and closed time probability density functions for this model by solving the matrix equations in terms of the rate constants. The resulting equations were used in LabVIEW 2 to determine the set of rate constants that yielded the maximum likelihood for the open and closed times observed with different experimental interventions.

### [ $\alpha$ -<sup>32</sup>P]8-N<sub>3</sub>ATP photolabeling

Photolabeling of membrane-associated CFTR was performed as previously described (Travis et al., 1993). Monolayer cultures of *Spodoptera frugiperda* (Sf9) cells were infected with a baculovirus containing the entire coding sequence for human CFTR (gift of R. J. Gregory and A. E. Smith, Genzyme Corp., Framingham, MA). Membranes were prepared by differential centrifugation and resuspended in 20 mM HEPES, pH 7.5, 50 mM NaCl, 3 mM  $\text{MgSO}_4$ , with 2  $\mu\text{g}/\text{ml}$  each of leupeptin, aprotinin, and pepstatin.

Photolabeling was performed in 30  $\mu\text{l}$  of the same buffer also containing 1 mM ATP, 30  $\mu\text{M}$  [ $\alpha$ -<sup>32</sup>P]8-N<sub>3</sub>ATP (6–12 Ci/mM) and other additions as indicated in the figures. The binding reaction was started by addition of Sf9 membranes (30  $\mu\text{g}$  protein) to tubes containing all other reaction components. After incubation for 1 min on ice, samples were irradiated at a distance of 3 cm for 1 min at 312 nm with a model UHV (680 microwatts/cm<sup>2</sup>) UV lamp to crosslink 8-N<sub>3</sub>ATP to CFTR. After irradiation, CFTR was solubilized and immunoprecipitated as described (Ostedgaard and Welsh, 1992; Travis et al., 1993) using antibodies raised against the R domain (M13–1, 0.3  $\mu\text{g}/\text{sample}$ ) and against the C terminus (M1–4, 10  $\mu\text{g}/\text{sample}$ ). Immuno-complexes were analyzed by SDS-PAGE and incorporation of [ $\alpha$ -<sup>32</sup>P]-8-N<sub>3</sub>-ATP was quantitated with an AMBIS radioanalytic imaging system (AMBIS Systems, Inc., San Diego, CA). Data are expressed as percent of radiolabel incorporation relative to control, which had no added P<sub>i</sub> or sulfate.

Results are means  $\pm$  SE of  $n$  observations. Statistical significance was assessed using a paired Student's *t*-test, or a one-sample *t*-test when the reference data had been normalized.

## RESULTS

### P<sub>i</sub> stimulates CFTR chloride channel activity

To determine whether intracellular P<sub>i</sub> would alter CFTR Cl<sup>-</sup> channel activity, we added 100 mM P<sub>i</sub> to the cytosolic surface of an excised inside-out membrane patch that had been phosphorylated by PKA. We were surprised to find that P<sub>i</sub> stimulated the activity of phosphorylated CFTR Cl<sup>-</sup> channels (Fig. 1 A). The increase in CFTR Cl<sup>-</sup> current was reversible upon removal of P<sub>i</sub> and occurred only in the presence, but not in the absence, of ATP, indicating that P<sub>i</sub> cannot substitute for ATP. Stimulation of CFTR channel activity by P<sub>i</sub> was concentration dependent (Fig. 1 B). There was no clear plateau to the dose-response curve, and the data could not be fit by kinetic models assuming a single binding or interaction site. An estimation of the EC<sub>50</sub> for P<sub>i</sub> stimulation is in the range of 5 mM P<sub>i</sub>. Therefore, for subsequent excised membrane patch experiments we used 5 mM P<sub>i</sub>. Experiments using the whole-cell patch configuration demonstrated that addition of 10 mM P<sub>i</sub> to the extracellular surface of the membrane did not potentiate CFTR Cl<sup>-</sup> currents ( $n = 3$ , data not shown), suggesting that stimulation is specific to the cytosolic surface.

We considered the possibility that intracellular P<sub>i</sub> might potentiate the inhibition of channel activity caused by ADP.

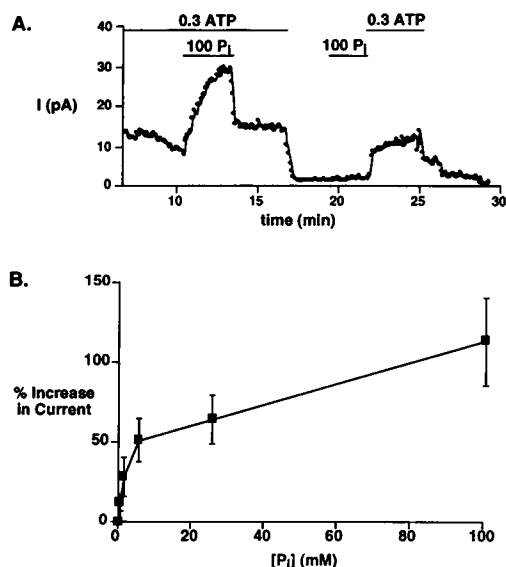


FIGURE 1 (A) Effect of P<sub>i</sub> (inorganic phosphate) on CFTR Cl<sup>-</sup> current. Data show the time course of current in an excised membrane patch from a HeLa cell transiently expressing wild-type CFTR. Before starting the time course, CFTR Cl<sup>-</sup> channels had been phosphorylated with cyclic AMP-dependent protein kinase (PKA) (75 nM) and 1 mM ATP (not shown). ATP (0.3 mM) and P<sub>i</sub> (100 mM) were present during times indicated by bars. (B) Effect of P<sub>i</sub> concentration on CFTR Cl<sup>-</sup> current. All values were determined in the presence of 0.3 mM ATP. Data points are mean  $\pm$  SE, three to six observations at each point; some error bars are smaller than data symbols.

To test this idea, we added 0.3 mM ADP to the cytosolic surface CFTR Cl<sup>-</sup> channels in the presence of 0.3 mM ATP. Consistent with previous results, addition of ADP decreased CFTR Cl<sup>-</sup> currents (Anderson and Welsh, 1992) (Fig. 2 A). Subsequent addition of 5 mM P<sub>i</sub> reversibly increased channel activity (Fig. 2 A). To determine whether the potentiation observed with P<sub>i</sub> was specific for P<sub>i</sub> or whether other divalent anions could potentiate as well as P<sub>i</sub>, we added sodium sulfate in the presence of 0.3 mM ATP (Fig. 2 B). We found that 5 mM sulfate did not increase CFTR Cl<sup>-</sup> current.

Fig. 3 summarizes the effect of P<sub>i</sub> and sulfate in the presence of intracellular ATP and ADP. Since the number of channels, hence current, varied widely from patch to patch, we quantitate the effect of P<sub>i</sub> as percent stimulation above the current supported by 0.3 mM ATP alone. Addition of 5 mM P<sub>i</sub> in the presence of 0.3 mM ATP significantly increased current ( $42.6 \pm 5.1\%$ ,  $n = 9$ ,  $p < 0.001$ ), whereas 5 mM sulfate produced a small but statistically significant decrease in activity ( $11.1 \pm 2.7\%$  decrease,  $n = 7$ ,  $p = 0.004$ ). In the absence of ATP, 5 mM P<sub>i</sub> was unable to support channel activity ( $n = 7$ ). When both 0.3 mM ADP and 0.3 mM ATP were present, 5 mM P<sub>i</sub> potentiated channel activity to a similar extent to that observed in the absence of ADP, although this stimulation did not achieve statistical significance ( $37.5 \pm 32.3\%$ ,  $n = 3$ ,  $p = 0.182$ ), current increased in all cases. Thus we found no evidence to suggest that ADP and P<sub>i</sub> to-

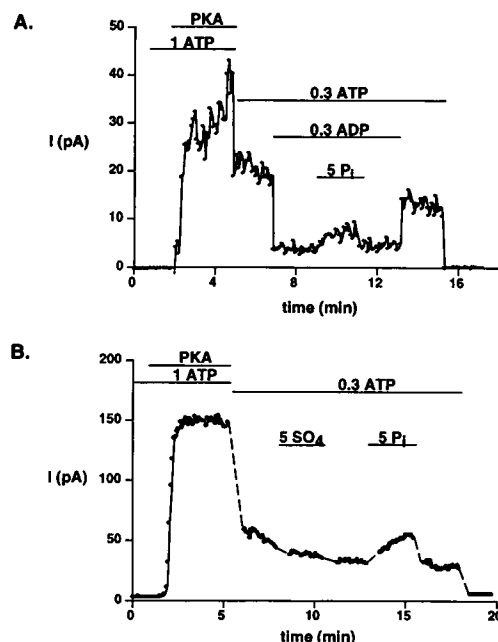


FIGURE 2 (A) Effect of P<sub>i</sub> in the presence of ADP. Data show time course of current in membrane patch excised from a HeLa cell transiently expressing wild-type CFTR. PKA (75 nM), ATP, ADP, and P<sub>i</sub> (at concentrations indicated in mM) were present during time indicated by bars. In this experiment data were not collected while solutions were changed. (B) Effect of sulfate on CFTR Cl<sup>-</sup> channel activity. Data show the time course of current in membrane patch excised from a HeLa cell transiently expressing wild-type CFTR. PKA (75 nM), ATP, sodium sulfate (SO<sub>4</sub>), and P<sub>i</sub> were added at concentrations indicated (in mM) during time indicated by bars. Dotted lines represent time during solution perfusion when data were not collected.

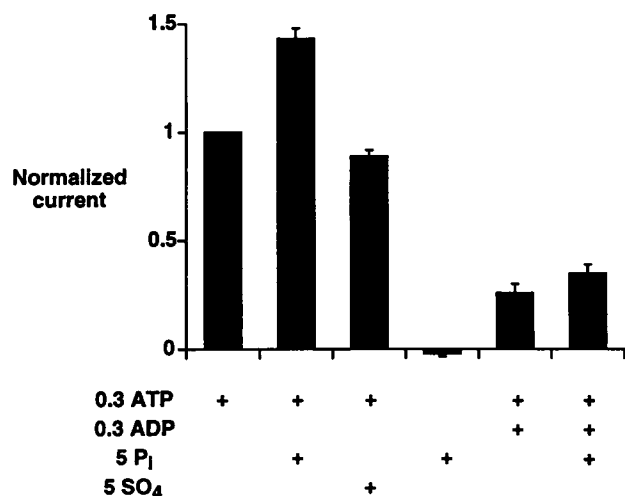


FIGURE 3 Effect of  $P_i$  and sulfate on CFTR  $Cl^-$  channel activity. Data are current relative to that supported by 0.3 mM ATP, normalized to 1. A plus (+) indicates the presence of ATP (0.3 mM), ADP (0.3 mM),  $P_i$  (5 mM), or sulfate (5 mM). Values are mean  $\pm$  SE of seven to nine observations for each condition except those including ADP, where  $n = 3$ . Data from membrane patches excised from HeLa cells, 3T3 fibroblasts, and C127 cells were similar and therefore were combined.

gether can produce an effect on channel activity that is different than the effect each has independently.

The current stimulated by  $P_i$  was due to activation of CFTR  $Cl^-$  channels as evidenced by several criteria: 1) there was a linear current-voltage relationship (not shown), 2) the current was  $Cl^-$  selective as determined from the reversal potential ( $E_{rev} = +32$ ,  $E_{Cl^-} = +27$  mV), 3) activation was reversible upon removal of ATP (e.g., see Figs. 1 and 2), 4)  $P_i$  did not stimulate current before channel phosphorylation by PKA ( $n = 3$ ), and 5) single-channel conductance and opening characteristics were consistent with those of CFTR.

### Effect of $P_i$ on single-channel properties

The macroscopic current ( $I$ ) measured in an excised membrane patch can be described in terms of single-channel characteristics as:  $I = i \cdot N \cdot p_o$ , where  $i$  is the amount of current conducted through a single channel,  $N$  is the number of active channels, and  $p_o$  is the open-state probability. To determine which of these parameters was increased by  $P_i$ , we performed experiments in which membrane patches contained either one or two active channels.

Fig. 4 A shows traces from an experiment in which only a single active CFTR  $Cl^-$  channel was observed. Fig. 4 B shows that addition of 5 mM  $P_i$  significantly increased  $P_o$ . No increase in the number of active channels was observed in the presence of 5 mM  $P_i$ , nor did the single-channel current amplitude change. Single-channel conductance was  $10.1 \pm 0.4$  pS ( $n = 5$ ) before addition of  $P_i$  and  $9.4 \pm 0.4$  pS ( $n = 4$ ,  $p = 0.145$ ) in the presence of 5 mM  $P_i$  (Fig. 4 C).

The increase in  $p_o$  caused by 5 mM  $P_i$  could be the result of an increase in the amount of time a channel remains open, a decrease in the amount of time it spends closed between

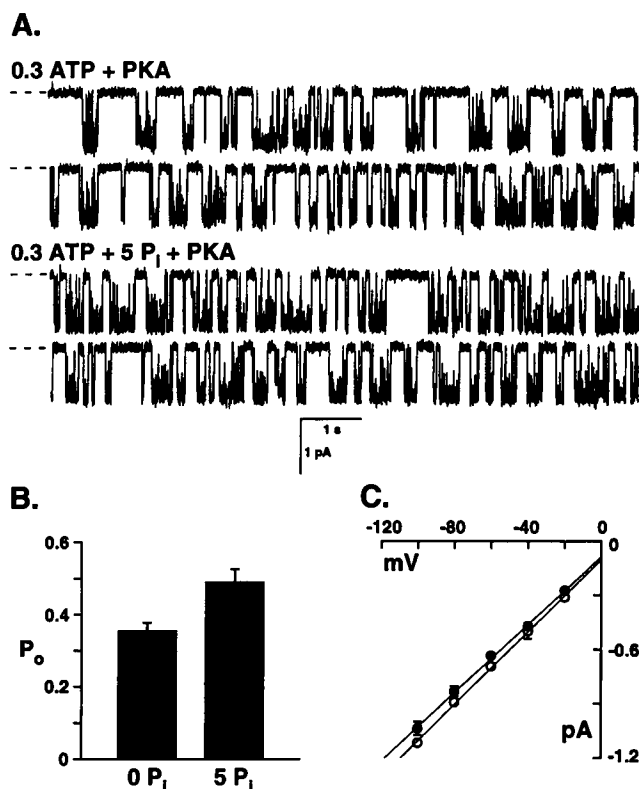


FIGURE 4 Effect of  $P_i$  on wild-type CFTR single-channel characteristics. (A) Traces from an excised inside-out membrane patch from a C127 cell containing a single, active channel. Dotted lines show closed-channel state, downward deflections correspond to openings. ATP and  $P_i$  concentrations (in mM) are indicated; PKA concentration was 75 nM. (B) Effect of 5 mM  $P_i$  on  $p_o$  in the presence of 0.3 ATP + 75 nM PKA. Data points are mean  $\pm$  SE. ( $n = 8$ ,  $p < 0.005$ ). (C) Effect of 5 mM  $P_i$  on single-channel current-voltage relationship. Data are in absence of  $P_i$  (○) and presence of 5 mM  $P_i$  (●), ( $g = 10.1 \pm 0.34$  and  $9.34 \pm 0.38$  pS,  $n = 5$  and 4, respectively,  $p = 0.145$ ).  $P_i$  did not change the reversal potential ( $p = 0.83$ ).

openings, or a combination of both. To determine how  $P_i$  was altering the gating of CFTR, we used maximum likelihood analysis and kinetic modeling. We chose this method over histogram fitting of open and closed times because this method provides more insight into the mechanism of channel activity, and it avoids errors inherent in data binning and curve fitting (Winter et al., 1994; McManus et al., 1987).

Maximum likelihood analysis and kinetic modeling have shown that the activity of single phosphorylated CFTR  $Cl^-$  channels can be described by a linear three-state model (Winter et al., 1994) (Fig. 5 A), although models with more states have been proposed based on histogram data from multichannel patches (Haws et al., 1992). Transitions between each state are described by the rate constants  $\beta_1$ ,  $\beta_2$ ,  $\alpha_1$ , and  $\alpha_2$ .  $C_1$  represents the long closed state between bursts of openings, and  $C_2 \leftrightarrow O$  represents the bursting state, where several channel openings (O) occur, which are separated by brief, flickery closures ( $C_2$ ) before the channel returns to the longer closed state. To determine which rate constants were altered by  $P_i$ , we performed kinetic analysis on data from four experiments in which only a single channel was present in

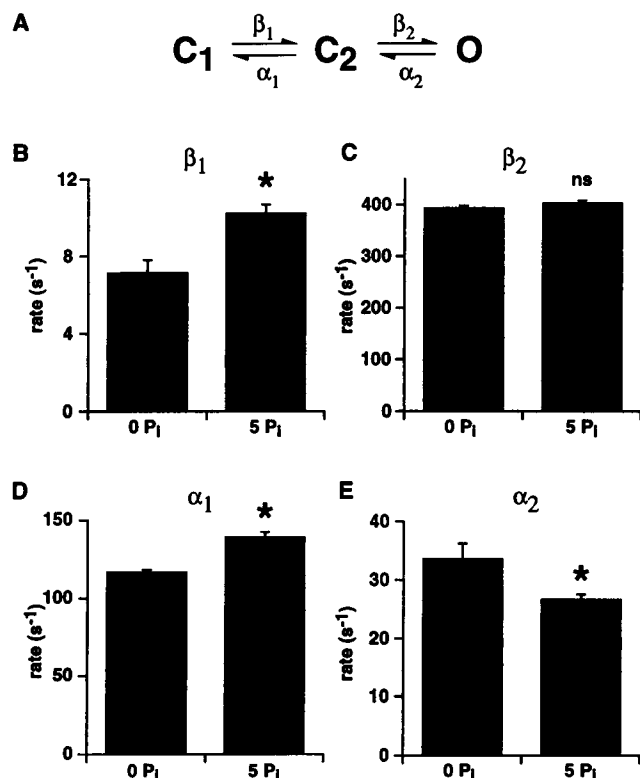


FIGURE 5 Effect of  $P_i$  on kinetically modeled rate constants. (A) The linear three-state model of CFTR channel activity denotes two closed states ( $C_1$  and  $C_2$ ), one open state (O), and the four rate constants ( $\beta_1$ ,  $\beta_2$ ,  $\alpha_1$ , and  $\alpha_2$ ). (B–E) rate constant values before and after addition of 5 mM  $P_i$ . Rate constants were derived as described in Materials and Methods from four experiments where the membrane patch contained only one active channel, in the presence of 0.3 mM ATP + 75 nM PKA. Asterisks indicate  $p < 0.05$ , while “ns” indicates that values in presence of  $P_i$  were not statistically different from control values.

the patch. We found that addition of  $P_i$  increased  $\beta_1$  and  $\alpha_1$ , decreased  $\alpha_2$ , and had no effect on  $\beta_2$  (Fig. 5, B–E).

These data indicate that  $P_i$  increased channel activity by increasing the rate of channel opening to the burst state while having very little effect on the bursts themselves. This is shown in the kinetic model by a 43% increase in  $\beta_1$ ; i.e.,  $P_i$  increased the rate of transition from the long closed state ( $C_1$ ) to the burst state (described by  $C_2$  and O). Although  $P_i$  changed both  $\alpha_1$  and  $\alpha_2$ , the effect of these changes on channel burst activity was trivial. For example, burst duration can be derived from the rate constant data as  $(\beta_2 + \alpha_2)/(\alpha_1 + \alpha_2)$ . Addition of 5 mM  $P_i$  produced reciprocal changes in  $\alpha_1$  and  $\alpha_2$  that were approximately equal and offsetting, such that when burst duration was calculated with the derived rate constants, there was less than a 2% difference in burst duration in the absence or presence of  $P_i$  ( $n = 4$ ,  $p = 0.015$ ). Similarly, the  $p_o$  within a burst is described by  $\beta_2/(\beta_2 + \alpha_2)$ . Since  $\alpha_2$  is one-tenth the magnitude of  $\beta_2$ , the 20% change in  $\alpha_2$  produced less than a 2% change in  $p_o$  within a burst ( $n = 4$ ,  $p = 0.299$ ).

The finding that bursts were essentially unaltered by  $P_i$  was confirmed by an independent determination of mean burst

duration and  $p_o$  within a burst using pCLAMP 6.0 software. Mean burst duration was determined with a  $t_c$ , the time that delineates intraburst gaps from interburst closures of 20 ms as described in Materials and Methods. Using this method of analysis, 5 mM  $P_i$  did not significantly change burst duration ( $162 \pm 7$  to  $171 \pm 6$  ms,  $n = 4$ ,  $p = 0.174$ ). Similarly,  $p_o$  within a burst changed less than 1% ( $0.956 \pm 0.003$  to  $0.964 \pm 0.005$ ,  $n = 4$ ,  $P = 0.011$ ). These data confirm that the primary effect of  $P_i$  was to increase the rate of channel transition to the burst state, with negligible effects on the bursts themselves.

### $P_i$ stimulates CFTR in the presence of 10 mM ATP

We considered the possibility that  $P_i$  might increase channel activity by increasing ATP concentration near the excised membrane patch. To test this possibility, we used 10 mM ATP to support channel activity. We chose this concentration because it is on the plateau portion of the ATP dose-response curve (Anderson et al., 1991a). Considering that half-maximal channel activity is supported by  $\sim 300 \mu\text{M}$  ATP, we predicted that at 10 mM ATP, ATP binding sites would be nearly saturated. If  $P_i$  exerted its stimulatory effect by altering ATP concentrations in close proximity to the membrane patch, we would expect that addition of  $P_i$  in the presence of 10 mM ATP would be without effect, since the channel is already maximally active. However, even in the presence of 10 mM ATP, addition of 5, 25, and 100 mM  $P_i$  still increased CFTR Cl<sup>-</sup> currents by  $28.5 \pm 0.1$ ,  $53.6 \pm 0.1$ , and  $93.4 \pm 0.2\%$  ( $n = 6, 3$ , and  $3$ , respectively). Fig. 6 shows a representative time course experiment.

### $P_i$ channel activity in CFTR containing a deletion in the R domain

We asked whether the effect of  $P_i$  was due to an interaction with the R domain. This seemed possible since phosphorylation of the R domain is required for channel activation

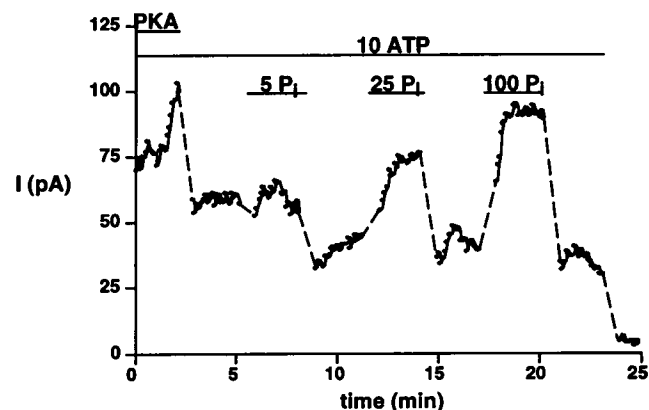


FIGURE 6 Effect of  $P_i$  on CFTR Cl<sup>-</sup> current in the presence of 10 mM ATP. PKA (75 nM), ATP (10 mM), and  $P_i$  (5, 25, and 100 mM) were present during time indicated by bars. Dotted line represents time during solution perfusion where data were not collected.

(Tabcharani et al., 1991; Berger et al., 1991; Cheng et al., 1991; Rich et al., 1993; Chang et al., 1993), and negative charge in the R domain increases  $p_o$  (Rich et al., 1993). Thus, it seemed possible that  $P_i$  might potentiate channel activity by interaction with the R domain, increasing negative charge and mimicking the effect of phosphorylation. To determine whether the R domain is necessary for  $P_i$  stimulation of CFTR  $Cl^-$  current, we examined two mutant CFTRs; CFTR $\Delta R$  and CFTR $\Delta R$ -S660A. In CFTR $\Delta R$ , amino acids 708–835 in the R domain have been deleted; this mutant has been shown to exhibit PKA-independent channel activity when ATP is present (Rich et al., 1991). In CFTR $\Delta R$ -S660A, amino acids 708–835 are deleted and serine 660 (a PKA phosphorylation site) is mutated to alanine. We found that for CFTR $\Delta R$ , either with or without phosphorylation by PKA ( $n = 3$  for each),  $P_i$  still potentiated currents in the presence of 0.3 mM ATP (Fig. 7). Stimulation was not dependent on the presence of serine 660, because we observed similar stimulation in the mutant CFTR $\Delta R$ -S660A ( $n = 6$ , not shown).

### $P_i$ but not sulfate decreases [ $\alpha$ - $^{32}P$ ]8- $N_3$ ATP binding to CFTR

To determine whether  $P_i$  was altering the interaction of nucleotides with CFTR, we measured the amount of [ $\alpha$ - $^{32}P$ ]8- $N_3$ ATP incorporation into membrane-associated CFTR in the presence and absence of  $P_i$ . We found that 10 and 100 mM  $P_i$  reduced the amount of radiolabel incorporation into CFTR (Fig. 8). In contrast, sulfate, which does not stimulate CFTR channel activity, had no effect on [ $\alpha$ - $^{32}P$ ]8- $N_3$ ATP labeling at either 10 or 100 mM. We initially considered that the decrease in binding might be due to the ability of  $P_i$  to complex  $Mg^{2+}$ . We calculated (Brooks and Storey, 1992) that addition of 100 mM sodium  $P_i$  to our binding reaction decreased the  $MgATP$  concentration (from 0.88 to 0.49 mM). This decrease in  $MgATP$  seems unlikely to explain the decrease in [ $\alpha$ - $^{32}P$ ]8- $N_3$ ATP binding because addition of sodium sulfate causes a similar decrease in  $MgATP$  concentration (from 0.88 to 0.53 mM); yet we observed no effect of sulfate on 8- $N_3$ ATP binding.

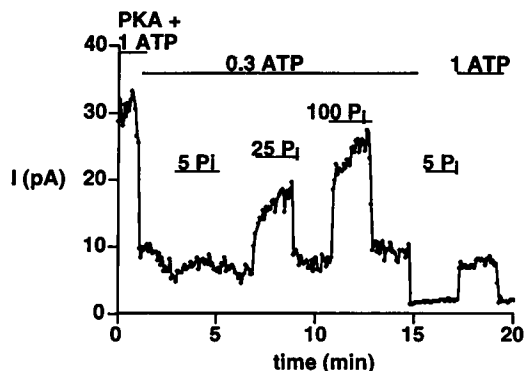


FIGURE 7 Effect of  $P_i$  on CFTR $\Delta R$   $Cl^-$  current. PKA (75 nM), ATP (0.3 or 1 mM), and  $P_i$  (5, 25, and 100 mM) were present during time indicated by bars. Data were not collected while solutions were changed.

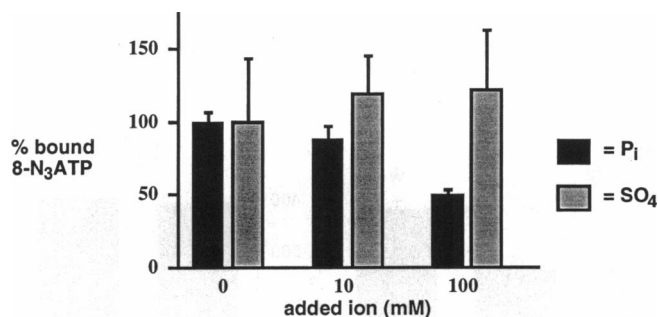


FIGURE 8 Effect of  $P_i$  on 8- $N_3$ ATP photolabeling of membrane-associated CFTR. Membranes from Sf9 cells infected with CFTR baculovirus were photolabeled with [ $\alpha$ - $^{32}P$ ]8- $N_3$ ATP in the presence of the indicated amounts of  $P_i$  or sulfate. Incorporation is expressed as percent of binding observed without added anion. Values are means  $\pm$  SE. ( $n = 6$ –11 for  $P_i$ ,  $n = 3$  for sulfate at each concentration).

## DISCUSSION

Our initial hypothesis was that  $P_i$ , applied to the cytosolic surface of CFTR  $Cl^-$  channels, might inhibit their activity. This was based on the suggestion that CFTR hydrolyzes ATP, and the observation that ADP, a product of ATP hydrolysis, inhibits the channel (Anderson et al., 1991a; Anderson and Welsh, 1992; Hwang et al., 1994; Baukrowitz et al., 1994). This hypothesis seemed reasonable because product inhibition of biological reactions is a common mechanism of regulation. For example, intracellular  $P_i$  has been shown to be one of the most important metabolic species responsible for decreasing contractile force in skeletal muscle (Ruegg et al., 1971; Dawson et al., 1978; Nosek et al., 1987). However, in contrast to our expectations, we found that  $P_i$  stimulated CFTR  $Cl^-$  channel activity. The stimulatory effect was only observed when  $P_i$  was added to the cytosolic surface of the phosphorylated channel, required the simultaneous presence of ATP, and was reversible.

To understand how  $P_i$  stimulated CFTR channel activity, we used the maximum likelihood analysis of single-channel data to model phosphorylated CFTR channel activity with the linear, three-state model shown in Fig. 5 (Winter et al., 1994). This model contains the minimal number of states required to describe the experimental data. However, more than one physical event, biochemical process, or conformational change might be contained within a single transition and hence many events may be described by a single rate constant. We found that the major effect of  $P_i$  was to stimulate entry of the channel into the burst state, a transition described by the forward rate constant  $\beta_1$ .  $P_i$  also increased the reverse rate constant  $\alpha_1$ , decreased the reverse rate constant  $\alpha_2$ , and had no effect on  $\beta_2$ . Although the changes in  $\alpha_1$  and  $\alpha_2$  were statistically significant, the effects on net channel activity were trivial and there was little effect on the properties of the bursts. We previously showed that  $\beta_1$ , the rate constant between  $C_1$  and  $C_2$ , is the only rate constant that is affected by ATP: as the ATP concentration increases,  $\beta_1$  increases (Winter et al., 1994). Thus  $\beta_1$  may describe the rate of ATP binding, the rate of ATP hydrolysis (if it occurs), and

any other steps through which the channel must traverse to arrive at C<sub>2</sub>. Our data suggest that P<sub>i</sub> may affect such a step.

We conclude that P<sub>i</sub> stimulates CFTR by interacting with the channel, and not by permeating through the ion conduction pathway for several reasons. First, P<sub>i</sub> did not increase single-channel conductance at 5 mM or even at 100 mM. Second, P<sub>i</sub> appears to compete with ATP binding to CFTR, suggesting that P<sub>i</sub> interacts with CFTR. Third, it appears that the 43% stimulation of current seen in excised macropatches is closely accounted for by the 38% increase in  $p_o$ , and the 43% increase in the opening rate constant  $\beta_1$ .

Where in CFTR Cl<sup>-</sup> channels does P<sub>i</sub> have its effect? We cannot be certain, but several considerations suggest that P<sub>i</sub> may interact with the NBDs. First, it seems unlikely that P<sub>i</sub> interacts with the R domain. Previous work indicates that phosphorylation of the R domain or mutagenic insertion of negatively charged residues opens the channel (Tabcharani et al., 1991; Berger et al., 1991; Cheng et al., 1991; Rich et al., 1993; Chang et al., 1993), suggesting that electrostatic interactions are important in removing the tonic inhibition imposed by the R domain. We considered that P<sub>i</sub> might interact noncovalently with positively charged residues to mimic the effect of phosphorylation and stimulate the channel. However, the data indicate that P<sub>i</sub> potentiated channel activity in two CFTR variants that lack much of the R domain. Moreover, sulfate (another divalent anion) did not stimulate CFTR, and P<sub>i</sub> had no effect in the absence of phosphorylation. In addition, in single-channel experiments where PKA was continually present and expected to maintain CFTR in a maximally phosphorylated state, P<sub>i</sub> still produced a 38% increase in  $p_o$ .

It seems unlikely that P<sub>i</sub> stimulates the channel through an interaction with the membrane-spanning domains. If that were the case, we would have expected to see some change in the conductive properties of the channel, but as stated above, single-channel conductance did not change, even in the presence of 100 mM P<sub>i</sub>.

Thus by exclusion, we speculate that P<sub>i</sub> may interact with the NBDs. This inference is supported by three additional observations. First, P<sub>i</sub> stimulated CFTR by increasing the same forward rate constant,  $\beta_1$ , that is affected by ATP, and we know that ATP interacts with the NBDs (Anderson et al., 1991a; Anderson and Welsh, 1992; Thomas et al., 1991, 1992; Hartman et al., 1992; Travis et al., 1993; Ko et al., 1994). Second, P<sub>i</sub> decreased 8-N<sub>3</sub>ATP binding, and previous work indicates that such ATP analogs interact with the NBDs. In fact, there is a precedent for P<sub>i</sub> binding at NBDs. For example, the F<sub>1</sub> ATPase has at least one high-affinity P<sub>i</sub> binding site (Penefsky, 1977; Kasahara and Penefsky, 1978). ADP, ATP, and AMP-PNP (adenylylimidodiphosphate) can compete with P<sub>i</sub> for binding to this site (Penefsky, 1977; Kasahara and Penefsky, 1978; Pougeois and Lauquin, 1985; Garin et al., 1989) suggesting that P<sub>i</sub> binds at or near a nucleotide binding site. Third, one would expect that if P<sub>i</sub> was interacting at the NBDs, there would be competition between P<sub>i</sub> and ATP for binding. The observation of a 29% increase in current observed in the presence of 10 mM ATP compared

with 43% with 0.3 mM ATP, although not statistically significant ( $p = 0.117$ ), is consistent with the notion that P<sub>i</sub> and ATP interact at the same site or sites.

How might P<sub>i</sub> activate the channel through the NBDs? It has previously been proposed that subsequent to phosphorylation, CFTR channel activity requires both binding and hydrolysis of ATP. It seems unlikely that P<sub>i</sub> increases the affinity of CFTR for ATP, i.e., stimulates ATP binding, because we found that P<sub>i</sub> stimulated the channel even in the presence of high concentrations of ATP, where we expect that ATP binding sites would be nearly saturated. Moreover, P<sub>i</sub> did not increase, but conversely decreased, 8-N<sub>3</sub>ATP photolabeling. Although these experiments do not address whether or not hydrolysis occurs, one could speculate on how P<sub>i</sub> may stimulate CFTR in the context of an ATP hydrolyzing process. Hydrolysis of ATP at one or both NBDs could yield bound ADP and P<sub>i</sub>. Alteration of the rate of P<sub>i</sub> dissociation by the principle of mass action and/or the exposure of a P<sub>i</sub> binding site in the NBDs at a particular step in channel gating may be related to the ability of P<sub>i</sub> to stimulate CFTR channel opening (or reopening) from a closed state. A recent study by Baukrowitz et al. (1994) using the P<sub>i</sub> analogs vanadate and beryllium fluoride has suggested that during the gating cycle, a P<sub>i</sub> binding site is exposed subsequent to hydrolysis of ATP and channel opening. It is possible that the stimulation we observe with P<sub>i</sub> is due to binding at this site, which is most likely an intimate part of the nucleotide binding domain.

Another possibility is that P<sub>i</sub> may stimulate in a purely allosteric manner at a site distinct from the NBDs with the decrease in 8-N<sub>3</sub>ATP being a coincident finding. For example, the rate-limiting step of channel activation in the presence of high concentrations of ATP might involve a process required to return the channel to a state where it is once again ready to bind ATP. If hydrolysis occurs, this rate-limiting step in the transition from C<sub>1</sub> to C<sub>2</sub> could involve the release of ADP from the NBDs so that ATP could once again bind. This notion is analogous to the situation with the F<sub>0</sub>-F<sub>1</sub> ATPase and G protein activity, where the rate-limiting step in nucleotide turnover is release of bound nucleotide (Boyer, 1989, 1993; Graziano et al., 1989). In fact, P<sub>i</sub> has been found to facilitate the release of bound nucleotide(s) from the F<sub>1</sub> ATPase (Beharry and Bragg, 1992). Because we cannot yet correlate physical events to different states of the model, we are at present limited to describing a possible order of events in channel activation that are consistent with our data, and we realize that there are other possible mechanisms not discussed here that may also be consistent with our findings.

It is interesting to note that Baukrowitz and co-authors found that the P<sub>i</sub> analogs orthovanadate and beryllium fluoride were potent stimulators of guinea pig cardiac CFTR channel activity (Baukrowitz et al., 1994). Contrary to our findings with P<sub>i</sub>, these compounds appeared to function by locking the channel in the bursting state. The difference between our two studies may be explained by the fact that although vanadate and beryllium fluoride are P<sub>i</sub> analogs and appear to bind to P<sub>i</sub> binding sites, once bound they appear to mimic the transition state of ATP hydrolysis,

suggesting that their studies are essentially arresting CFTR channel activity at this state in the gating cycle, while our kinetic assessment examines the effects of  $P_i$  on all steps of channel gating.

It is interesting to speculate whether  $P_i$  might regulate CFTR in vivo. Cellular concentrations of  $P_i$  range from ~100 to 30 mM, depending upon the measurement technique ( $^{31}\text{P}$ -NMR vs. chemical extraction) and the status of the tissue before the measurement (resting vs. fatigued) (Balaban, 1984, 1990; Thompson and Fitts, 1992). Perhaps the most accurate evaluation is with  $^{31}\text{P}$ -NMR in intact tissue, which estimates that intracellular free  $P_i$  is in the range of 100  $\mu\text{M}$ , or approximately one-tenth the concentration of ATP (Balaban, 1984). These  $P_i$  values are in the range at which we observed an effect on channel activity (100  $\mu\text{M}$   $P_i$  produced a  $12.5 \pm 5\%$  increase), and more importantly, occur on a steep portion of the dose response curve where small changes in  $P_i$  could yield substantial changes in CFTR activity. However, further experiments are needed to determine whether intracellular  $P_i$  regulates CFTR channels in vivo.

In summary, our data show that  $P_i$  stimulates CFTR  $\text{Cl}^-$  channel activity predominantly by increasing the rate at which a channel enters the bursting state and establishes  $P_i$  as a previously unrecognized regulator of CFTR. Although the relative importance of  $P_i$  in regulating the channel in intact epithelia is uncertain, it seems likely that  $P_i$  may prove to be a useful reagent in further understanding the mechanisms of CFTR  $\text{Cl}^-$  channel regulation.

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