Tolbutamide Causes Open Channel Blockade of Cystic Fibrosis Transmembrane Conductance Regulator Cl^- Channels

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ABSTRACT Cystic fibrosis transmembrane conductance regulator (CFTR) is an epithelial Cl^- channel that is regulated by protein kinase A and cytosolic nucleotides. Previously, Sheppard and Welsh reported that the sulfonylureas glibenclamide and tolbutamide reduced CFTR whole cell currents. The aim of this study was to quantify the effects of tolbutamide on CFTR gating in excised membrane patches containing multiple channels. We chose tolbutamide because weak (i.e., fast-type) open channel blockers introduce brief events into multichannel recordings that can be readily quantified by current fluctuation analysis. Inspection of current records revealed that the addition of tolbutamide reduced the apparent single-channel current amplitude and increased the open-channel noise, as expected for a fast-type open channel blocker. The apparent decrease in unitary current amplitude provides a measure of open probability within a burst ($P_{o\ Burst}$), and the resulting concentration-response relationship was described by a simple Michaelis-Menten inhibition function. The concentration of tolbutamide causing a 50% reduction of $P_{o\ Burst}$ (540 ± 20 μM) was similar to the concentration producing a 50% inhibition of short-circuit current across T84 colonic epithelial cell monolayers (400 ± 20 μM). Changes in CFTR gating were then quantified by analyzing current fluctuations. Tolbutamide caused a high-frequency Lorentzian (corner frequency, $f_c > 300$ Hz) to appear in the power density spectrum. The f_c of this Lorentzian component increased as a linear function of tolbutamide concentration, as expected for a pseudo-first-order open-blocked mechanism and yielded estimates of the on rate (k_{on} = 2.8 ± 0.3 μM^{-1} s^{-1}), the off rate (k_{off} = 1210 ± 225 s^{-1}), and the dissociation constant (K_D = 430 ± 80 μM). Based on these observations, we propose that there is a bimolecular interaction between tolbutamide and CFTR, causing open channel blockade.

INTRODUCTION

Cystic fibrosis transmembrane conductance regulator (CFTR) is an ATP-regulated Cl^- channel and a member of the ATP-binding cassette (ABC) superfamily of proteins. It was recently reported that the sulfonylurea receptor (SUR) that regulates ATP-sensitive K^+ channels (K_{ATP}) is a member of this same superfamily of proteins (Aguilar-Bryan et al., 1995; Inagaki et al., 1995). CFTR Cl^- channels are present in the apical membranes of pancreatic, intestinal, and airway epithelial cells, where there are known defects in cAMP-regulated anion and fluid secretion in cystic fibrosis (CF) (Gray et al., 1988; Tabcharani et al., 1990; Quinton, 1990). CFTR-like channels have also been identified in the myocardium (Bahinski et al., 1989; Ehara and Ishihara, 1990; Nagel et al., 1992). These cardiac Cl^- channels are thought to accelerate repolarization during the cardiac action potential but have an uncertain relationship to CF (Bahinski et al., 1989). Given the diversity of locations at which CFTR resides, the pharmacology of this ion channel has wide clinical implications.

Sulfonylureas are potent blockers of SUR/K_{ATP} in pancreatic islet cells, myocardium, and smooth muscle (Ashcroft and Ashcroft, 1992; Edwards and Weston, 1993). Previously, Sheppard and Welsh (1992) reported that the sulfonylureas tolbutamide and glibenclamide reduced CFTR Cl^- current, as measured by the whole-cell configuration of the patch-clamp technique. The K_i of tolbutamide for inhibition of Cl^- current was estimated to be 150 μM with modest negative cooperativity (Hill coefficient = 0.8). Although tolbutamide alters the gating of K_{ATP} channels by binding to SUR, these drugs are also known to affect the activity of intracellular enzymes, including protein kinase A (Okuno et al., 1988; Caro, 1990; Edwards and Weston, 1993). Thus, there is some uncertainty regarding the molecular mechanism mediating the tolbutamide-induced reduction in whole-cell current. Understanding the mechanism by which tolbutamide reduces ion transport may lead to new insights regarding the molecular biophysics of CFTR, including the states of particular domains that participate in gating (e.g., the required phosphorylation state of the protein, requirements for concurrent presence of nucleotides) and the identification of the kinetic state that is most affected. Based upon these observations, comparisons could then be made regarding the relationship of CFTR to other ATP-sensitive channels. Therefore, we decided to identify and quantify possible sulfonylurea-CFTR interactions in excised membrane patches.

Because most excised membrane patches from CFTR-transfected L cells contain multiple channels (Venglarik et al., 1994b; Schultz et al., 1995b), there are two advantages that favor the use of a weak blocker (i.e., tolbutamide) for

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kinetic analysis. First, a weak open-channel blocker will cause an apparent reduction in single-channel current amplitude due to the effect of the low-pass filter (Miller, 1982). The normalized unitary current amplitude provides a measure of the blocker-induced reduction in open probability within a burst ($P_{o\ Burst}$) that is independent of the number of active channels and the initial P_o. Second, weak open-channel blockers are favored in current fluctuation analysis because they introduce high-frequency Lorentzian components that can be clearly distinguished in the power density spectra (PDS) (Li and Lindemann, 1983; Van Driessche and Van Deynse, 1990; Venglarik et al., 1994b). Thus, the objective of this study was to identify and quantify possible effects of tolbutamide on CFTR gating in excised membrane patches containing multiple CFTR channels. The data presented in this report show that tolbutamide caused a fast-type open channel blockade of CFTR channels. Some of our results have been reported previously in abstract form (Venglarik et al., 1993a, 1994a).

MATERIALS AND METHODS

Cell culture

T84 human colonic epithelial cells were studied between passages 36 and 76. These cells were maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium, supplemented with 14 mM HEPES and 10% calf bovine serum as previously described (Dharmsathaphorn et al., 1985; Mandel et al., 1986). Unless otherwise specified, all media and reagents were obtained from Sigma Chemical Co. (St. Louis, MO). T84 cells were grown to confluence, dispersed with 0.05% trypsin (Gibco, Grand Island, NY) in a Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline containing 1 mM EDTA, and plated 1:1 on 30-mm Millicell-HA filters (Millipore, Bedford, MA). The resulting monolayers were used for Ussing chamber experiments 2–3 weeks after passage.

Mouse L cells transfected with human CFTR (passages 11–60) were maintained in Dulbecco's modified Eagle's medium (Gibco) containing 10% fetal bovine serum (HyClone, Logan, UT) as described previously (Yang et al., 1993; Venglarik et al., 1994b). Cells were grown to 90% confluence, dispersed with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline containing 1 mM EDTA, and plated onto collagen-coated plastic coverslips for patch-clamp studies.

Transepithelial voltage clamp measurements

Filters containing confluent T84 cell monolayers were cut from the plastic rings and mounted in Ussing chambers (area = 0.5 cm^2) with a small quantity of silicone grease. The water-jacketed Ussing chambers were similar to those used previously to study ion transport across rat colon (Bridges et al., 1983). The temperature was maintained at 37°C, and the solutions in both compartments were stirred and gassed with air. The bathing solutions contained (in mM): 120 NaCl, 4.5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 D-glucose, 10 HEPES-NaOH (pH 7.4). Short-circuit current (I_{sc}) measurements were obtained with a voltage clamp (model 616C; University of Iowa Department of Bioengineering) as described previously (Venglarik and Dawson, 1986). Based on unidirectional tracer fluxes, the cyclic AMP-stimulated I_{sc} across T84 colonic monolayers is due to net Cl^- secretion (Dharmsathaphorn et al., 1985) and provides an indirect measure of CFTR activity (Mandel et al., 1986; Tabcharani et al., 1990). Forskolin (10 μM) was used to increase cellular cyclic AMP levels (Bridges et al., 1983) and was added as a small volume of a 10 mM stock solution in DMSO. Because tolbutamide is a weak acid, 100 mM stock solutions contained 0.1 N NaOH to neutralize the pH.

Patch-clamp methodology

Excised inside-out membrane patches were obtained as previously described (Venglarik et al., 1994b). Briefly, coverslips containing L cells were transferred to a heated chamber (35–37°C) on the stage of an inverted microscope. Pipettes were fabricated from Kimax-51 glass (Kimble), using a two-stage puller (PP-83; Narishige USA, Greenvale, NY) and had a resistance of 2–5 MΩ when filled with a standard solution containing (in mM): 150 *N*-methyl-D-glucamine-HCl, 1 $CaCl_2$, 2 $MgCl_2$, and 10 1,3-bis[tris(hydroxymethyl)-methylamino]propane-HCl (pH 7.4). Current measurements were obtained with an Axopatch 200A amplifier (Axon Instruments, Sunyvale, CA), which employs a low-noise integrating headstage. Currents were monitored with a digital oscilloscope and stored on VHS videotape using pulse code modulation (model 200; Vetter, Rebersburg, PA). Electrical potentials are reported with respect to the pipette interior according to the usual convention, such that positive (i.e., "outward") currents are consistent with increases either in the outward flow of cations from the bath into the pipette or with the inward flow of anions from the pipette to the bath. Patches were excised into a solution containing (in mM): 140 NaCl, 5 KCl, 2 $MgCl_2$, 10 NaF, 0.5 EGTA, 0.2 $CaCl_2$ and 10 1,3-bis[tris(hydroxymethyl)-methylamino]propane-HCl (pH 7.4). F^- was included as a nonspecific inhibitor of phosphatases that might be active on patch excision and can lead to channel inactivation (Tabcharani et al., 1991), although we have previously reported that similar outcomes are seen in the absence of F^- (Schultz et al., 1995b, 1996a). The bathing solution also contained 0.3 mM ATP to maintain CFTR activity (Anderson et al., 1991; Venglarik et al., 1994b). Cyclic AMP-dependent protein kinase (PKA) (10 nM; Promega, Madison, WI) was added after excision to increase CFTR activity (Hwang et al., 1993; Tabcharani et al., 1991). As previously reported, most CFTR channels remained active for the duration of patch viability (up to 60 min), despite the fact that the PKA was washed out in less than 90 s by continuous perfusion (Venglarik et al., 1994b; Schultz et al., 1995b). Inward current is expected to be dominated by the flow of Cl^- because the pipette did not contain permeant cations. Thus, CFTR channels were identified by their current amplitude and by their response to PKA addition.

Kinetic analysis

The digitized current records were played back, low-pass filtered with an 8-pole Bessel filter (902LPF, Frequency Devices, Haverhill, MA), and acquired with a TL-1 DMA interface, pClamp software (version 5.5.1; Axon Instruments, Foster City, CA) and an IBM-compatible 486-DX computer. The length of recording, filter cutoff, frequency and acquisition rate were dependent on the type of analysis being performed (see below). The pClamp files were analyzed using BioPatch software (version 3.21; Molecular Kinetics, Pullman, WA). All figures were constructed using SigmaPlot software (version 5.0; Jandel Scientific, San Rafael, CA).

Two types of analyses were performed. First, we measured the mean amplitude of control ($i_{control}$) and fast-blocked channels (i_{block}) from amplitude histograms as described previously (Venglarik et al., 1993b). The ratio of $i_{block}/i_{control}$ is related to the underlying change in $P_{o\ Burst}$ for an open-blocked mechanism (Miller, 1982). Ten to forty seconds of record was used to construct amplitude histograms. These recordings were heavily filtered by a low-pass 8-pole Bessel filter (−3 db = 15 Hz) to reduce the control and blocker-induced noise and resolve the small changes in the unitary current amplitude. Second, we used spectral analysis to quantify tolbutamide-induced changes in CFTR gating (for reviews see DeFelice, 1981; Van Driessche and Van Deynse, 1990). Digital files were reacquired at a sampling rate of 10 kHz while being filtered at 5 kHz. These records were divided into nonoverlapping segments containing 8192 data points,

and the PDS was calculated for each block using BioPatch software. Spectra from each set of blocks (60–174 spectra) were averaged to increase precision. These data were fit to Lorentzian type functions of the following form:

$$S_f = S_0/(1 + (f/f_c)^2). \quad (1)$$

S_0 is the zero frequency asymptote and f_c is the corner frequency where S_0 has been decreased by half. Fits were performed in BioPatch. The corner frequency is related to a set of opening (r_+) and closing (r_-) rates:

$$2\pi f_c = r_+ + r_-. \quad (2)$$

It is important to distinguish between the transition rates and the underlying chemical rate constants because they are not always equivalent. Lindemann and Van Driessche (1977) showed that the chemical rate constants for the open-blocked model can be derived by plotting $2\pi f_c$ as a function of blocker concentration and fitting the expected result to the observed outcome. This approach has since been applied to quantify the rate constants for other models (Li and Lindemann, 1983; Venglarik et al., 1994b; Schultz et al., 1995b).

RESULTS

Tolbutamide reduced the cyclic AMP-activated I_{sc} across T84 colonic epithelial cells

Ussing chamber experiments were performed to provide support for the hypothesis that tolbutamide blocks CFTR in intact epithelial tissue, and to gain further insight regarding the concentration-response relationship. The data in Fig. 1 show that tolbutamide reduced the forskolin-stimulated I_{sc} across T84 colonic epithelial cell monolayers in a concentration-dependent fashion and that the effect was consistent with a simple Michaelis-Menten inhibition function. Fig. 1 *A* illustrates the current record from a representative experiment. Forskolin (10 μM) was added to solutions bathing both faces of the T84 monolayer at the time indicated to increase cellular cAMP and activate I_{sc}. As mentioned previously, the cAMP-stimulated I_{sc} is due to net transepithelial Cl^- secretion (Dharmsathaphorn et al., 1985; Mandel et al., 1986). Stepwise addition of tolbutamide caused a concentration-dependent reduction in I_{sc}. Ouabain (100 μM) or furosemide (200 μM; not shown) was added to the serosal bathing solution to abolish any remaining Cl^- current. In control experiments, forskolin-stimulated I_{sc} was stable, in that 87 ± 2% of the peak current remained 30 min after the addition of forskolin (n = 20). The effect of tolbutamide was rapidly reversed upon replacement of the bathing solutions with solutions containing no tolbutamide (not shown). Normalized data from Fig. 1 *A* and five similar experiments are summarized in Fig. 1 *B*. The solid line shows the best fit of a simple Michaelis-Menten inhibition function (i.e., 1 − [tolbutamide]/(K_i + [tolbutamide])) to these data. Initially, the data were analyzed, allowing a Hill coefficient to float, and a value of 0.83 ± 0.03 (r = 0.98) was derived. However, because this value approached 1 (as it did in the analysis of channel inhibition in excised patches; see below), further analysis constrained the Hill coefficient to unity, and a K_i of 400 ± 20 μM was predicted. Although the interpretation of these results is confounded by the presence of two membranes in series, the I_{sc} measurements provide evidence that tolbutamide inhibits CFTR in colonic epithelial cells. Furthermore, these results provide insight regarding the range of tolbutamide concentrations for patch-clamp experiments (see below).

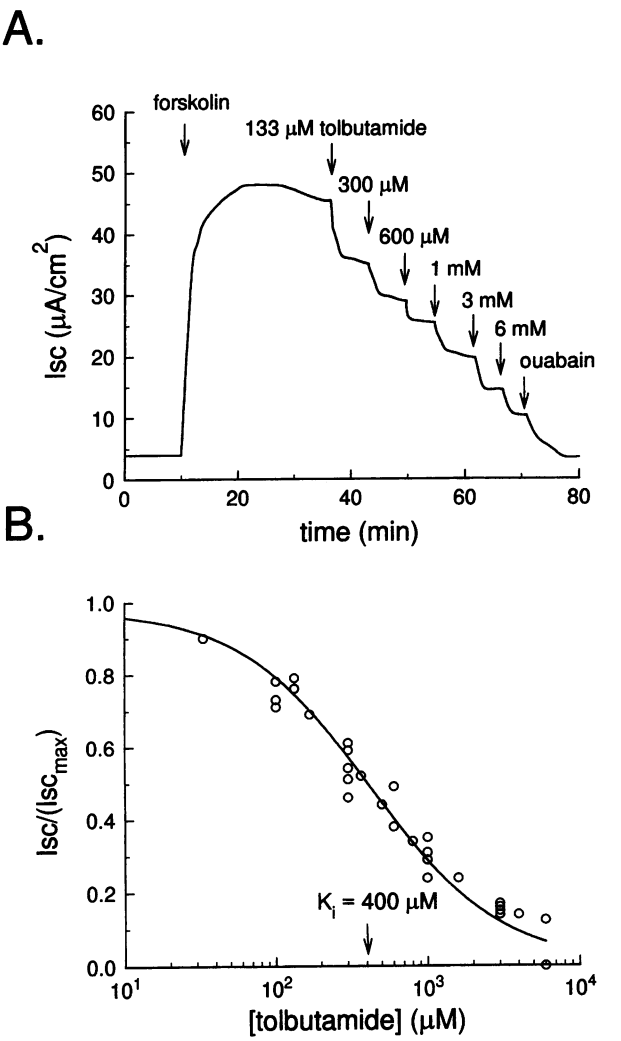

FIGURE 1 Effect of tolbutamide on short-circuit current (I_{sc}) across a T84 colonic epithelial cell monolayer. (*A*) Representative experiment. (*B*) Summary of the results from six such experiments. T84 cell monolayers were mounted in modified Ussing chambers and voltage clamped as described in Materials and Methods. Forskolin (10 μM) was added to both the mucosal and serosal sides to increase intracellular cAMP and to activate a chloride secretory current. Tolbutamide was then added in increasing increments, which produced a stepwise decrease in I_{sc}. The concentrations indicated are the final concentrations of tolbutamide. Serosal ouabain (100 μM) was used to abolish the remaining I_{sc} due to Cl^- secretion. The normalized concentration response for the change in I_{sc} (*B*) was fit to a simple Michaelis-Menten function, using a nonlinear curve-fitting routine (Statgraphics, STSC). The solid line illustrates the best fit of the data (K_i = 400 ± 20 μM).

Effect of tolbutamide on CFTR channel activity

Occasionally we obtained single-channel recordings of CFTR activity in excised membrane patches ($n = 3$). Fig. 2 illustrates the effect of 200 μM tolbutamide on a representative single channel. The CFTR channel in this patch inactivated immediately upon excision and was reactivated by brief exposure to 10 nM PKA (not shown). This observation and the single-channel current amplitude (−0.80 pA at −80 mV) are the results expected for CFTR. Addition of tolbutamide reduced the current amplitude and increased the open-channel noise. This result is suggestive of fast-flickery open-channel blockade. Similar effects were observed in 17 other experiments involving multichannel patches. The relationship between tolbutamide concentration and open probability within a burst ($P_{o\ Burst}$) as measured by the change in the unitary current amplitude is described in the following section. We did not quantify the effect on gating using event duration analysis because brief events (i.e., <2 ms) cannot be properly resolved due to the poor signal-to-noise ratio. Instead, we derived the transition rates by analyzing PDS as described in a subsequent section.

Tolbutamide reduces CFTR open probability

The apparent reduction in unitary current amplitude caused by a fast open-channel blocker is directly related to the underlying change in channel open probability within a burst ($P_{o\ Burst}$) (Miller, 1982). Therefore we measured unitary currents of heavily filtered records (−3 db at 15 Hz) with amplitude histograms. This cutoff frequency was needed to resolve changes in the small current amplitude and virtually eliminated the background and blocker-induced noise (not shown). Fig. 3 summarizes these data and shows that the normalized decrease in CFTR amplitude ($i_{block}/i_{control}$) was concentration dependent. Furthermore, the inhibition was well described by a simple Michaelis-

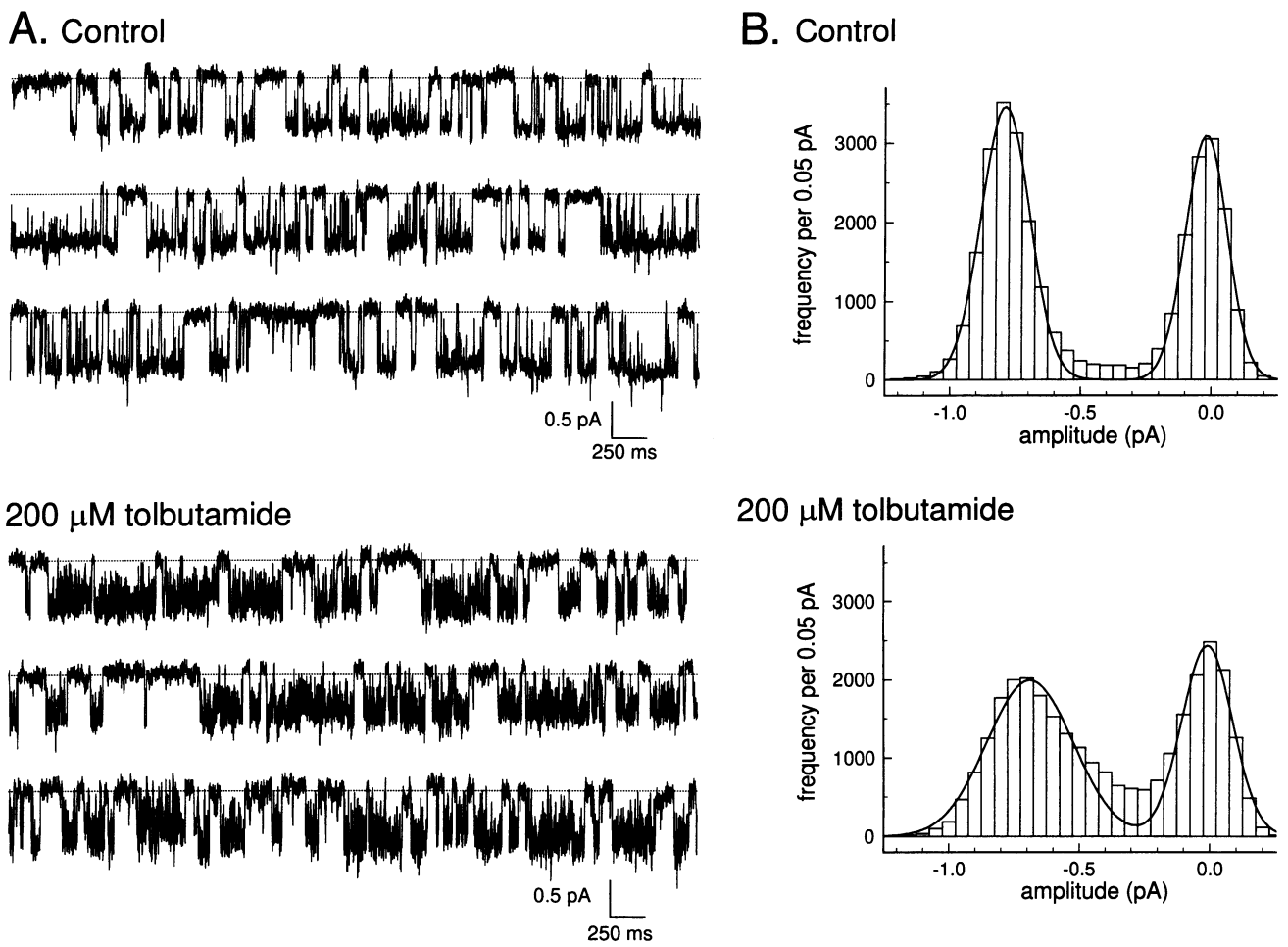

FIGURE 2 Current records illustrating CFTR channel activity before (control) and after the addition of 200 μM tolbutamide (*A*) and the corresponding amplitude histograms (*B*). The dashed line indicates the closed state of the channel. Each line contains 5 s of recording, and a total of 15 s is shown before and after tolbutamide treatment. The amplitude histograms shown in *B* were constructed from the recordings in *A* and were fit to Gaussian functions (*solid lines*). The 0.4-ml bath was constantly perfused at a rate of 1.2 ml/min and contained 0.3 mM ATP throughout the experiment. Two to three minutes was allowed to elapse to permit the composition of the bathing solution to become stationary. Conditions: −80 mV; 140 NaCl; bath, 140 *N*-methyl-D-glucamine-HCl pipette; cutoff frequency, 300 Hz; sample rate, 1000 Hz. All points were plotted.

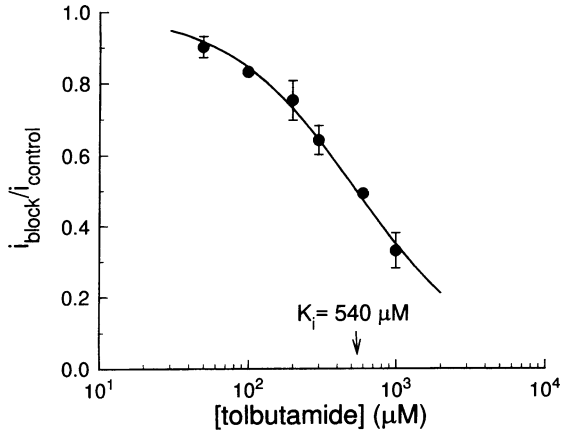

FIGURE 3 Concentration-response relation for tolbutamide inhibition of open probability within a burst ($P_{o\ Burst}$). $P_{o\ Burst}$ was calculated as $i_{block}/i_{control}$ as described in Materials and Methods. These data were derived from the amplitude histograms of recordings filtered at 15 Hz and acquired at 250 Hz. Each point represents the mean and SD of at least three experiments. Unless otherwise shown, SD falls within the area of the symbol. These data were fit to a simple Michaelis-Menten inhibition function using a nonlinear curve-fitting routine (Statgraphics, STSC; K_i = 540 ± 20 μM). The solid line illustrates the best fit to the data set. Because of the reduction in current amplitude, it was not possible to obtain accurate estimates of $i_{block}/i_{control}$ at concentrations of tolbutamide > 1 mM. Experimental conditions are described in Materials and Methods and in the legend for Fig. 2.

Menten function (solid line) with a K_i of 540 ± 20 μM. The Hill coefficient was constrained to unity because initial analysis revealed that it was not different from 1 (0.96 ± 0.04; r = 0.99). This result is suggestive of a simple bimolecular open-blocked mechanism.

Kinetic analysis of the tolbutamide-induced change in CFTR gating

Spectral analysis was employed to quantify tolbutamide-induced changes in CFTR gating. PDS calculated from current records before and after tolbutamide addition are illustrated in Fig. 4. The solid lines show the best fits of these spectra to Lorentzian functions. Both spectra are dominated by a single Lorentzian component at frequencies less than 30 Hz, as described previously (Venglarik et al., 1994b; Fischer and Machen, 1994; Schultz et al., 1995b). The control spectra contain an additional Lorentzian component (f_c = 100 Hz), which has very little power and is presumably due to the brief closures described by Haws et al. (1992). Tolbutamide (1 mM) caused a high-frequency Lorentzian component to appear in the PDS (f_c = 650 Hz), which is the result expected for an open-block mechanism. At high concentrations of tolbutamide (i.e., ≥300 μM), the PDS were equally well fit by calculations employing either two or three Lorentzian components. Therefore all spectra were fit to two components. It was not possible to clearly resolve the blocker-induced Lorentzian component at low concentrations of tolbutamide (i.e., ≤200 μM) and these data were excluded from the kinetic analysis (refer to Fig. 5).

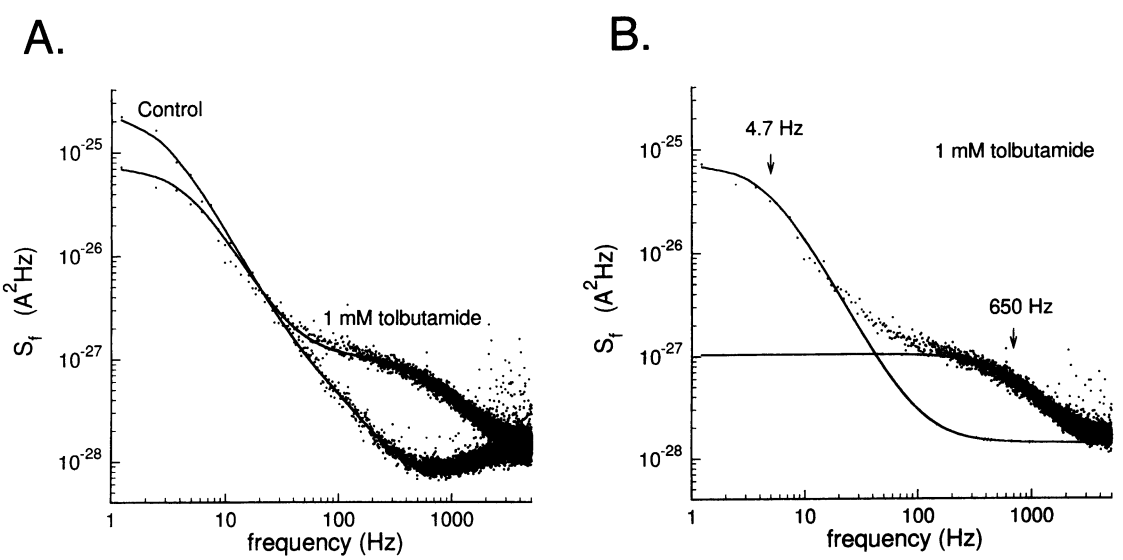

FIGURE 4 Representative power density spectra (PDS) before and after addition of tolbutamide. PDS were constructed as described in Materials and Methods. Records were filtered at 5 kHz and sampled at 10 kHz. The spectra shown are the average of 87 (control) and 97 (1 mM tolbutamide) spectra. The solid lines shown in *A* illustrate the best fit of these data to two Lorentzian components (see Eq. 1) plus a component of frequency-independent noise. The parameters of the control spectra were f_{c1} = 2.7 Hz, S_{01} = 2.5 × 10^{-25} A^2Hz, f_{c2} = 100 Hz, S_{02} = 4.2 × $10^{-28}A^2Hz$, offset = 6.7 × $10^{-29}A^2Hz$. The parameters of the spectra obtained in the presence of tolbutamide were f_{c1} = 4.7 Hz, S_{01} = 7.3 × $10^{-26}A^2Hz$, f_{c2} = 650 Hz, S_{02} = 9.0 × $10^{-28}A^2Hz$, offset = 1.4 × $10^{-28}A^2Hz$. Although the increase in the f_c of the low-frequency component was a consistent observation, we did not quantify this result because of the variance of these data and the possibility of aliasing. This observation is not expected to alter the interpretation of the effects of tolbutamide on the high-frequency Lorentzian component. (*B*) Relative contribution of the two Lorentzian components to the PDS in the presence of 1 mM tolbutamide (*solid lines*). The corner frequencies (f_c) are indicated by the arrows.

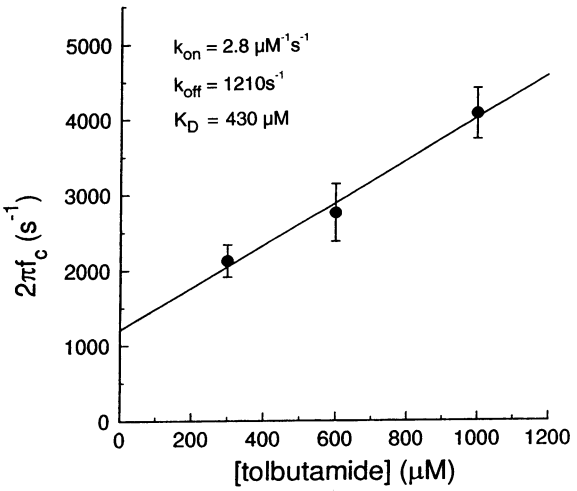

FIGURE 5 The plot of $2\pi f_c$ as a function of tolbutamide concentration. Each point represents the mean and SD of at least three experiments that were analyzed as described in Fig. 4. The solid line was obtained by simple linear regression of the averaged data. Based on the closed-open-blocked mechanism, the slope of the line represents the blocker on rate (k_{on} = 2.8 ± 0.3 μM^{-1} s^{-1}), and the intercept is the sum of the blocker off rate (k_{off}) and the closing rate (k_{close}). Presuming that $k_{off} \gg k_{close}$, then $k_{off} \approx$ 1210 ± 230 s^{-1}. The ratio of k_{off}/k_{on} can be used to calculate the K_i (430 μM).

The representative spectrum shown in Fig. 4 provides evidence for the following model:

Closed↔Open↔Blocked

It is possible to construct a more detailed model for CFTR gating that includes many additional closed states (Haws et al., 1992; Hwang et al., 1993; Venglarik et al., 1994b; Schultz et al., 1995b). In the model shown above, the longer states are grouped together (closed) and infrequent brief closures are ignored (i.e., bursting = open). This simplification is possible because the nucleotide concentration and phosphorylation status of the channel are constant in these experiments. Our previous study of CFTR fluctuations under these conditions indicated that the single low-frequency Lorentzian component ($f_c \approx$ 1 to 5 Hz) in the PDS corresponds to the burst duration and interburst interval (Venglarik et al., 1994b). In addition, there is relatively little power associated with the behavior within a burst in excised patches (Fisher and Machen, 1994; Schultz et al., 1995b; also refer to Fig. 4).

We obtained additional support for this simple model and derived estimates of the kinetic constants by plotting $2\pi f_c$ as a function of tolbutamide concentration (Fig. 5). A straight line with a positive slope was fitted to the data, which is the result expected for an open-blocked transition (Lindemann and Van Driessche, 1977). Based on rate theory, the slope of the line corresponds to the on rate (k_{on} = 2.8 ± 0.3 μM^{-1} s^{-1}), and the ordinate intercept is the sum of the off rate (k_{off}) and the closing rate (k_{close}) for a closed-open-blocked mechanism. Assuming that $k_{off} \gg k_{close}$, then $k_{off} \approx$ 1210 ± 230 s^{-1}. The ratio of k_{off}/k_{on} can be used to calculate the K_D (430 ± 80 μM). It is likely that $k_{off} \gg k_{close}$, because this K_D is smaller than the estimate obtained from amplitude analysis (540 ± 20 μM), and the k_{off} is substantially greater than the k_{close} (6.5 s^{-1}), which we previously reported for CFTR in these conditions (Venglarik et al., 1994b; Schultz et al., 1995b). Furthermore, the K_i derived from analysis of channel activity in excised membrane patches is essentially the same as the K_i estimated from I_{sc} measurements (400 ± 20 μM). As previously stated, the effects of tolbutamide at the tissue level could result from the sum of interactions at multiple functional binding sites within the cell. However, the close agreement with values derived from channels in excised membrane patches suggests that CFTR is the primary site of action in the intact tissue.

DISCUSSION

Herein we provide a quantitative description of the interaction between tolbutamide and CFTR in excised membrane patches. Earlier reports identified the inhibition of chloride currents by tolbutamide in whole-cell recordings from CFTR-transfected cells (Sheppard and Welsh, 1992). We extend this observation by demonstrating that tolbutamide directly interacts with the open state of CFTR in excised membrane patches to cause a fast-flickery type of channel block. Tolbutamide was employed for the present study because it is a low-affinity ligand that causes a type of block that can be readily quantified by fluctuation analysis. Our observations allow us to confirm the relatively low potency of tolbutamide (K_D = 430 μM) and to determine the channel state with which tolbutamide interacts.

Glibenclamide, another sulfonylurea, is a more potent blocker of CFTR chloride channels. Sheppard and Welsh (1992) reported that glibenclamide was 7 to 8 times as effective as tolbutamide at inhibiting whole-cell CFTR current. Our work has shown that glibenclamide is 10 to 30 times as effective as tolbutamide at blocking CFTR in excised membrane patches (DeRoos et al., 1993a,b; Schultz et al., 1996b). The potencies of these sulfonylureas in affecting CFTR closely parallels their relative binding potencies to SUR (Ashcroft and Ashcroft, 1992), suggesting that the relevant pharmacophore is similar for the two proteins. Although CFTR and SUR are members of the same protein family and share characteristics such as nucleotide regulation and sulfonylurea binding, it remains important to note that the binding affinities for these sulfonylureas are 10,000 times higher for SUR than for CFTR. Thus, the sulfonylurea-binding sites on CFTR and SUR have similarities; however, a component of the binding site on SUR allows for a more stable interaction with glibenclamide and tolbutamide. A second class of sulfonylureas, diaryl-sulfonylureas, appears to selectively bind to CFTR, thus providing direction for development of ligands selective for CFTR (Schultz et al., 1995a).

It has been reported that sulfonylureas interact with a regulatory protein and not directly with either CFTR (Hongre et al., 1994) or K_{ATP} (Ashford et al., 1994). However,

Aguilar-Bryan et al. (1995) reported that SUR cloned from HIT T15 cells is, like CFTR, a member of the ABC superfamily of proteins. This parallel between the SUR and CFTR further supports the hypothesis of a direct ligand-receptor relationship between the sulfonylureas and CFTR. Indeed, we have recently shown that the photoactivatable radiolabeled sulfonylurea, [^{125}I]iodo-glibenclamide, blocks CFTR channel gating and, when photoactivated, radiolabels the CFTR protein (Singh et al., 1995).

There is a need to develop selective, high-affinity ligands for CFTR because no such compounds are available. Scientifically, both openers and blockers would be useful for definitively resolving the effects of CFTR at the tissue or organ level. Clinically, compounds of specific interest would be expected to directly and selectively bind to CFTR to alter either the trafficking or gating characteristics of mutant forms of the protein and thus provide relief from symptoms of the disease. Earlier reports indicated that sulfonylureas both permeate the cell membrane (Rabe et al., 1995) and alter CFTR gating (Sheppard and Welsh, 1992; Devor et al., 1995), making them candidate compounds for the development of specific ligands. Our data indicate that the block of CFTR by tolbutamide is due to a direct interaction with the channel protein, because we were able to show reversible, concentration-dependent effects in excised patches of cell membrane.

It is important to note that tolbutamide interacts with the open state of the CFTR channel. In the kinetic schemes that have been proposed, the open-state conformation is a nucleotide-bound form. It remains unclear whether simple nucleotide association and dissociation regulate entry to and exit from the open state or if ATP hydrolytic steps are required for gating (Schultz et al., 1995b, 1996a; Gunderson and Kopito, 1995; Hwang et al., 1994). Nonetheless, tolbutamide interacts with the nucleotide-bound, open state of the channel and precludes it from closing, i.e., tolbutamide introduces a brief (~800 μs) nonconducting state of CFTR that prolongs the duration of the nucleotide-bound form. Preliminary evidence indicates that ΔF508-CFTR has a reduced affinity for ATP that results in a lower likelihood that it will be in the nucleotide-bound form, which can open (Schultz et al., 1994a,b). Thus, stabilization of the nucleotide-bound form of the channel has important therapeutic implications. Future studies will be directed to define compounds which, like tolbutamide, stabilize the open conformation of the channel, but without interrupting ion permeation. Because of the extensive work completed on sulfonylureas for the treatment of diabetes and bacterial infections (Ashcroft and Ashcroft, 1992), a wealth of related compounds are available for further scrutiny. Analysis of this pharmacopeia will likely provide insights into the chemical nature of the sulfonylurea-binding site on CFTR and may result in the identification of therapeutic agents.

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