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Modification of Gating of an Airway Epithelial Chloride Channel by 5-Nitro-2-(3-Phenylpropylamino)Benzoic Acid (NPPB)

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Summary. 5-Nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) has been shown to produce a reduction in channel open probability in a number of epithelial chloride channels. We have investigated its action on a calcium-dependent airway epithelial chloride channel, which shows voltage-dependent gating following incorporation into planar phospholipid bilayers. Since the channel demonstrates a distinct subconductance state at approximately one-third of the fully open level, both 50 and 20% threshold analysis (TA) have been used to describe channel kinetics. Lifetime analysis using 50% TA in the absence of NPPB showed that two open and three closed lifetimes provided the optimal fit. Similar analysis using 20% TA required only two closed lifetimes and allowed for tentative assignment of tau values to either substate or fully closed events. NPPB (10–100 μM) produced a concentration-related shift of the normal voltage dependence of gating, with more negative holding potentials being required to produce a given channel open probability than in the absence of NPPB. Ten μM NPPB produced a similar shift in lifetime values. However, addition of 50 μM NPPB produced a unique, single open lifetime. No evidence was found for NPPB acting as a direct voltage-dependent blocker of chloride conductance.

Key Words trachea · epithelium · channel · NPPB · gating

Introduction

A number of studies have established NPPB as a potent and relatively specific inhibitor of epithelial chloride channels (Greger, Schlatter & Gögelein, 1987; Hayslett et al., 1988). The compound is generally classified as a channel 'blocker' and is frequently used as such in the characterization of chloride channels. However, one study using HT_{29} cells found that no additional time constants appeared following addition of NPPB. Thus, NPPB may alter rate constants of transitions between existing states, and therefore, influence channel gating rather than act as a channel blocker (Dreinhöfer, Gögelein & Greger, 1988).

We have recently described the basic properties of a calcium-dependent airway epithelial chloride channel, showing voltage-dependent gating following incorporation into planar phospholipid bilayers (Alton et al., 1991). In the present study we describe the effects of NPPB on this channel and suggest the principal action of this agent is to influence the gating of the channel. The observed effect is very similar to that of ATP on this channel, suggesting a similar mode of action for these two negatively charged ions.

Materials and Methods

The methods for preparation of membrane vesicles from sheep tracheal epithelium and the technique for single-channel characterization have been described in detail (Alton et al., 1991) and are only summarized.

Preparation of Membrane Vesicles

Membrane vesicles were prepared from sheep tracheae as described by Langridge-Smith, Field and Dubinsky (1983). Tracheae were transported from a local abbatoir in ice-cold, aerated, buffered Tyrode's solution. On arrival in the laboratory, the epithelial lining was removed by scraping and homogenized. Following isolation of a mixed membrane pellet by centrifugation, basolateral membranes were precipitated by incubation with 10 mM $MgCl_2$ for 1 hr. Mixed membranes were separated by centrifugation, and apical and basolateral membranes were suspended in a cryoprotectant solution of 400 mM sucrose to a final protein concentration of approximately 5 mg/ml. Aliquots were snap-frozen in liquid nitrogen and stored at −80°C.

Single-Channel Technique

Apical membrane vesicles were incorporated into planar phospholipid bilayers using standard techniques (Miller, 1982). The *cis* chamber, internal volume of 0.8 ml, contained a 200-μm hole joining this chamber with a well of 5.0 ml (*trans* chamber). Ag/AgCl electrodes placed in KCl wells connected both the *cis* and *trans* chambers to an operational amplifier. External voltage

commands provided by an Amstrad CPC 6128 computer linked to a digital-to-analog converter were applied to the *cis* chamber, the *trans* chamber being held at ground potential. The signal was filtered using a Krohn-Hite 4-pole low-pass filter before display on an oscilloscope. Unfiltered data were stored on FM tape for further analysis.

Bilayers were formed from phosphatidylethanolamine and phosphatidylserine (70 : 30%), and chambers were filled with 200 mM NaCl in 10 mM HEPES-Tris, pH 7.2. Membrane vesicles were added to the *cis* chamber in the presence of 2 mM Ca^{2+} and increasing osmotic gradients.

Experimental Protocol

Following incorporation of a channel(s) a clamp voltage of −30 mV was applied to the bilayer. At this voltage channel open probability is maximal (Alton et al., 1991) and the presence of more than one channel is readily apparent. All incorporations of multiple channels were discarded. In the presence of a single channel (n = 23), the *cis* chamber was perfused with 600 mM NaCl and 2 mM Ca^{2+} added to this chamber.

NPPB was dissolved in dimethyl sulfoxide (DMSO) to produce a 100-mM stock solution. A 10-mM solution was obtained by dilution in 60% DMSO and 30% HEPES-Tris. Thereafter all dilutions were made using HEPES-Tris. DMSO, in equivalent volumes, has been previously shown to have no effect on the kinetics of this channel (Alton et al., 1991).

Following perfusion of the *cis* chamber 2–5 min recordings were made at clamp voltages of −30 and −60 mV for lifetime analysis. NPPB (10 μM) was then added to the chamber, and the recordings were repeated. Thereafter, 30-sec recordings of channel activity at a range of clamp voltages from −90 to +60 mV were made, preceded at each voltage by a short period of clamping at −30 mV to standardize open probability. This protocol was repeated for increasing concentrations of NPPB (50 and 100 μM), with chamber washout between each concentration. We have previously established the dose-related response of this channel to NPPB, which acts from the *cis* side only (Alton et al., 1991).

Data Analysis

For analysis of channel open probability (P_o), data were digitized (2 kHz), filtered (100 Hz) using an 8-pole Bessel filter and analyzed using 'Satori' (Intracel, Cambridge, UK). Such relatively harsh filtering was necessary for this part of the analysis due to the small channel conductance at positive holding potentials. The methods for analysis of the channel have been previously described in detail (Alton et al., 1991). Briefly, the channel commonly displays a subconductance level at approximately one-third of the fully open level. To enable separate analysis of the contribution of this substate to channel kinetics, data were analyzed using both 20 and 50% amplitude TA. Using 50% TA, thus treating the substate as a closed level, P_o was calculated from the proportion of time the channel spent in the open state compared to the total recording time. P_o was similarly obtained using 20% TA, and therefore, the percentage of time spent in the substate (substate %) could be calculated from the difference between the two (20% TA P_o − 50% TA P_o) for each voltage. The total time spent in the substate (substate time) was calculated from substate % × total recording time. The number of substate events (substate number) was obtained from the difference in event numbers using the two types of threshold analysis (50% TA event number − 20% TA event number). The mean dwell time of the channel in the substate (substate dwell time) was calculated from the total time spent in the substate divided by the total number of substate events. An 'event' describes the transition between the open and closed states irrespective of the threshold used for analysis.

For lifetime analysis, data obtained using both 50% TA and 20% TA were digitized (3 kHz) and filtered (1.5 kHz) using an 8-pole Bessel filter, stored in sequential files and displayed in non-cumulative histograms. Individual lifetimes were fitted to a probability density function (PDF) using the method of maximum likelihood (Colquhoun & Sigworth, 1983). Events of duration shorter than 0.6 msec were incompletely resolved and were not included in the analysis. A missed-events correction for these events was therefore applied (Colquhoun & Sigworth, 1983) and a likelihood ratio test was used to compare fits of 1–3 exponentials (Blatz & Magleby, 1986).

Analysis of Thermodynamics of Gating

For a voltage-dependent reaction, the Gibbs 'internal free energy of opening' (ΔG_i) and the 'effective gating charge' (z) can be calculated from

$$P_o = P_m[1 + \exp(\Delta G_i/RT + zFV/RT)]^{-1} \quad (1)$$

where: P_o = steady-state open probability, P_m = maximal open probability, V = membrane potential, F = Faraday's constant, R = gas constant, and T = temperature (K).

In its linearized form

$$\ln([P_m/P_o] - 1) = \Delta G_i/RT + zFV/RT \quad (2)$$

and a plot of $\ln([P_m/P_o] - 1)$ against voltage allows for calculation of ΔG_i and z from the ordinate intercept and slope, respectively.

Results

Characteristics of the Channel in the Absence of NPPB

We have previously shown (Alton et al., 1991) that as the holding potential is reduced from −60 to −30 mV the following changes occur: (i) Mean open time measured using 50% TA is approximately halved, accompanied by a small increase in 50% TA event number. (ii) Mean open time measured using 20% TA is reduced, although less so than when measured using 50% TA; 20% TA event number remains unchanged. (iii) No change occurs in mean closed times measured using either 50% TA or 20% TA. (iv) The number of entries of the channel into the substate increases. (v) The substate dwell time remains unchanged. (vi) As a consequence the percentage of time the channel spends in the subconductance state increases. (vii) There is little change in the overall open probability of the channel mea-

Table 1. Lifetime distributions, using 50% TA, in the absence of NPPB and following addition of 10 and 50 μM NPPB[a]

	Open lifetime distribution		Closed lifetime distribution	
	Time constant	Area	Time constant	Area
Control				
−60 mV	0.8	0.29	1.0	0.80
	35.4	0.71	2.8	0.17
			90.6	0.03
−30 mV	0.6	0.41	0.8	0.84
	36.2	0.59	2.7	0.15
			60.4	0.01
10 μM NPPB				
−60 mV	1.3	0.42	0.9	0.72
	28.0	0.58	2.4	0.26
			20.7	0.02
−30 mV	0.7	0.35	0.8	0.81
	18.1	0.65	3.6	0.17
			29.8	0.02
50 μM NPPB				
−60 mV	6.4	1.0	1.1	0.43
			1.9	0.55
			7.8	0.03
−30 mV	4.0	1.0	1.0	0.43
			3.6	0.41
			10.3	0.16

[a] Probability density functions (PDF) were obtained according to $f(t) = a_1(1/\tau_1)\exp(-t/\tau_1) + \ldots + a_n(1/\tau_n)\exp(-t/\tau_n)$ where a = respective area, τ = respective time constant and t = time. Area indicates the relative areas of the PDF described by the time constants.

Table 2. Lifetime distributions, using 20% TA, in the absence of NPPB and following addition of 10 and 50 μM NPPB[a]

	Open lifetime distribution		Closed lifetime distribution	
	Time constant	Area	Time constant	Area
Control				
−60 mV	1.5	0.32	1.0	0.93
	241.7	0.68	58.1	0.07
−30 mV	1.3	0.36	0.9	0.89
	145.9	0.64	9.0	0.11
10 μM NPPB				
−60 mV	2.4	0.40	0.9	0.93
	120.6	0.60	6.0	0.07
−30 mV	1.1	0.19	1.0	0.77
	43.3	0.81	3.3	0.23
50 μM NPPB				
−60 mV	5.3	0.61	1.2	0.92
	20.6	0.39	4.9	0.08
−30 mV	1.7	0.06	1.3	0.63
	7.1	0.94	4.6	0.37

[a] Probability density functions were obtained as described in Table 1.

sured using either 50% TA or 20% TA (since the substate contributes only approximately 8% of total channel P_o).

In the present study, lifetime analysis in the absence of NPPB was undertaken both to extend these observations and to provide a baseline for comparison with the NPPB effect. Using 50% TA mean values for lifetime analyses at −60 and −30 mV for three channels are shown in Table 1. Two open lifetimes provided the best fit at either voltage. The effect of decreasing clamp voltage was to increase the percentage of time spent in the shorter lifetime. The increase in substate events at −30 mV, in comparison to that seen with a holding potential of −60 mV, is not accompanied by an alteration in the longer open time. Thus, it is likely that these increased number of closings to the substate do not occur within long openings. For the closed times the data were best fitted by three exponentials with little change in values with clamp voltage.

To attempt to correlate channel closing events to the substate or to the fully closed level with these lifetimes, a similar analysis was undertaken using 20% TA (Table 2). At both voltages the best fit to open times was again provided by two exponentials. As predicted from the previously described changes in channel kinetics, treating the substate as an opening (20% TA) produced a large increase in the time constant of an open lifetime (tau 2) at either clamp voltage.

For the closed times, only two exponentials are now required to fit the data (Table 2). Thus, it is likely that the shortest closed time constant (approximately 1.0 msec) represents full closing events. The intermediate time constant (approximately 2.8 msec) probably reflects closings to the substate, a value which disappears using 20% TA. Although we have no direct evidence, it is possible that the longest time constant (approximately 60–90 msec) using 50% TA, represents closings to the substate which then dwell in the substate before moving to the fully closed level. Such events are occasionally seen with this channel in keeping with the small percentage of the total that this lifetime comprises. If this is correct, 20% TA would be predicted to only 'see' a shortened version of these events in comparison to that at 50% TA.

Effect of NPPB on Channel Open Probability

Figure 1 shows the effects of 10 and 50 μM NPPB on the channel at a holding potential of −30 mV. At 10 μM there appears to be an increase in the frequency of events, both to the substate and to the fully

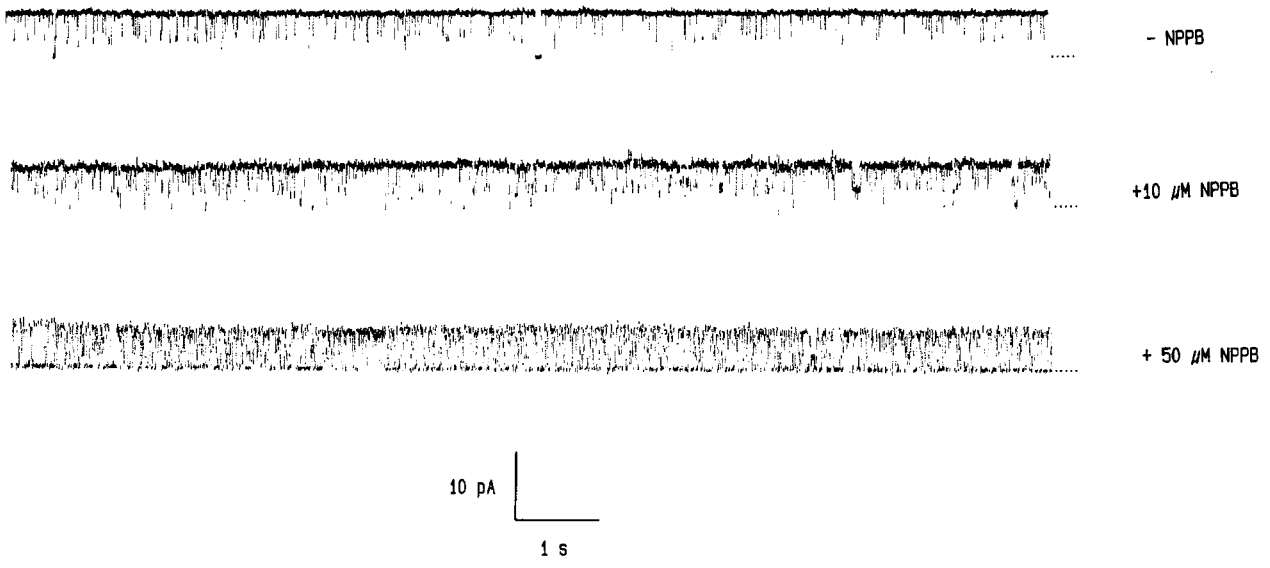

Fig. 1. Effects of 10 and 50 μM NPPB on current fluctuations at a holding potential of −30 mV. Recordings were made under the following conditions in this and the following figures: *cis*, 600 mM NaCl, 2 mM $CaCl_2$; *trans*, 200 mM NaCl; and are shown low-pass filtered at 600 Hz. Dotted lines indicate the fully closed level.

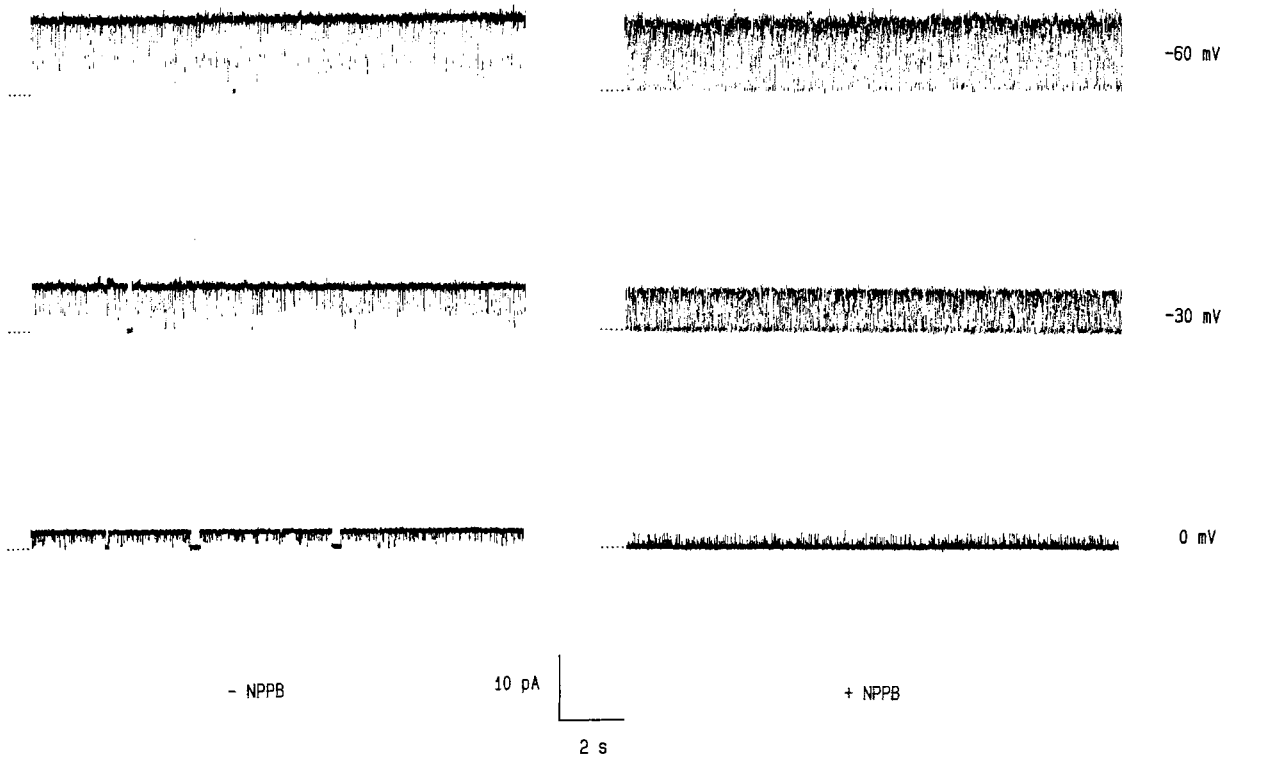

Fig. 2. Single-channel fluctuations at the indicated holding potentials (in mV) in the presence and absence of NPPB (50 μM) filtered at 600 Hz. Dotted lines indicate the closed channel level.

closed level. Fifty μM NPPB produces channel 'flickering' while higher concentrations produce long events at the closed level, with only occasional openings which are either not fully resolved or only reach the substate level.

Figure 2 exemplifies the effects of voltage on the NPPB (50 μM)-induced changes at a range of holding potentials. The effects of NPPB were principally apparent at low negative holding potentials. Since the channel is predominantly closed at posi-

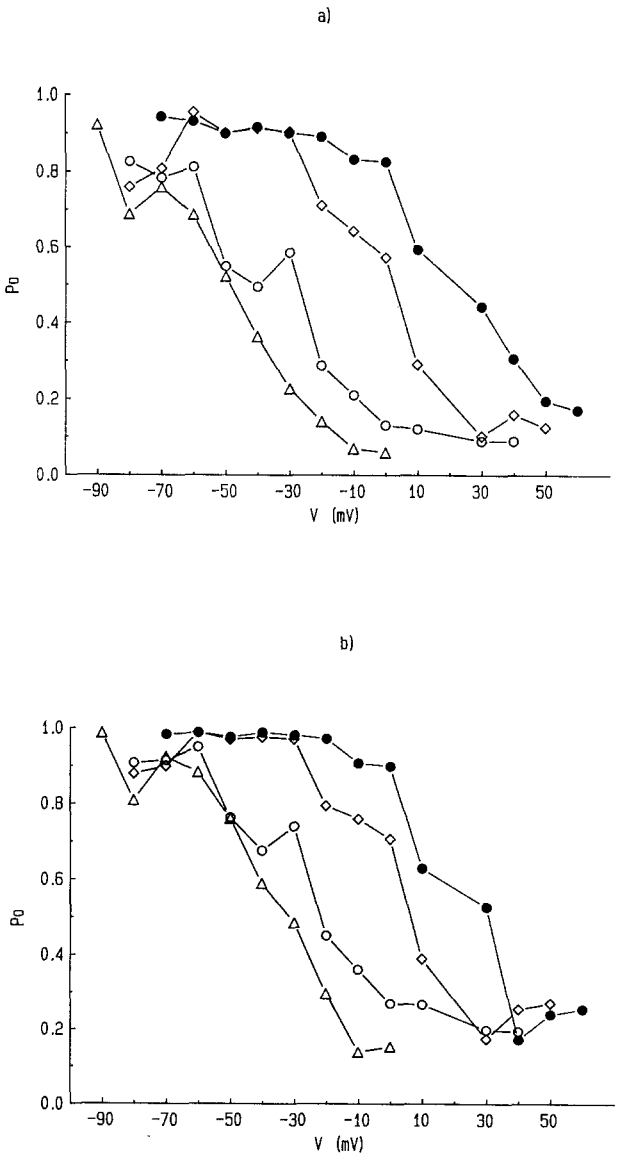

Fig. 3. (*a*) The effect of applied voltage on the probability of the channel being open (P_o) using 50% TA: (●), mean P_o values for channels in the absence of NPPB (n = 3–20); (◇), 10 μM NPPB (n = 2–5); (○), 50 μM NPPB (n = 2–4); and (△), 100 μM NPPB (n = 2–3). SEM are not shown for clarity of presentation in this and the following figures. (*b*) The effect of applied voltage on the probability of the channel being open using 20% TA. Notation is as in *a*.

tive holding potentials, quantification of an effect at these voltages was not possible. At high negative potentials the effect of NPPB appeared to diminish.

The effects of NPPB on channel open probability measured using either 50% TA or 20% TA are shown in Fig. 3*a* and *b*, respectively. NPPB produced a concentration-related shift of the normal voltage dependence of channel open probability, with more negative holding potentials being required to elicit the open probability seen in the absence of NPPB. Using 50% TA, and thus treating substate events as closings, a relatively greater effect of NPPB is seen (Fig. 3*a*) in comparison to that with 20% TA (Fig. 3*b*), suggesting an increased occupancy of the substate.

Using 50% TA, NPPB produces a dose-related reduction in the mean open time of the fully open state, with increasingly greater effect as the holding potential becomes more negative (Fig. 4*a*). The effect of NPPB on event number using 50% TA appears twofold (Fig. 4*b*). Thus, the voltage at which the maximum event number occurs, becomes more negative with increasing concentrations of NPPB. Furthermore, the maximum number of events is increased in a concentration-related manner, with the curve describing the relationship between voltage and event number becoming increasingly 'bell-shaped.' Thus, at the holding potentials at which a maximum effect of NPPB on P_o is seen (approximately +10 to −50 mV), opening events are shorter in duration and more frequent. However, at holding potentials more negative than this range, openings continue to be of short duration but their number returns towards that seen in the absence of NPPB.

Using 50% TA, NPPB shows little effect on mean closed time (Fig. 4*c*). However, given the complex effect of voltage on event number noted above, at the voltages where NPPB maximally reduces P_o, there will be a marked increase in the number of events at the closed level, although these are of unchanged mean duration. At more negative holding potentials, the number of closing events returns towards control levels.

The increase in the number of 'closings' caused by NPPB may relate either to an increase in the number of events to the substate, the fully closed level, or both. To distinguish these possibilities Fig. 4*d* and *e* show the effects of clamp voltage on mean open time and event number, respectively, using 20% TA. Again, at a given holding potential, NPPB produces a dose-related reduction in mean open time and a similar effect on event number to that described above for 50% TA. The mean duration of events to the fully closed level is again unaltered (Fig. 4*f*). Thus, the effects of NPPB include an increase in the number of fully closed events.

To directly assess the effects of NPPB on the subconductance state, Fig. 5*a* shows that there is a concentration-related change in the substate event number. The pattern of this is very similar to that described above for the event number using either 50% TA or 20% TA. At the highest concentration of NPPB studied (100 μM), it was not possible to accurately assess substate event number at voltages ranging from +10 to −30 mV, since incomplete openings occurred which registered using 20% TA, but not at 50% TA. Substate dwell time (Fig. 5*b*)

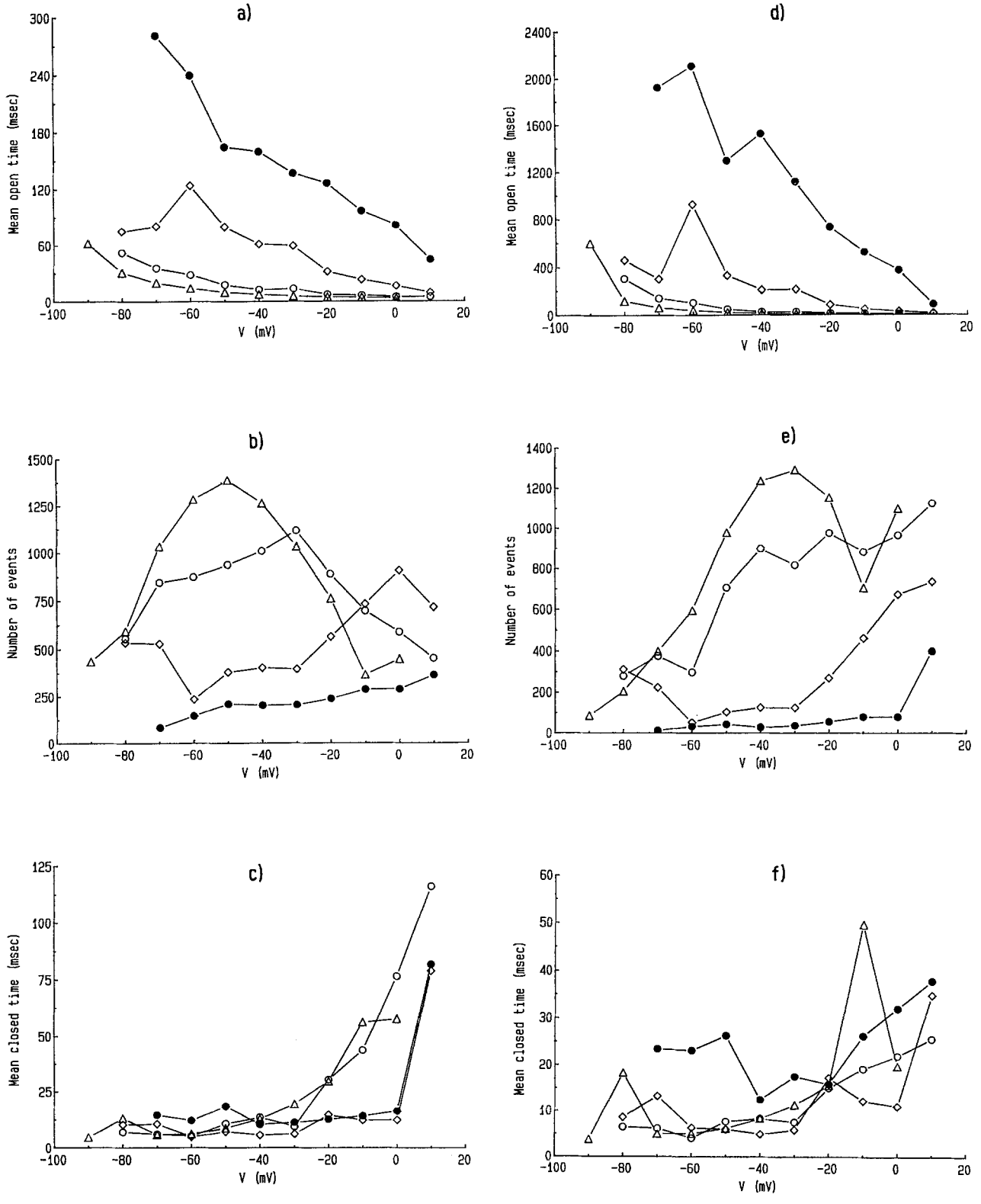

Fig. 4. The effect of voltage on the following measured parameters: (*a*) mean open time measured using 50% TA, (*b*) total number of events using 50% TA, (*c*) mean closed time using 50% TA, (*d*) mean open time using 20% TA, (*e*) total number of events using 20% TA, and (*f*) mean closed time using 20% TA. Parameters were derived as described in Materials and Methods. Points are mean values of 2 to 23 separate determinations at each voltage. (●), in the absence of NPPB; (◇), 10 μM NPPB; (○), 50 μM NPPB; and (△), 100 μM NPPB.

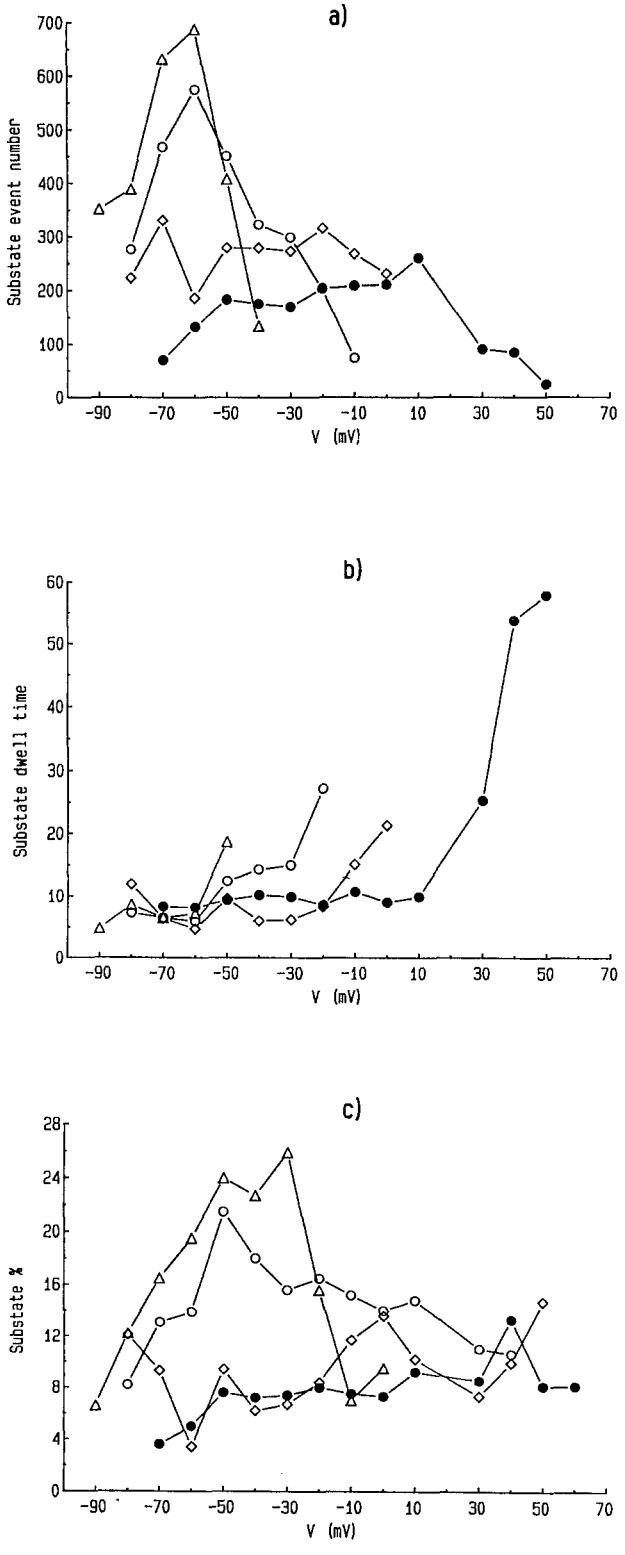

Fig. 5. The effect of voltage on the following measured parameters: (*a*) number of substate events, (*b*) dwell time in substate, and (*c*) mean percentage time spent in substate. Parameters were derived as described in Materials and Methods. Points are mean values of 2 to 23 separate determinations at each applied voltage. (●), in the absence of NPPB; (◇), 10 μM; (○), 50 μM NPPB; and (△), 100 μM NPPB.

again demonstrates that NPPB causes a concentration-related shift of the effects of holding potential toward more negative voltages. However, within the voltage range at which the principal effects of NPPB on P_o are seen (+10 to −50 mV), little change in the duration of substate events was seen at any NPPB concentration.

As expected, therefore, from the combination of these changes there is a concentration-related increase in the percentage of time the channel spends in the substate in the presence of NPPB (Fig. 5*c*), over the range of holding potentials at which NPPB is maximally effective. Thus, the effects seen using 50% TA are representative of an increase in events both to the subconductance and fully closed levels. This pattern of events is that seen at more positive holding potentials in the absence of NPPB (Alton et al., 1991), in agreement with the described shift of the various parameters.

NPPB is negatively charged and may therefore act as a voltage-dependent blocker. Figure 6 shows plots of normalized channel open probability against holding potential in the presence of increasing concentrations of NPPB using either 50% TA or 20% TA. The voltage dependence of the normalized P_o is in the opposite direction to that expected of a negatively charged ion, with maximal P_o at the most negative holding potentials. Furthermore, NPPB had no observable effect on single-channel conductance, measured at either −30 or −60 mV (e.g., Fig. 2). We conclude that NPPB is unlikely to act as a voltage-dependent blocker of this channel.

The above data suggest that the effect of NPPB is on channel gating, partly by altering the voltage dependence of gating seen in the absence of NPPB. We were, therefore, interested to know whether NPPB altered the gating charge of the channel. An estimate of the thermodynamics of the NPPB effect and any resulting change in the gating charge can be obtained from the Boltzmann equation. The Gibbs 'internal free energy of opening' for the channel derived from Fig. 7*a* in the absence of NPPB is −0.17 kcal/mol, the negative value indicating that at the reversal potential it is thermodynamically favorable for the channel to be in the open state. In contrast, with increasing NPPB concentrations, the free energy reverses in sign (10 μM: 0.51 kcal/mol, 50 μM: 1.46 kcal/mol, 100 μM: 2.47 kcal/mol) in keeping with the observed changes in channel kinetics. The slope of the curves in Fig. 7*a* provides an estimate of the effective gating charge for the channel. In the absence of NPPB a minimum of 1.4 elementary charges must move across the entire voltage drop or a higher number move partway across. The positive value indicates a net movement of positive charge towards the *cis* side of the membrane. With increas-

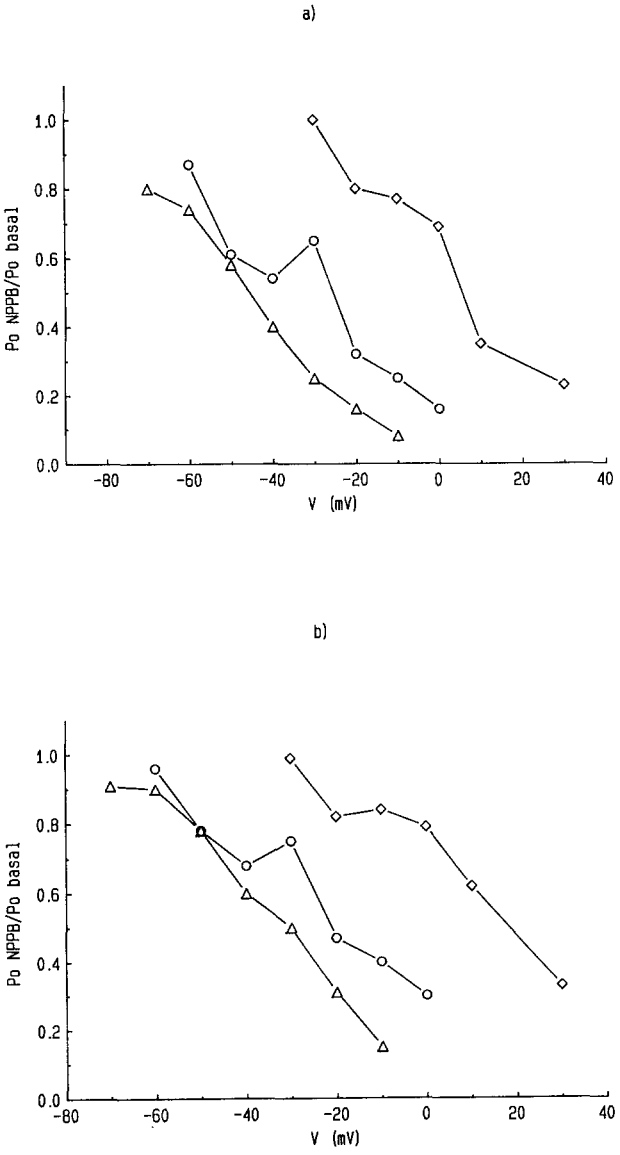

Fig. 6. Effect of voltage on normalized channel open probability (P_o NPPB/P_o basal) following addition of 10 μM (◇), 50 μM (○) and 100 μM (△) NPPB. Points are mean of 3 to 20 separate determinations at each voltage. (*a*) 50% TA; (*b*) 20% TA.

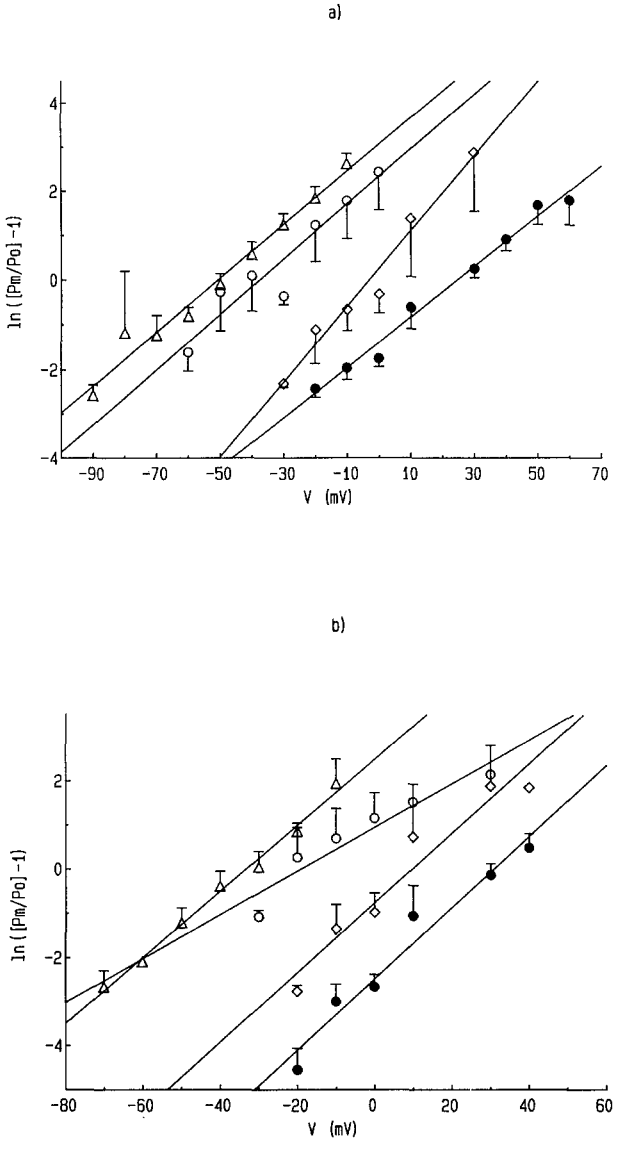

Fig. 7. The effect of voltage on $\ln([P_m/P_o] - 1)$ in the absence of NPPB (●) or following addition of 10 μM (◇), 50 μM (○) or 100 μM (△) NPPB. Points are mean values of 2 to 23 separate determinations at each voltage. (*a*) 50% TA; (*b*) 20% TA.

ing concentrations of NPPB no consistent alteration in this gating charge was seen, with values ranging from 1.4–2.1.

Using 20% TA (Fig. 7*b*), ΔG_i follows the same pattern as for 50% TA. However, values are consistently smaller than those obtained using 50% TA (control: −1.1 kcal/mol, 10 μM NPPB: 0.43 kcal/mol, 50 μM NPPB: 1.07 kcal/mol, 100 μM NPPB: 2.1 kcal/mol). Since, using this threshold, entry into the subconductance state (from the fully open level) does not register as a closing, more negative values for ΔG_i at the reversal potential would be consistent with these predictions. Using 20% TA, values for the effective gating charge were not consistently different to those using 50% TA, ranging from 1.3 to 1.8.

Effect of NPPB on Lifetime Analysis

10 μM

At this NPPB concentration two open time constants are seen at both holding potentials using 50%

TA (Table 1). The values show little change from those seen without the drug, but the relative percentages of the areas are now shifted so that at −60 mV, values are very similar to those seen at −30 mV prior to NPPB addition. The values at −30 mV show a shortened second open time. As shown in Fig. 4*a*, in the presence of NPPB (10 μM) mean open time at −60 mV decreases, presumably since the channel now spends a higher proportion of time in the short open state. At −30 mV the above-noted reduction in mean open time relates to a reduction in the duration of both lifetimes. This in turn presumably relates to an increase in the number of events (Fig. 4*b*) at −30 mV. For the closed lifetimes (Table 1), values at either holding potential are changed little from control values (i.e., three exponentials are required), with only a shortening of tau 3 being seen. Whether this reflects an increased frequency of one-third events is only conjecture.

Using 20% TA (Table 2), there are, as expected, two open times, the second of which is markedly longer than that seen using 50% TA. Again, at −60 mV values for the time constants and the percentages of the area are as previously seen at −30 mV in the absence of NPPB. Also, as for 50% TA, both tau values are shorter at −30 than at −60 mV. Two closed times provide the best fit for the data with the short tau 2 previously seen at −30 mV now being found at the −60-mV holding potential.

50 μM NPPB

At the higher concentration of NPPB, a marked difference is seen in lifetimes, with only one open time constant being required to fit the data using 50% TA (Table 1). However, three closed times (Table 1) are still required with tau 3 shortened once again. Using 20% TA (Table 2), two exponentials provide the best fit to open times, in keeping with the visualization of channel kinetics at this drug concentration. Thus, openings are seen which do not reach the 50% threshold and are thus registered only at 20% TA. The tau 1 values at both −60 and −30 mV correspond reasonably well with those found using 50% TA. Finally, two closed time constants are seen, with little difference in values compared to those found with 10 μM NPPB.

Discussion

A number of studies have described the effects of NPPB on chloride channels from a variety of epithelia (Greger et al., 1987; Hayslett et al., 1988). NPPB typically induces channel 'flickering' which is generally interpreted as a blocking phenomenon. However, one study has examined the kinetics of this effect using HT_{29} colonic carcinoma cells (Dreinhöfer et al., 1988). In the presence of NPPB (1–50 μM) no additional time constants were seen in comparison to control values. It was therefore hypothesized that NPPB was influencing rate constants of the transitions between existing conformational states, rather than inducing a blocked state, suggesting that NPPB acts on channel gating rather than as a blocker.

We have previously described the effects of voltage on the gating of a calcium-dependent airway epithelial chloride channel (Alton et al., 1991). Since we used NPPB as part of the characterization of this channel and observed the 'flickering' effects noted above, we were interested to see if NPPB principally affected gating, acted as a blocker, or did both in this channel.

NPPB carries a negative charge at a carboxylate group, which is ionized at physiological pH (Greger, 1990). Thus, a voltage-dependent blocking effect would be a potential mechanism of action. However, we found no evidence of this, normalized channel open probability (Fig. 6) demonstrating the opposite relationship expected of a voltage-dependent blocker. Furthermore, NPPB showed no effect on single-channel conductance.

Figures 3–5 clearly indicate that the principal effect of NPPB is on channel gating, producing a concentration-related shift of the normal voltage dependence of gating towards more negative holding potentials. Support for this interpretation comes from an examination of open lifetimes at low NPPB concentrations (10 μM), at which tau values equivalent to an approximately 30-mV leftward shift were seen.

NPPB may alter the gating of the channel in a number of ways. By combining with the charge sensing apparatus of the channel, NPPB could alter its response to an imposed voltage change within the membrane. Calculation of the effective gating charge in the absence of NPPB suggests that a charge of 1.4 needs to be moved across the entire voltage drop or suitable combinations of larger charge across a shorter distance. However, in the presence of NPPB the magnitude of the effective gating charge remains unchanged, suggesting that NPPB does not act in this way.

A second possible mechanism by which NPPB could alter channel gating is through an effect on surface charge, as the strength of the electric field within the membrane relates to the potential profile across it (Hille, 1984). By binding to the channel protein, NPPB would alter this gradient for a given imposed clamp voltage. While we have no direct evidence to support this hypothesis, the strong cal-

cium dependence of channel open probability in the presence of a negatively charged bilayer suggests that surface charge may be able to influence the gating of this channel. This is further supported by the observation that channel open probability, in the presence of NPPB, rises with increasingly negative clamp voltages. Thus, the magnitude of the field within the membrane would increase with the applied voltage for a constant NPPB effect on surface charge.

At concentrations of 50 μM or greater, NPPB produced the typical flickering phenomenon described in several studies. Furthermore, we saw a very similar effect to that described by Dreinhöfer et al. (1988) namely a reduction to one open time constant. At least three explanations are possible for these observations in the context of the likely effect of NPPB on gating. In the presence of 50 μM NPPB, a clamp voltage of −30 mV is 'seen' by the channel as a membrane potential of approximately +10 mV. Since we were unable to undertake lifetime analysis at such positive holding potentials, we cannot exclude the possibility that at such voltages a single open time constant is sufficient to fit the data. However, the 'flickering' phenomenon seen in the presence of NPPB is not apparent, in its absence, at these voltages.

Secondly, NPPB may increase the transition rate between open states and the subconductance or closed states. The effects of increasingly negative clamp voltages would then be envisaged as increasing the transition rates in the opposite direction, P_o of the channel depending on a balance between the two. This hypothesis alone is unlikely to explain the observed effects, since NPPB does not produce similar changes in the measured parameters at different holding potentials, nor is there any shortening of tau 1 following the addition of 10 μM NPPB.

Finally, the binding of NPPB to the channel may induce a conformational change in the protein to produce a unique open state. Support for this hypothesis is provided by the complex effect of increasing concentrations of NPPB on the number of transitions between the open and closed states (Fig. 4*b* and *e*). A simple effect of NPPB on surface potential would be expected to move the relationship between voltage and event number toward more negative potentials for a given effect. However, NPPB also produces a marked increase in the peak event number, suggesting that changes in surface potential are insufficient to explain the observed effects. Furthermore, channel mean open time, even at high negative clamp voltages, does not return to values seen in the absence of NPPB (Fig. 4*a* and *d*). Similar limitations of alterations in membrane potential to explain changes in channel gating have been well described (Hille, 1984) and suggest that an additional interaction of the agent with the channel protein probably occurs. Finally, the asymmetry of the action of NPPB, which was effective only when applied to the *cis* surface of the channel, may suggest a specific site for combination of NPPB with the channel protein. We have previously shown (Alton et al., 1991) that ATP produces very similar effects to those seen with NPPB on this channel and is also only effective from the *cis* surface. Thus, large negatively charged ions may be able to alter gating by binding to a site outside the voltage drop of the channel.

Our previously reported use of both 50% TA and 20% TA to describe the kinetics of a channel demonstrating a subconductance state at the one-third open level is supported by lifetime analysis using this method. We have been able tentatively to assign types of closing events to the measured time constants and have demonstrated predicted changes in open times when values are compared using both types of threshold analysis. Furthermore, the shift in channel open probability with 10 μM NPPB was demonstrated as an equivalent shift in channel open lifetimes using this form of analysis. However, such a dual-threshold approach will require further validation to confirm whether it provides a reasonable estimate of rate constants for the open, closed and subconductance states.

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