0026-895X/98/020397-10$3.00/0

MOLECULAR PHARMACOLOGY, **54:**397–406 (1998).

Ethanol Potentiation of Calcium-Activated Potassium Channels Reconstituted into Planar Lipid Bilayers

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Received February 27, 1998; Accepted May 11, 1998

This paper is available online at http://www.molpharm.org

ABSTRACT

We examined the actions of ethanol on the single channel properties of large conductance Ca^{2+}-activated K^+ (BK) channels isolated from skeletal muscle T-tubule membranes and incorporated into planar lipid bilayer membranes. We have taken advantage of this preparation, because it lacks most elements of cellular complexity, including cytoplasmic constituents and complex membrane lipid composition and architecture, to examine the minimum requirements for the effects of alcohol. Clinically relevant concentrations (25–200 mM) of ethanol increased the activity of BK channels incorporated into bilayers composed of phosphatidylethanolamine (PE) alone or PE and phosphatidylserine. The potentiation of channel activity by ethanol was attributable predominantly to a decrease in the average amount of time spent in closed states. Ethanol did not significantly affect the current amplitude-voltage relationship for BK channels, indicating that channel conductance for K^+ was unaffected by the drug. Although base-line characteristics of BK channels incorporated into bilayers composed only of PE differed from those of channels in PE/ phosphatidylserine in a manner expected from the change in bilayer charges, the actions of ethanol on channel activity were qualitatively similar in the different lipid environments. The effects of ethanol on single channel properties of BK channels in the planar bilayer are very similar to those reported for the action of ethanol on neurohypophysial BK channels studied in native membrane, and for cloned BK channels expressed in *Xenopus laevis* oocytes, which suggests that ethanol's site and mechanism of action are preserved in this greatly simplified preparation.

Clinically relevant concentrations of ethanol (10–100 mM) rapidly and reversibly potentiate the activity of BK channels in excised patches of neurohypophysial terminal membrane (Dopico *et al.*, 1996), as well as in oocytes expressing cloned BK channels (Dopico *et al.*, 1998). Functionally, this increase in neurohypophysial BK conductance will hyperpolarize nerve terminals in the posterior pituitary and, coupled with inhibition of voltage-gated Ca^{2+} channels (Wang *et al.*, 1991, 1994), will decrease the secretion of vasopressin, leading to diuresis, a consequence of acute ethanol ingestion. The molecular site of action of ethanol has been a hotly debated topic and is difficult to determine in a complex cellular environment. Therefore, we report here the study of ethanol's actions at the single channel level, on T-tubule BK channels reconstituted into planar bilayer membranes of known composition. T-tubule BK channels are a convenient model for studying alcohol action because they reliably fuse into planar bilayers, where they have been well characterized (Moczydlowski and Latorre, 1983a; Moczydlowski *et al.*, 1985).

This research was supported by National Institutes of Health Grants AA08003 and AA05542 (S.N.T) and AA05460 (B.C.).

Until recently, ethanol had been considered to modulate ion channels primarily by disordering the bulk membrane lipid (Seeman, 1972; Deitrich *et al.*, 1989). More recently, emphasis has shifted to interactions between ethanol and membrane proteins. Use of cloned channels and site-directed mutagenesis has shown that alcohol's effects depend on channel subunit composition (Masood *et al.*, 1994; Chu *et al.*, 1995), protein sequence (Wafford *et al.*, 1991), and individual amino acids (Covarrubias *et al.*, 1995). However, membrane lipids still may play an important role in ethanol's modulation of ion channel function. This possibility is particularly relevant in the case of BK channels, whose activity is known to be influenced by the cholesterol/phospholipid ratio in both natural membranes (Bolotina *et al.*, 1989) and artificial bilayers (Chang *et al.*, 1995), by the type of phospholipid constituting the bulky lipid of the bilayer (Moczydlowski *et al.*, 1985), and by fatty acids, normally present in natural membranes, that modulate BK channel activity, most probably by a direct interaction with the channel protein (Kirber *et al.*, 1992).

Biological membranes are highly organized structures with nonrandom distribution of lipids. For example, some

ABBREVIATIONS: BK, large conductance Ca^{2+}-activated K^+; PS, 1-palmitoyl-2-oleoyl phosphatidylserine; PE, 1-palmitoyl-2-oleoyl phosphatidylethanolamine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; $[Ca^{2+}]_{ic}$, calcium concentration in the intracellular chamber; I, current amplitude; V, voltage.

lipid species preferentially distribute into the extracellular leaflet of the membrane, whereas others are found predominantly in the intracellular leaflet, forming vertical "transbilayer" domains (Gennis, 1989). Lipids also preferentially cluster within a bilayer leaflet to form lateral domains (Welti and Glaser, 1994). Formation of lateral domains can result from the juxtaposition of coexisting areas of gel- and fluid-phase lipids, the nonrandom mixing between different lipid species, or the presence of cholesterol, Ca^{2+}, or proteins. A number of studies have demonstrated that alcohols have selective actions on vertical and lateral domains. For example, ethanol selectively increases the fluidity of the extracellular leaflet in synaptic plasma membranes, an effect attributable to differences in transbilayer cholesterol distribution (Schroeder *et al.*, 1988; Wood *et al.*, 1989). Using fluorescence photobleaching recovery techniques in Aplysia neurons, ethanol was shown to increase the diffusion of the probe rhodamine-phosphatidyl-ethanolamine more than the probe 1-acyl-2-(6-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)]aminohexanoyl) phosphatidylcholine, which suggests that ethanol's actions on membrane proteins, such as gated ion channels, might be dependent upon the existence of dissimilar lateral domains (Treistman *et al.*, 1987). More detailed studies with respect to alcohol action on lateral domains have been conducted in model membranes. For example, ethanol's ability to disorder model membranes is enhanced by gangliosides (Harris *et al.*, 1984) and phospholipid polyunsaturation (Ho *et al.*, 1994), but is antagonized by cholesterol (Chin and Goldstein, 1981). Consequently, native membrane domains rich in gangliosides and polyunsaturated phospholipids, but low in cholesterol, would presumably be particularly sensitive to perturbation by ethanol (Deitrich *et al.*, 1989). Computer modeling studies also suggest that ethanol preferentially accumulates in domains with special packing properties favoring the intercalation of alcohols (Jorgensen *et al.*, 1993). Membrane proteins in this region would be exposed to concentrations of ethanol higher than that in the bulk membrane (Goldstein, 1984). One approach to evaluate the role of membrane organization and domain asymmetry is to study ethanol's action on ion channels incorporated into model planar bilayers that lack the heterogenous organization found in native membranes. The extra- and intracellular leaflets of these model bilayers are identical with respect to composition and fluidity, because these membranes rarely exhibit transbilayer phospholipid flip-flop (Hall and Latorre, 1976), and are formed from phospholipids extensively mixed in decane. Because nonrandom mixing of dissimilar lipids is one driving force for the formation of lateral domains (Welti and Glaser, 1994), domain formation in these model bilayers is reduced by casting bilayers from a limited number of lipid types. Lastly, this preparation also significantly minimizes the presence of other potential targets for the modulatory action of ethanol on BK channels, including intracellular components and intact cytoskeletal elements.

Materials and Methods

Preparation of skeletal muscle T-tubule membrane vesicles. T-tubule membrane vesicles from rat skeletal muscle were prepared as stated in Moczydlowski and Latorre (1983b). Membrane vesicles were resuspended in 300 mM sucrose and stored at −80°. The protein concentration was ~ 3.0 mg/mL as determined by Bradford assay.

Electrophysiology. Single-channel events were recorded at a bandwidth of 10 kHz with either a Dagan 8900 (Dagan Corp, Minneapolis, MN) or an EPC-7 (List Electronics, Darmstadt, Germany) patch clamp amplifier and stored on videotape using a pulse code modulator (Sony, Tokyo, Japan). Data were re-acquired at 5 kHz, low-pass filtered at 1 kHz with an eight-pole Bessel filter (Model 902LPF; Frequency Devices, Haverhill, MA), and analyzed using the pClamp suite of programs (ver. 6.02; Axon Instruments, Burlingame, CA). Lipids were dried under N_2 gas and resuspended in decane (10 mg/mL) before experiments. For experiments using PS and PE planar bilayers, PE and PS were mixed in a ratio of 3:1 (w/w). The experimental chambers (graciously provided by Dr. Christopher Miller, Howard Hughes Medical Institute, Brandeis University) were milled from delrin (Patriot Plastics, Woburn, MA), and consisted of an upper "*cis*" intracellular chamber (700 μl and connected to the headstage input) and a lower "*trans*" extracellular chamber (400 μl and referred to ground) that are separated by a plastic coverslip containing a small hole formed by the technique described by Wonderlin (Wonderlin *et al.*, 1990). Planar bilayers (~50–200 pF) were formed by painting the lipid mixture across the small hole in the coverslip. Bilayer capacitance was monitored by noting the current across the bilayer in response to a triangular wave (20 mV/25 ms). Within the limits of our resolution, ethanol had no obvious effect on bilayer capacitance (data not shown). Incorporation of T-tubule BK channels was accomplished by dropping ~ 0.5–1.5 μl of membrane preparation (into the *cis* well) directly onto the preformed bilayer in the presence of an osmotic gradient:intracellular chamber (*cis*) hyperosmotic with respect to the extracellular (*trans*) chamber. Because Ca^{2+} was present only in the intracellular chamber, only those channels that inserted with their Ca^{2+} sensor facing the intracellular chamber were activated, and thus, recorded. Solutions consisted of (extracellular) 0.35 mM KOH, 10 mM HEPES, 0.1 mM EGTA, pH, 7.2; (intracellular) 300 mM KCl, 1.05 mM $CaCl_2$, 10 mM HEPES, 1 mM EGTA, pH 7.2 (free $[Ca^{2+}]_{ic}$, 50 μM). Appropriate amounts of EGTA (100 mM stock) were directly added to the intracellular chamber, and mixed using a Pipetteman, to produce the desired ~3–8 μM free $[Ca^{2+}]_{ic}$. This range allowed us to work at low values of NP_o to avoid a "ceiling" effect, which could occur with a channel activator, such as ethanol. Free $[Ca^{2+}]_{ic}$ was calculated with the aid of a computer program based on Fabiato's calculations (Fabiato, 1988). Ethanol or a concentrated stock of urea, used as a control, was added in a similar manner to the intracellular chamber to yield the desired final concentration. Iberiotoxin was diluted with extracellular solution from a frozen stock to the desired final concentration (10 nM) and applied by superfusion of the extracellular bilayer chamber.

Ethanol's action on channels reconstituted in PE/PS bilayers was studied under different experimental conditions. In five of 11 cases, the bilayer was held at 0 mV and the free $[Ca^{2+}]_{ic} \approx 3$ μM. In the remaining six cases, the bilayer was held at values between −40 and +20 mV and the free $[Ca^{2+}]_{ic} \approx 3$–8 μM. Ethanol's effect was not different among these experiments and the data were pooled. Experiments that showed run-up or run-down of channel activity during the control period (in the absence of ethanol) were discarded. Moreover, experiments that showed increasing levels of channel openings during the run, consistent with the insertion of further channels, were also discarded. In some experiments, these were minimized by immediately collapsing the osmotic gradient after the initial insertion of channels into the bilayer. Enhancement of channel activity cannot be attributable to additional channel fusion in the presence of ethanol because drug effects were observed in bilayers containing only one level of channel openings at $P_o \sim 1$. Experiments were conducted at room temperature (~22°).

Data analysis. As an index of channel activity we use NP_o, the product of the number (N) of channels contained in a bilayer multiplied by an individual channel's probability of being open (P_o). The product NP_o was obtained from all-points histograms that were constructed from 20–140 sec of actual recording time. Durations of

open and closed times were measured with half-amplitude threshold analysis. Dwell-time data are plotted with a logarithmic time axis along the abscissa and a square-root ordinate exhibiting the number of events in each bin, according to the method of Sigworth and Sine (1987). Using this transformation, each exponential component has a peak at the value of its time constant. A maximum-likelihood minimization routine was used to fit the distribution of open and closed times. Determination of the minimum number of exponential terms for adequate fit was established using a standard *F* statistic table (significance level <0.01). BK channels occasionally entered a long closed state that could last for seconds. Because these events are probably caused by Ba^{2+} blockade (Neyton, 1996), long closed events were excluded from the analysis in both the absence and presence of ethanol. Slope conductances were obtained from linear regression fitting of the unitary I-V relationships. For each condition, I values at a given V corresponded to 2–9 different bilayers. A standard *F* statistic table was also consulted when slope conductances were compared. Data are presented as the mean ± standard error. On occasion, where stated, comparisons between individual means were analyzed with Student's *t* test.

Chemicals. All salts were obtained from Sigma (St. Louis, MO). Decane was obtained from Aldrich (Milwaukee, WI). HEPES (ultra pure) and ethanol (deionized, 100% purity) were obtained from American Bioanalytical (Natick, MA). Iberiotoxin was obtained from Alamone Labs (Jerusalem, Israel). PE and PS were obtained from Avanti Polar Lipids (Pelham, AL).

Results

Ethanol enhances the activity of BK channels incorporated into PE/PS planar bilayers. Our recording conditions were designed to selectively record BK channels, excluding other channel types. Several pieces of evidence confirm that the overwhelming majority of channels in our T-tubule recordings are BK channels. The channels under study are: 1) Ca^{2+}-dependent (i.e., channel activity increased with increases in $[Ca^{2+}]_{ic}$); 2) voltage-sensitive (i.e., channel activity increased with depolarization); 3) of large unitary conductance (>200 pS, using K^+ as the permeant ion); and 4) blocked when 10 nM iberiotoxin, a specific blocker of BK channels (Galvez *et al*., 1990), was applied to the extracellular side of the bilayer (data not shown).

Ethanol applied to the "intracellular" side of the bilayer increased the activity of BK channels in a concentration-dependent manner. The response of a single BK channel in a PE/PS bilayer to increasing ethanol concentrations (25–100 mM) is shown in Fig. 1. Ethanol's action cannot be attributed to an increase in osmolarity since the addition of urea (50 and 100 mM) to the intracellular side of the bilayer failed to potentiate channel activity (five of five cases). Channel activity was increased in 11 of 16, unchanged in four of 16, and decreased in one of 16 cases, by the alcohol. When ethanol (100 mM) was present on the "extracellular" side of the bilayer, channel activity was also increased (two of two cases), demonstrating that the drug is active from either side of the bilayer. In these two experiments, the P_o value was increased 26- and 3.3-fold. Because we did not further characterize the action of ethanol applied to the extracellular face, it is unknown whether the concentration dependency for ethanol applied to the extracellular side differs from that for the cytoplasmic side.

The data from the 11 of 16 cases where enhancement of channel activity was observed in response to ethanol applied intracellularly are summarized in Fig. 2. No clear saturation of the ethanol effect was observed in the concentration range shown, making the determination of a reliable EC_{50} value impossible. Higher concentrations of ethanol were difficult to test due to bilayer breakdown.

For these experiments, the I-V relationship in the absence or presence of high ethanol concentrations (75–200 mM), which were shown previously to markedly increase channel activity (see Fig. 2), were obtained under conditions of non-symmetric K^+ (intracellular $[K^+]$ = 300 mM, extracellular $[K^+]$ = 0.35 mM). Both in the absence and presence of ethanol, the I-V relationship was linear between −40 and +20 mV. The slope conductance was 245.3 and 268.2 pS in the absence and presence of ethanol, respectively. This difference was not statistically significant ($F(1, 3) = 1.4$, $p > 0.05$). Thus, as previously reported for native and cloned BK channels expressed in natural membranes (Dopico *et al.*, 1996, 1998), ethanol does not significantly affect BK channel conduction for K^+ in this minimal system.

Ethanol alters the gating of BK channels. An ethanol-dependent increase in unitary BK channel activity might be explained by an increase in the total amount of time a channel spends in open states (i.e., an increase in the mean open time), a decrease in the total amount of time a channel spends in closed states (i.e., a decrease in the mean closed time), or both. To address this issue, dwell-time distribution analyses were performed on data from the experiment shown in Fig. 1. The distribution of openings could be well-fitted with the sum of two exponential functions, evidence of at least two open states in the absence or presence of the alcohol (Fig. 3). At all concentrations tested, ethanol slightly increased the relative amount of time spent in the long open state. The changes induced by the drug on both the duration and the relative contribution of each component to the total time spent in the open state can be observed in Fig. 3. The minor changes produced by ethanol in both duration and relative contribution of long openings to the total time spent in the open state resulted in an overall increase in the channel mean open time (calculated by summing the products of the individual components of the open time distribution, τ_{o1} and τ_{o2}, and their respective contribution to the total fit, obtained from Fig. 3) at all concentrations tested: the control mean open time (12.0 msec) was increased by 5.2, 18.5, 46.7, and 27.9% in the presence of 25, 50, 75, and 100 mM ethanol, respectively. This influence of ethanol on the mean open time of BK channels reconstituted in PE/PS bilayers is similar to that observed when ethanol potentiation of BK channels was studied using either neurohypophysial terminals or cloned mslo channels expressed in oocytes (Dopico *et al.*, 1996, 1998).

The distribution of closed times, both in the presence and absence of ethanol, could also be best fitted by two components, indicating the existence of at least two closed states. Fig. 3 shows that ethanol produced a concentration-dependent, marked reduction in both the duration and the relative contribution to total closed time, of the longer closures. These changes resulted in a marked reduction of the channel mean closed time; the control mean closed time (38.3 msec) was decreased by 67.4, 91.6, 98.3, and 98.1% in the presence of 25, 50, 75, and 100 mM ethanol, respectively. Together, the data shown in Fig. 3, and the calculated mean open and closed times, indicate that ethanol increases the P_o of BK channels incorporated into PE/PS bilayers primarily by a concentra-

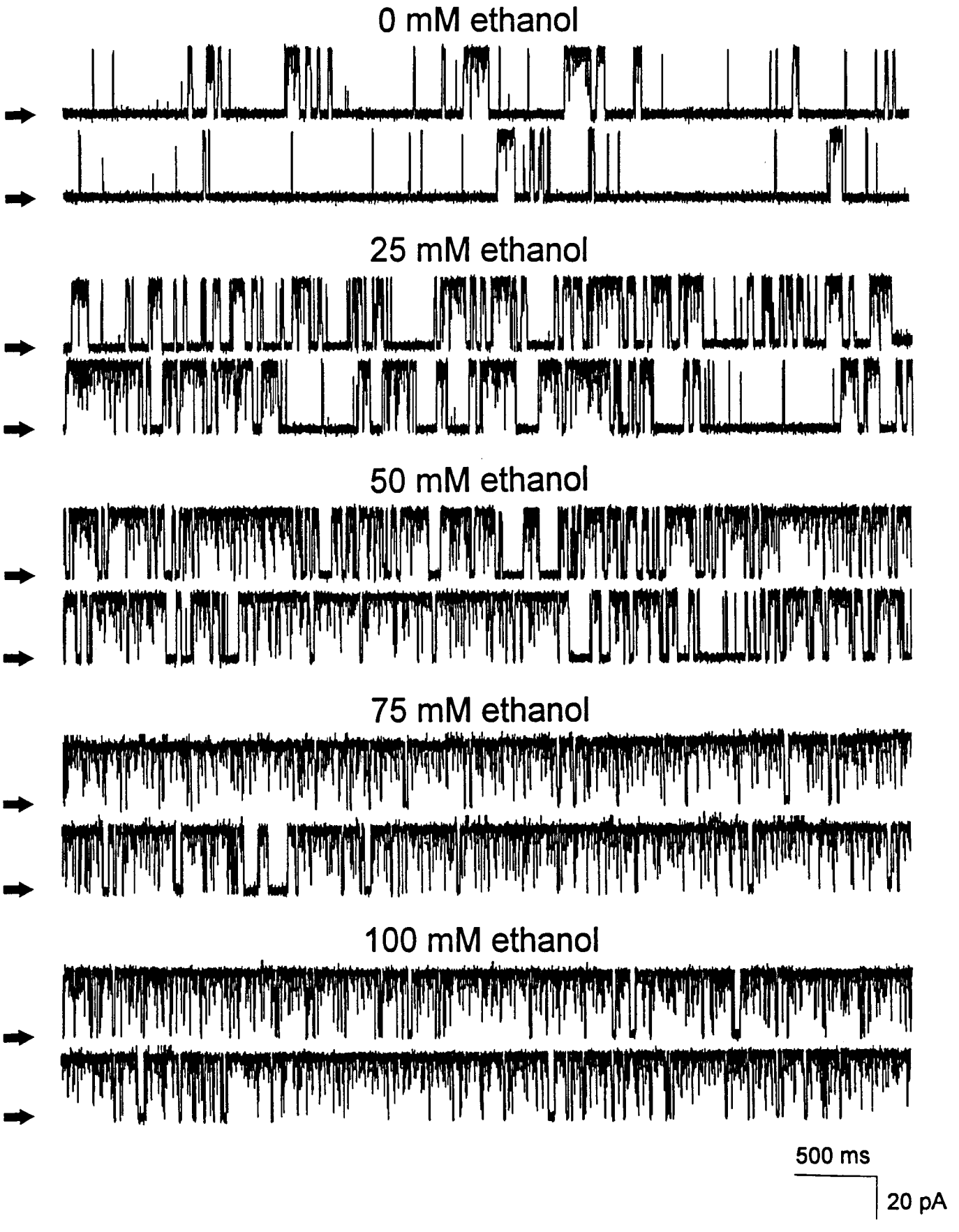

Fig. 1. Ethanol enhances the activity of a single BK channel incorporated into a PE/PS bilayer in a concentration-dependent manner (holding potential = 0 mV, ~8.1 μM free $[Ca^{2+}]_{ic}$). *Arrows*, closed levels.

tion-dependent reduction of the channel mean closed time that targets, in particular, the long closed state of the channel. This result was observed in two of two experiments where detailed kinetic analyses were performed (Table 1).

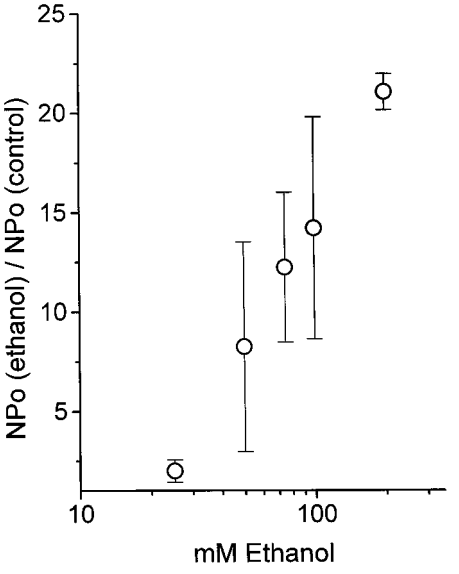

Fig. 2. Cumulative concentration-response relation for ethanol's activation of BK channels. The data are from 11 bilayers with either a single channel or two channels.

The similarities between the actions of ethanol on reconstituted BK channel gating and previous data obtained with either native or cloned BK channels studied in natural membranes (Dopico *et al.*, 1996, 1998; Table 1) indicate that at least some modifications of channel gating processes by the drug are preserved in this minimally reduced preparation.

Ethanol enhances the activity of BK channels incorporated into PE planar bilayers. One advantage of the use of the planar bilayer technique is the ability to examine the effect of lipid environment on the action of ethanol. T-tubule BK channels can also be readily reconstituted in bilayers cast from PE alone. The absence of PS from these model membranes not only alters the surface charge, but also further simplifies the lateral domain organization present in comparison to the PE/PS bilayers used in the previous experiments. Domain formation due to the immobilization of negatively charged PS species can be induced by the interaction of Ca^{2+} with acidic lipids (Welti and Glaser, 1994). PE/PS but not PE bilayers would, therefore, presumably contain these Ca^{2+} induced lipid domains. To assess the potential role of these Ca^{2+} induced domains, we tested the ethanol sensitivity of BK channels incorporated into one-component PE bilayers. Furthermore, because channels are reconstituted by fusing membrane vesicles with the preformed bilayer, native lipid is also necessarily introduced into the bilayer in the process. Comparison of channel characteristics in bilayers composed solely of the uncharged PE, with the behavior

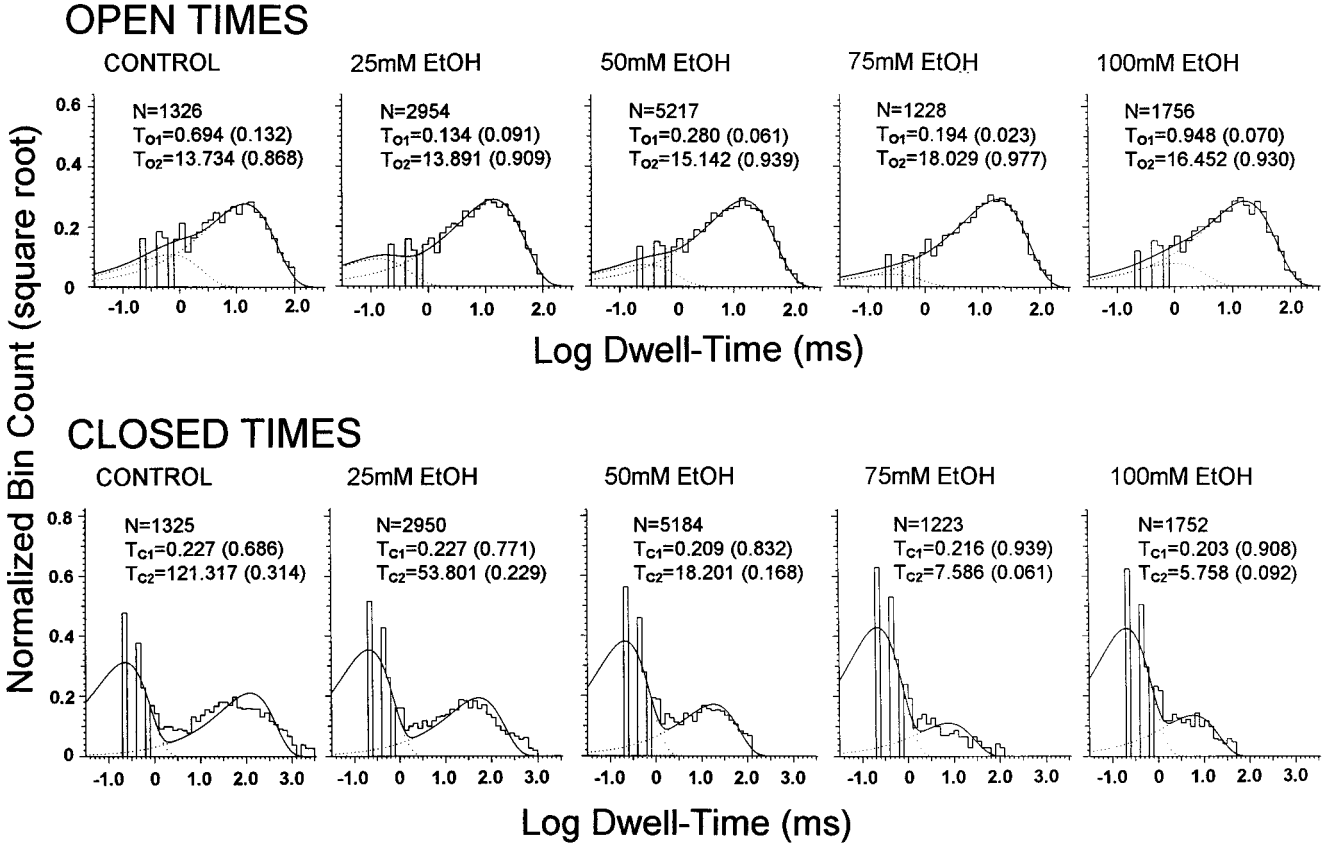

Fig. 3. Open and closed time distributions in the absence or presence of ethanol. The figure shows data from a representative BK channel reconstituted in a PE/PS bilayer, before and after exposure to different concentrations of ethanol. Each *panel* shows the total number of events (*N*), the duration of each particular component (*T*, in msec), and the relative contribution of each particular component to the total fit (in *parentheses*). The number of events were normalized before applying a Sigworth and Sine transformation (Sigworth and Sine, 1987), which allows a better resolution of individual components. *Dotted lines*, individual fitted components; *solid line*, composite fit.

observed in PE/PS bilayers containing the negatively charged lipid, PS, can provide crude confirmation that native lipids carried with the incorporated channel exchanged with the planar bilayer lipid. If the native lipid persisted as a stable complex surrounding the reconstituted channel, we might predict channel behavior to be identical in the two lipid mixtures.

The behavior of BK channels in charged PE/PS bilayers clearly differed from that observed for channels in uncharged PE bilayers in two ways, under set conditions (free $[Ca^{2+}]_{ic}$= 20 μM; $V_M = -20$ mV). First, the open probabilities for BK channels in PE bilayers were lower than that for channels in PE/PS bilayers (Fig. 4). The mean P_o for channels in PE bilayers was 0.48 ± 0.12 ($n = 10$), whereas that for channels in PE/PS bilayers was 0.95 ± 0.02 ($n = 5$). Second, the slope conductance for BK channels in PE/PS bilayers (287.8 pS) was significantly greater than that for BK channels in PE bilayers (226.8 pS) ($F(1, 3) = 23.5, p < 0.05$). A surface charge effect produced by the fixed negative charge on PS is known to lead to local accumulation of cytosolic Ca^{2+} and K^+ (Moczydlowski *et al.*, 1985). The local increase in cytosolic Ca^{2+} activates BK channels, whereas the local increase in cytosolic K^+ increases the driving force for the permeant ion to flow outward, explaining the differences in P_o and slope conductance, respectively. The wide spread in the distribution of open probabilities seen for channels in PE is probably caused by native differences in Ca^{2+} sensitivity among individual BK channels (Moczydlowski *et al.*, 1985; Latorre, 1994). No such spread was observed for channels in PE/PS bilayers because inherent differences in Ca^{2+} sensitivity are masked by a maximum P_o of close to 1 (Fig. 4). In summary, the data are consistent with extensive exchange between native and planar bilayer lipid, and elimination of native lipid domain structure.

Despite the base-line differences in parameters of channel function, ethanol effects on BK channels inserted into PE bilayers are remarkably similar to that found for channels in PE/PS bilayers. Ethanol increased BK channel activity without altering the magnitude of the unitary current (Fig. 5). Although the degree of augmentation by 50 mM ethanol (4.3 ± 1.5 fold, $n = 6$) was lower than that observed for channels in PE/PS bilayers, this difference was not significant (Student's t test, $p > 0.05$). Open dwell-time histograms for the experiment (Fig. 5) were well fitted with two components in the presence or absence of ethanol, evidence for at least two open states available to the channel (Fig. 6). Ethanol (50 mM) increased the contribution of the long openings (τ_{o2}) to the total time spent in the open state, from 70.0 to 85.8%, without significantly affecting the durations of either the short (τ_{o1}) or long component (τ_{o2}) (Fig. 6). The mean open time (3.6 ms) was increased by 20.5% in the presence of ethanol (4.3 msec). Thus, the actions of ethanol on open times are qualitatively similar in PE and PE/PS bilayers.

Closed dwell-time histograms for the experiment shown in Fig. 5 were also well fitted with two components in the presence or absence of ethanol, evidence for at least two closed states available to the channel (Fig. 6). In comparison with the effects seen on the open times, the actions of ethanol on closed times were more complex. First, ethanol decreased the contribution of the long closings (τ_{c2}) to the total time spent in the closed state from 53.3 to 39.9% (Fig. 6). Second, whereas the duration of τ_{c1} was unaffected by ethanol, the longer closed component was shortened by alcohol (44.8 msec in the absence of ethanol and 13.3 msec in the presence of ethanol) (Fig. 6). Taken together, the mean closed time (24.0 msec) was reduced by 77.2% in the presence of alcohol, to 5.5

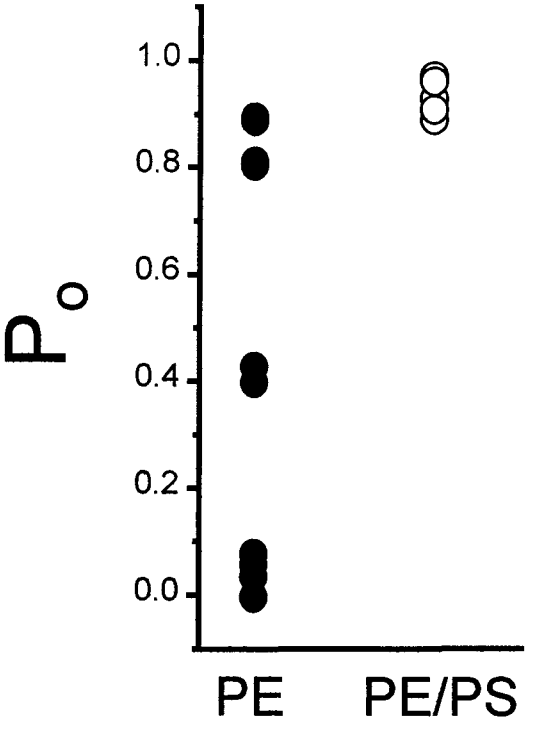

Fig. 4. Reconstituted BK channel activity is modulated by the bilayer lipid. The P_o of single BK channels under identical conditions (20 μM free $[Ca^{2+}]$, holding potential, −20 mV) are plotted according to the lipid mixture in which the channel was studied, either PE ($n = 10$) or PE/PS ($n = 5$).

TABLE 1

Comparison of ethanol effects on BK channel gating in various preparations (results are expressed as percentages of pre-ethanol values).

Measurements were performed before and during exposure (≤1 min) to 50 mM ethanol. Each value is the mean ± standard error of 6–20 determinations; each determination was obtained in a different patch/bilayer. In cases in which $n = 2$, both the average and the individual values (in parentheses) are given. In all cases, the free $[Ca^{++}]_{ic}$ was adjusted such that the NP_o was <0.5. The compositions of the solutions used are described in Materials and Methods or in the references cited.

	I/O Patches		T-tubule BK Channel	
	Neurohypophyseal BK channels[a]	α mslo (mbr5) in *Xenopus* oocytes[b]	PE/PS bilayer	PE bilayer
NP_o	457.8 ± 60.3	268.7 ± 27.8	825.6 ± 528.5	426.1 ± 154.3
t_o	128.7 (72.6, 175.4)	159.6 ± 22.4	106.3 (94.1, 118.5)	131.1 (120.5, 141.5)
t_c	58.3 (49.3, 67.2)	54.2 ± 4.8	24.5 (8.4, 39.7)	40.6 (22.8, 58.5)

NP_o, steady state activity; t_o, mean open time; t_c, mean closed time.
[a] From Dopico *et al.*, 1996.
[b] From Dopico *et al.*, 1998.

msec. These findings indicate that in a one component PE bilayer, as in PE/PS membranes, the enhancement of channel activity by ethanol is caused predominantly by a decrease in the overall amount of time a channel spends in closed states. This effect was found in two of two experiments in which detailed kinetic analyses were performed (Table 1). That ethanol's action on BK channel activity is observed in an uncharged one-component bilayer suggests that Ca^2-induced domains are not necessary for ethanol's actions on these channels.

Discussion

The finding that reconstituted ion channels are ethanol-sensitive, in a manner similar to that of native channels (Dopico *et al.*, 1996) and cloned channels expressed in oocytes (Dopico *et al.*, 1998), is strong evidence that the minimal elements required for drug action are the ion channel proteins and their surrounding lipids. In the simplest case, ethanol's action was observed in bilayers containing only PE. This observation indicates that other lipids, such as cholesterol and negatively charged phospholipids, whose presence is known to modulate BK channel activity (Moczydlowski *et al.*, 1985; Bolotina *et al.*, 1989; Chang *et al.*, 1995), and whose headgroups might be targets for ethanol modulation of ion channel function (Abadji *et al.*, 1994), are not necessary for ethanol's potentiation of BK channels. In general, incorporation of ion channels into planar bilayers likely removes a majority of the modulators of channel activity present *in situ*. Although previous evidence suggests that vertical and lateral organization of lipids in biological membranes plays an important role in the actions of alcohols on ion channels (Treistman *et al.*, 1987; Schroeder *et al.*, 1988; Wood *et al.*, 1989), our results suggest that complex lipid architecture is not obligatory for ethanol's potentiation of BK channels. Several pieces of evidence from this study and the literature indicate

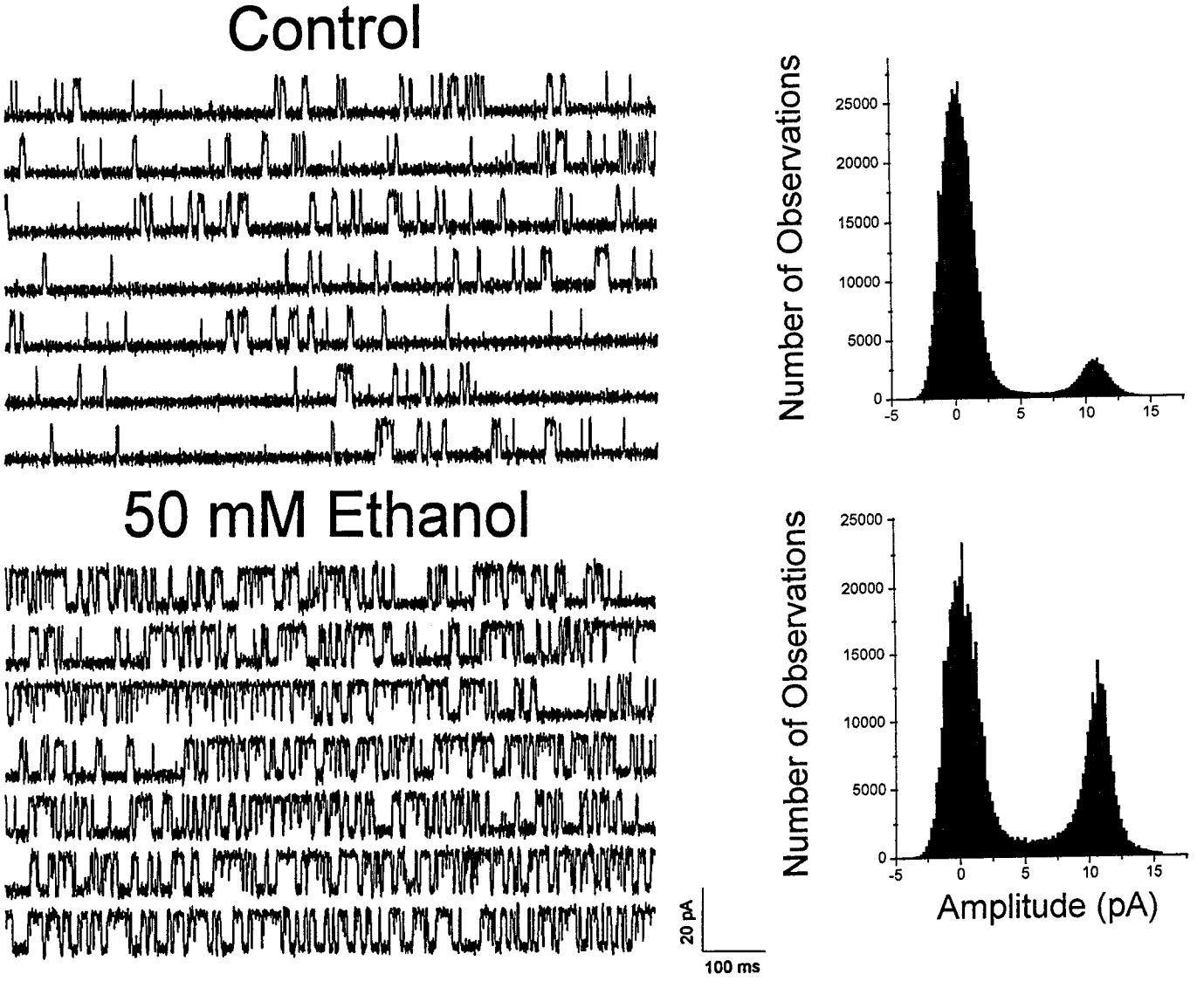

Fig. 5. Ethanol increases the activity of a single BK channel incorporated into a PE bilayer without affecting the magnitude of the unitary current. *Upward deflections* in the current record represent channel openings. In this experiment, the slope conductance was 356.5 pS in symmetric 300 mM KCl, the holding potential was +30 mV, and free $[Ca^{2+}]$ was ~ 8.1 μM. Ethanol (50 mM) increased the P_o from 0.097 to 0.313. The unitary current was 10.5 pA in the absence or presence of ethanol. The goodness of fit of the Gaussian distributions was $r = 0.9$ for control and $r = 0.87$ for ethanol.

that exchange between native lipid associated with the incorporated channel exchanges with the planar bilayer lipid. First, BK channels were modulated by the amount of fixed charge present in the bilayer, with channels in neutral PE bilayers exhibiting lower P_o and conductance values than channels in negatively charged PE/PS bilayers. These data are qualitatively identical to those of Moczydlowski *et al.* (1985) and indicate that replacement of native with bilayer lipid is extensive, possibly complete. This interpretation is buttressed by evidence in the literature. For example, the activity of nystatin, a peptide that requires ergosterol to form channels, is lost when membrane vesicles containing nystatin and ergosterol are incorporated into ergosterol-free membranes, presumably because of diffusion of ergosterol away from the channel complex (Woodbury and Miller, 1990). ESR studies of reconstituted nicotinic acetylcholine receptors indicate that the lipid at the protein/lipid boundary is relatively motionally restricted, but, nevertheless, can exchange with the bulk lipid. This exchange rate is rapid, on the order of 10^7/sec, and is slowed by high protein/lipid ratios (Ellena *et*

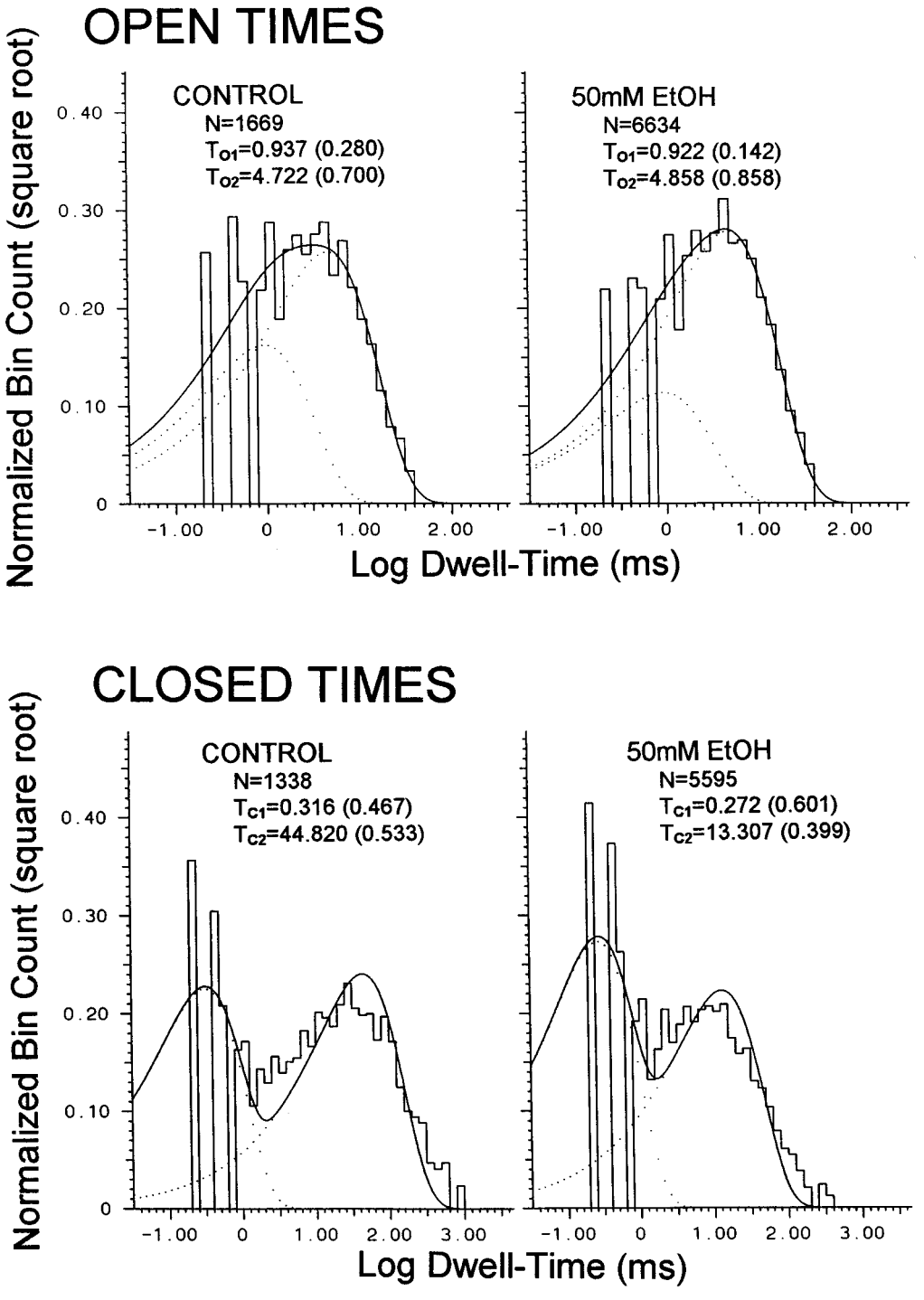

Fig. 6. Open and closed time distributions in the absence or presence of 50 mM ethanol for the experiment displayed in Fig. 5. Each panel shows the total number of events (*N*), the duration of each particular component (*T*, in msec), and the relative contribution of each particular component to the total fit (in *parentheses*). The number of events was normalized before applying a Sigworth-Sine transformation. *Dotted lines*, individual fitted components; *solid line*, composite fit.

al., 1983; Barrantes, 1989). We might expect this exchange to be faster in our system because the protein/lipid ratio is likely to be far lower than in biological membranes. Thus, the data strongly suggest that the bilayer lipid substitutes for the native lipid immediately surrounding incorporated channels, greatly reducing the level of transverse and lateral membrane heterogeneity.

It has been reported that neuronal (Reinhardt *et al.*, 1991), muscle (Toro *et al.*, 1990), and cloned (Esguerra *et al.*, 1994) BK channels remain functionally coupled to kinases and G proteins after incorporation into planar bilayers. Although it is unknown whether these modulators are present in our preparation, it is unlikely that these proteins are involved in ethanol's action here, because ATP and GTP, which are required for the function of these modulatory proteins, were absent from our bathing solutions.

The effect of ethanol on channel gating was strikingly similar for channels inserted into either PE/PS or PE bilayers. Ethanol increased the relative proportion of long openings, without changing their duration, which resulted in a mild increase in the channel mean open time. In addition, the drug markedly reduced the mean closed time, with this being the major determinant of ethanol-induced channel activation. These findings parallel those observed for the action of ethanol on neurohypophysial BK channels studied *in situ* (Dopico *et al.*, 1996), and cloned (α subunit, *mslo*) BK channels expressed in *Xenopus laevis* oocytes (Dopico *et al.*, 1998), suggesting that the activation of all BK channels by ethanol share site(s) and mechanism(s) of action. A comparison of ethanol's actions on gating properties of BK channels in these preparations and in the bilayers reported in this article can be seen in Table 1. The T-tubule BK channels examined in the present study were less ethanol-sensitive than neurohypophysial and mslo BK channels. Although we were not able to calculate a reliable EC_{50}, it is higher than the EC_{50} values of ~22 and 24 mM obtained in neurohypophysial and cloned (mslo) BK channels (Dopico *et al.*, 1996, 1998). T-tubule, neurohypophysial, and cloned (*mslo*) BK channels are not identical proteins, as evidenced by their differing sensitivity to activation by $[Ca^{++}]_{ic}$ and to charybdotoxin block (Latorre, 1994). Thus, quantitative differences in ethanol sensitivity might be attributed to differences in protein structure. Alternatively, differences might also result from the loss of a membrane-associated factor, whether lipid or protein, that influences ethanol's potency.

In conclusion, our results demonstrate that ethanol affects native BK channels *in situ*, cloned BK channels (*mslo*) expressed in oocytes, and reconstituted BK channels in a similar manner, suggesting that at least some site(s) and mechanism(s) of ethanol action for these channels are identical and are preserved in a simple model membrane. Because the bulk membrane environment is different for channels in planar bilayer, neurohypophysial, and oocyte membranes, and because the qualitative actions of ethanol were very similar in PS-containing and PS-deficient bilayers, these data are consistent with a site of action that is either on the protein itself or at a site very close, perhaps in the lipid-protein interface. Freely diffusible cytoplasmic factors, intracellular proteins, and complex lipid architecture are not obligatory for ethanol's potentiation of BK channel activity.

Acknowledgments

We wish to thank Drs. Irwin Levitan and Christopher Miller for their important assistance with setting up the planar bilayer experiments.

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