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Characterization of a Delayed Rectifier K^+ Channel in NG108-15 Neuroblastoma *X* Glioma Cells: Gating Kinetics and the Effects of Enrichment of Membrane Phospholipids with Arachidonic Acid

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Summary. A voltage-sensitive K^+ channel with characteristics of the delayed rectifier was studied in NG108-15 cells using the cell-attached patch-clamp technique. The primary conductance of the channel was 18 pS, but occasional openings to a subconductance state were observed. The average latency to first opening of the channel was about 4 msec. Based on about 20,000 channel openings, the open time probability density function (pdf) required at least three exponentials (time constants of about 0.2, 3 and 9 msec) to achieve an adequate fit to the data. The closed time pdf required at least six exponentials to describe the data (time constants ranging from 0.093 to 440 msec). Thus, the channel exists in at least three open and six closed states. The ensemble average describing the inactivation of the channel was well fit by two exponentials with time constants of 170 msec and 4.2 sec. To examine the effect of changes in membrane lipid composition on the properties of the channel, the phospholipids of the cells were enriched with polyunsaturated fatty acids. In patches from 20:4-enriched cells the conductance, mean first latency, and open-time pdf were similar to control cells. However, the open state probability was increased from 0.25 to 0.44 and the mean closed time was decreased from 20 to 9 msec. The closed time pdf exhibited a higher proportion of closing events associated with short time constants, i.e., the probability of the channel closing into a long-lived closed state was decreased. The decay phase of the ensemble average also was changed; the proportion of the curve described by the slower time constant was almost doubled. Thus, the delayed rectifier from NG108-15 cells can exist in at least three open and six closed states, and changes in membrane lipid composition may have subtle effects on the gating kinetics of the channel.

Key Words K^+ channels · delayed rectifier · neuroblastoma cells · membrane lipids · voltage-gated channels

Introduction

Several different types of voltage-activated K^+ channels have been identified and studied using both gating current and single channel measurements [for reviews *see* Bezanilla (1985) and Latorre, Coronado and Vergara (1984)]. One of the most thoroughly studied channel types is the delayed rectifier; the single-channel properties of these channels have been determined both in native membranes (Conti & Neher, 1980; Llano & Bezanilla, 1985) and after reconstitution into black lipid membranes (Coronado, Latorre & Mautner, 1984). In both cases the delayed rectifier displays a conductance of 15–20 pS with bursting kinetics resulting from multiple closed states. The complexity of the gating process has been confirmed by the gating current measurements of White and Bezanilla (1985).

Single-channel measurements also have detected a variety of different K^+ channels in cultured mammalian cells. Ebihara and Speers (1984), using PCC4 embryonal carcinoma cells, described a channel of conductance 10 pS, exhibiting multiple closed states, which they identified as a delayed rectifier. Misler and Falke (1985) detected a 20–25 pS K^+ channel in NIE-115 neuroblastoma cells. More recently, Rogawski (1985) has described a 15–20 pS voltage-activated K^+ channel in cultured hippocampal neurons, and four different K^+ channels were described by Hoshi and Aldrich (1986) in PC12 pheochromocytoma cells. Finally, Solck and Aldrich (1986) observed a 15–20 pS K^+ channel in dissociated *Drosophila* CNS neurons. Thus, as was predicted by whole cell and extracellular measurements, many different types of single K^+ channels do occur. The channel corresponding most closely to the classical delayed rectifier has a conductance of 15–20 pS and complex gating kinetics with multiple closed states. Since these gating properties will ultimately control the amount of current flowing through the channels, it is important to obtain a detailed understanding of the gating of the channels

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and factors which may influence or alter the gating process. A logical question to ask is whether the gating characteristics result solely from the inherent properties of the channel protein or also are influenced by the nature of the lipid environment in which the channels reside.

To study the gating process of voltage-activated K^+ channels and the role of membrane lipids in gating, we have used a simple, well-characterized mammalian neuronal tissue culture cell line, the NG108-15. This neuroblastoma *x* glioma hybrid cell line expresses many of the properties of normal neurons (for reviews *see* Hamprecht, 1977; McGee, 1980), and under appropriate conditions the cells form functional neuromuscular synapses in culture (Nelson, Christian, & Nirenberg, 1976; Christian et al., 1977). After differentiation by conditions which elevate intracellular cAMP, many of the cells are electrically excitable based on studies with intracellular recording techniques. Of particular interest, the membrane lipid composition of the cells is easily manipulated simply by growing the cells in the presence of specific lipid precursors. For example, under normal conditions oleic acid, with a single double bond, is by far the predominant unsaturated fatty acid in the membrane phospholipids. Addition of low concentrations of polyunsaturated fatty acids to the growth medium causes a profound increase in the phospholipid content of polyunsaturated fatty acids at the expense of oleic acid (McGee, 1981; Saum, McGee & Love, 1981; Love, Saum & McGee, 1985). Recently, we have shown, using intracellular recordings, that exposure of the cells to these unsaturated fatty acids caused a decrease in the rate of rise of Na^+ action potentials generated by the cells but not Ca^{2+} action potentials (Love et al., 1985). The decreased rate of rise appeared to correlate with a decreased number of voltage-activated Na^+ channels, but the recording techniques used were not sufficiently sensitive to determine if the properties of the individual channels were altered. We report now a continuation of these studies with a characterization of a voltage-activated K^+ channel on NG108-15 cells and an initial study of the effects of membrane enrichment with arachidonic acid (20:4) on the gating properties of the channel.

Materials and Methods

Growth of Cells and Lipid Modification

Neuroblastoma *x* glioma hybrid cells (NG108-15) were grown in Dulbecco's modified Eagle's medium and treated with 1 m*M* N^6, $O^{2'}$-dibutyryl cyclic AMP for 5–10 days as previously described (Saum et al., 1981; Love et al., 1985). The method for enrichment of cells with arachidonic acid (20:4) also has been described (McGee, 1980). Briefly, a sterile solution of BSA to which 20:4 had been complexed was added to the culture medium (at the time of subculture to 35-mm dishes) such that the final concentration of 20:4 was 40 μM. Under these conditions the free (unbound) concentration of 20:4 is below 1 μM. Four to eight days later cells were used for patch-clamp recording after replacing the growth medium with a balanced salt solution, pH 7.4, containing (in mM) 150 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 1.2 $MgSO_4$, 10 glucose and 10 HEPES.

Patch Clamp Recordings of Voltage-Sensitive K^+ Channels

Standard patch-clamp electrodes were filled with the same salt solution as was bathing the cells unless indicated. All recordings were obtained in the cell-attached configuration and measured as outward currents. (Detached patches almost always exhibited one or many spontaneous channels, which usually prevented analysis of voltage-activated channels.) Electrical activity was measured with a List EPC 7 patch-clamp amplifier in the voltage-clamp mode, and data was recorded on a Sony VCR using a pulse code modulation unit modified to give a uniform frequency response from DC to 20 kHz (Lamb, 1985).

The resting membrane potential (*Vm*) for each cell was determined at the end of the experiment using the current clamp mode, by giving a brief, strong suction on the electrode to break the patch and immediately recording *Vm*. This technique was successful about 70% of the time, but the tight seal sometimes broke during the procedure. The average *Vm* obtained for control cells was -52 ± 2 mV (SEM, $n = 14$) and -53 ± 1 mV ($n = 39$) for 20:4-enriched cells. These values were used in calculations of absolute patch potentials.

Many patches contained one or more of the voltage-sensitive K^+ channels. Initial experiments showed them to be partially or totally inactivated at normal resting membrane potentials. To remove inactivation the patch potential was hyperpolarized to -120 mV, which was adequate to ensure removal of inactivation in all cases. Generally, depolarizations to patch potentials of -50 mV or less failed to elicit channel openings, whereas greater depolarizing potentials produced immediate responses in patches containing voltage-sensitive K^+ channels. To determine channel conductance, four to six depolarizing steps to patch potentials of -40 to $+30$ mV were applied for 10 sec separated by 10 sec of repolarization.

Four patches, two each from control and 20:4-enriched cells, contained a single voltage-sensitive K^+ channel and were sufficiently stable to permit long recordings and kinetic analysis. Data were collected using 40 depolarizing steps to 0 mV applied for 20 sec, separated by 10-sec periods of repolarization. Under best conditions patches were held for 2 hr or more with no apparent change in their properties.

Data Reduction and Analysis

Recorded data were filtered with a cutoff frequency of 3 kHz, and digitized and stored using a PDP 11/34 computer. Digitization intervals of 200 and 50 μsec were used for amplitude measurements and kinetic measurements, respectively. Blocks of digitized data were sequentially displayed on a Tektronix video storage terminal. Channel amplitudes were measured for at least 30 individual openings. Kinetic measurements were performed

using a modification of the dual threshold crossing algorithm previously described (Gration et al., 1982; Kerry et al., 1986). The modification provided an interactive element whereby the user was able to verify each channel event and to intervene where necessary. The stimulus artifact was used to define time = 0. Channel openings and closings were measured manually until the capacitative currents had dissipated and a stable baseline was attained. In about 18% of the depolarizations the latency (time to first opening) was too short to be resolved from the artifact, although it was obvious that the channel was already open. This minimum resolution time was estimated to be about 1 msec, but the latencies were recorded as 0 msec.

After stabilization of the baseline, channel openings and closings were measured automatically. Thresholds for opening and closing were set at 50 and 25% of peak conductance, respectively. Correction for baseline drift was made periodically during the course of analysis. Using this procedure, closings and openings of duration greater than 150 μsec were reliably measured. An average of about 10,000 channel openings from 40 depolarizations was obtained from each of the four patches containing a single voltage-sensitive K^+ channel. The kinetic analyses presented are based on these four patches.

It is useful to define some terms used in the analysis of channel kinetics (*see* Fig. 1). A sweep is the period between the onset (t = 0 sec) and the end (t = 20) of depolarization. An experiment consists of an ensemble of approximately 40 sweeps. Within a sweep, the latency (t_{lat}) is the time from t = 0 until the first channel opening. A cluster is the group of channel events initiated by the first opening and terminated by the final (long) closing, its duration is denoted by t_{cls}. For a given ensemble (experiment) the mean latency $\langle t_{lat} \rangle$ and mean cluster length $\langle t_{cls} \rangle$ were obtained by averaging across all sweeps. The mean values of other overall measurements (ie., m_o, m_c, and p_o) were all derived from dwell times within clusters for a given ensemble and are therefore derived from about 10,000 events.

Open and closed time histograms were derived from all channel open and closed times, exclusive of the latency and the final closed time. In constructing histograms, exponentially increasing time intervals were used to adequately represent both long and short events in the same distribution. All distributions are shown as probability density functions (pdfs), obtained by normalizing histograms to unit area (Colquhoun & Sigworth, 1983). Ensemble averages of the time dependence of channel open probability (Aldrich & Yellen, 1983) were derived from the lists of channel open and closed times, and were also sampled using exponentially increasing time intervals. The open and closed time distributions and the decay phases of the ensemble averages were all fitted using standard curve-fitting algorithms. The latency pdfs were fitted using the maximum likelihood procedure (Everitt & Hand, 1981; Colquhoun & Sigworth, 1983).

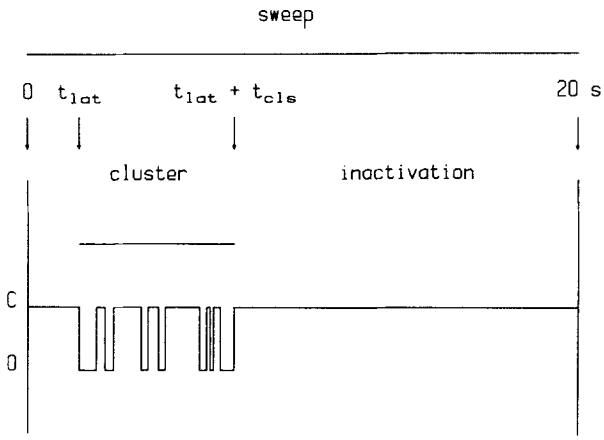

Fig. 1. Cartoon of a single depolarization taken from an ensemble. The open and closed states of the channel are indicated by O and C, respectively. The horizontal bars indicate the lengths of the sweep and of the cluster of channel activity (t_{cls}). The latency (t_{lat}) is also indicated. The remaining overall kinetic parameter (*see* Table 1) are defined as follows

$$m_o = T_o/N;\ m_c = T_c/n;\ P_o = m_o/(m_o + m_c)$$

where T_o is the total time spent open during clusters for the entire ensemble, T_c is the total time spent closed during clusters (i.e., the total time spent closed excluding the latency and inactive period) for the entire ensemble, and N is the total number of single-channel openings for the ensemble

Results

Enrichment of Membrane Phospholipids with Arachidonic Acid

The phospholipid fatty acid composition of the particular cells used in these studies was not determined. Many previous experiments had shown that the fatty acid composition of control cells and the changes induced by exposure to exogenous fatty acids were highly reproducible during several years of experimentation involving a variety of sources and batches of fetal bovine serum as well as many different batches of cells. Under the conditions used in these experiments, phospholipids from control cells would contain about 39% oleic acid (18 : 1), 5% arachidonic acid (20 : 4) and 11% other polyunsaturated fatty acids. The exposure to 20 : 4 decreases the content of 18 : 1 to about 18%, increases 20 : 4 to 21% and has little effect on the amount of other polyunsaturated fatty acids (12%) (Saum et al., 1981). Thus, cells exposed to 20 : 4 are highly enriched with 20 : 4, predominantly at the expense of 18 : 1.

General Description of the Voltage-Sensitive K^+ Channel

Shown in Fig. 2 are representative traces from control and 20 : 4-enriched patches. Both complete sweeps (digitized at 3-msec intervals to allow display) and expanded regions are presented. As can be seen, depolarization resulted in rapid initiation of unitary conductance channel activity (channels open downward). The activity usually persisted for several seconds until appearing to enter an inactive state. Continued depolarization beyond the 20 sec

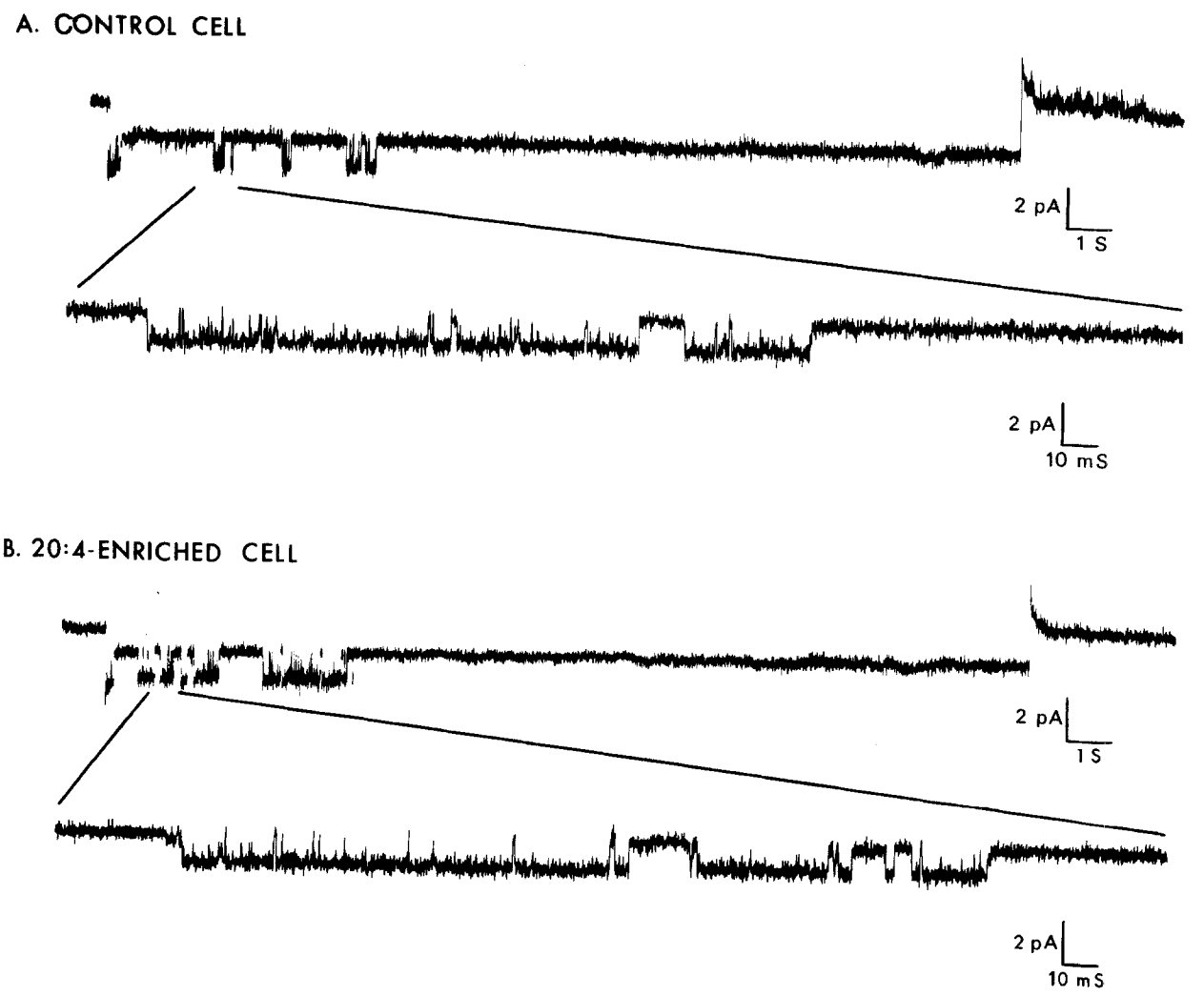

Fig. 2. Two recordings (*A*, control; *B*, 20 : 4-enriched) of a single-channel opening in response to step depolarization (*see* Materials and Methods). In both cases, after a short lag (latency), there is a cluster of channel openings (downward deflections), followed by a long inactive period extending to the end of the sweep. The expanded sections show the complex kinetics within each cluster

pulse duration failed to induce any additional channel openings. The duration of the clusters ranged from a few msec to several sec. Occasionally the channel was still in the open state when the pulse ended. Inspection of the expanded region revealed complex kinetic behavior within a cluster of openings. Although the unitary conductance was predominant, occasional sub-conductances were observed (less than 3% of the channel openings). An example of a particularly high frequency of subconductances is shown in Fig. 3. These openings were not believed to represent a different channel because they were never observed in the absence of, or adding to, the full conductance state. The subconductance state was seen in both control and 20 : 4-enriched patches and no obvious difference in their frequency was observed. However, a detailed analysis of their frequency was not made. For the purposes of kinetic analysis both full and partial conductances were treated as channel openings.

The voltage dependence of channel gating was investigated by application of depolarizing pulses (10 sec duration) to various patch potentials (V_d) from a holding potential of -120 mV. Shown in Fig. 4 are the summed records from three depolarizations, separated by 10-sec repolarizations, to the indicated V_d for both a control and 20 : 4-enriched patch containing a single channel. In both cases, the degree of channel activation increased with greater depolarization. At $V_d = -23$ mV, little or no channel activity was detected. At about 0 mV, initial channel activity is followed by prolonged inactivation. For depolarizations to $V_d \geq +30$ mV, channel inactivation is incomplete, the channel remaining active throughout the 10-sec pulse.

Voltage-dependence of gating was studied in

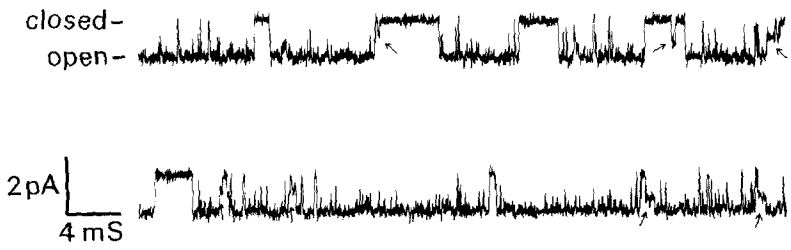

Fig. 3. Examples of subconductance states of the voltage-sensitive K^+ channel. During clusters of single-channel openings, excursions to a subconductance level were occasionally observed. Although brief, these excursions were of sufficient duration to be easily resolved from full conductance openings truncated by the frequency response of the amplifier

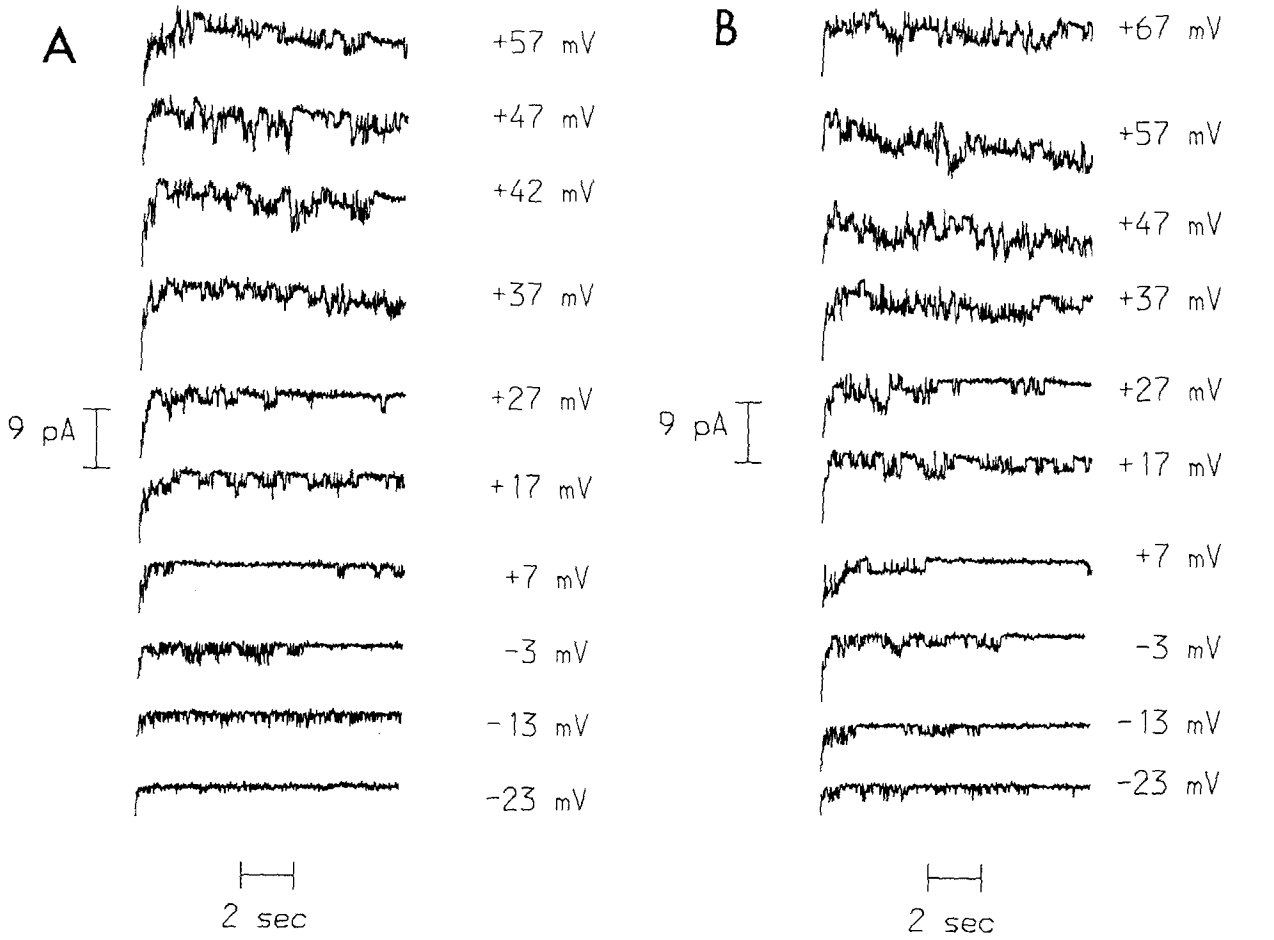

Fig. 4. Channel openings in response to depolarizing pulses, to membrane potentials (V_d) as given on the right-hand side of the traces, for a control (*A*) and 20 : 4-enriched (*B*) patch. The traces represent the sum of those obtained in response to the application of three consecutive 10-sec depolarizations separated by 10-sec repolarization. Thus, each trace may be regarded as that which would be obtained from a patch containing three K^+ channels

more detail for the control patch. Ensemble averages of the single-channel currents were derived from 40 pulses each for $V_d = +7$ and $+37$ mV (Fig. 5). (Note that these ensemble averages should be distinguished from the ensemble averages obtained from the idealized single-channel records which are used in the analysis of channel kinetics described below.) The major difference between the two potentials is the incomplete inactivation of the channel at the more depolarized potential. At $V_d = +7$ mV the current eventually decays to $P_o = 0.0$, whereas at V_d +37 mV the ensemble average levels out at about $P_o = 0.25$. A similar result was obtained for the 20 : 4-enriched patch (*not shown*).

Analysis of Current-Voltage Relationships

The current-voltage relationship for the voltage-sensitive K^+ channel was investigated to determine the single channel conductance and reversal poten-

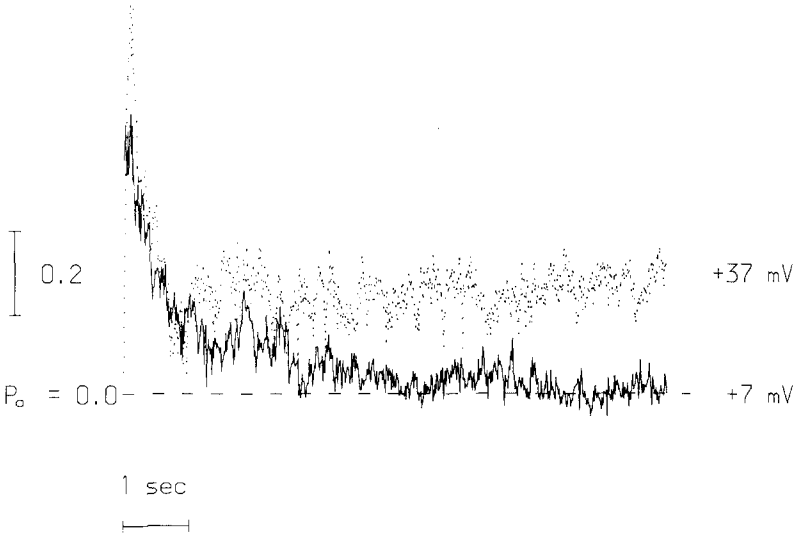

Fig. 5. Ensemble average currents obtained for depolarizations to V_d = +7 mV (solid line) and V_d = +37 mV (broken line) for a control patch containing one K^+ channel. The number of traces averaged were 46 and 48, respectively. The traces have been corrected for capacitative transients by subtraction of fits to null sweeps. They have been normalized by dividing by the single-channel current amplitude to give estimates of $p_o(t)$

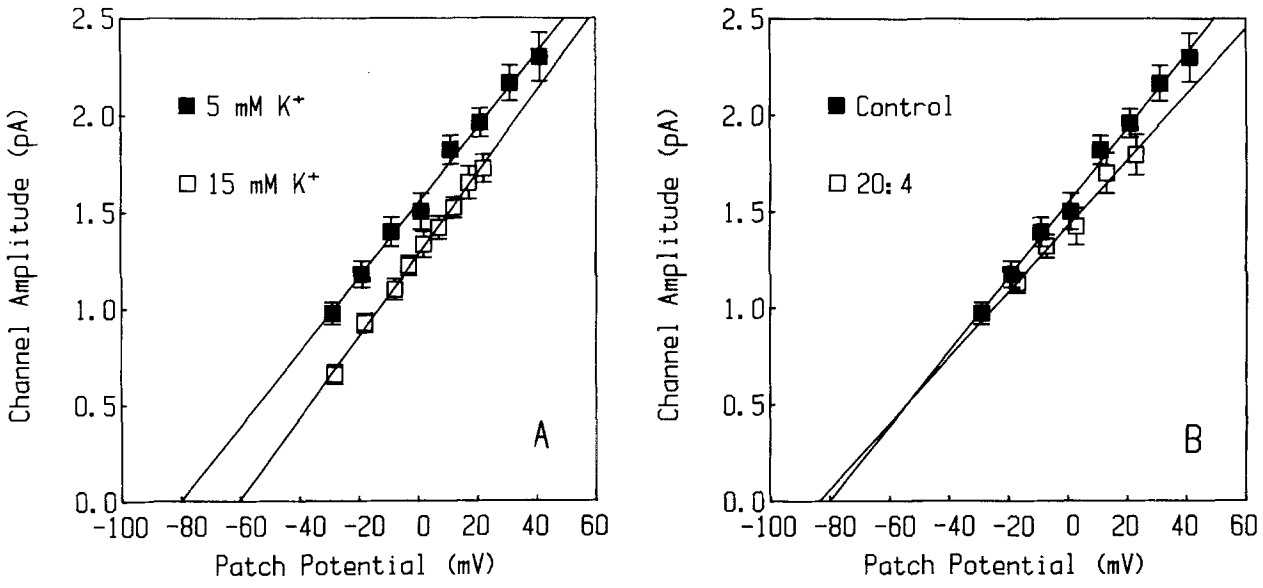

Fig. 6. Current-voltage relationships for the voltage-sensitive K^+ channel. The absolute patch potential was determined by breaking the patch at the end of the experiment and measuring the resting membrane potential as described in Materials and Methods. (*A*) Current-voltage relationships for cell-attached patches in which the pipette contained 5 or 15 mM K^+. The elevated concentration of K^+ shifted the reversal potential in the manner expected for a K^+-selection channel (*see* text). The single-channel conductances were 19.3 pS and 21.4 pS for 5 and 15 mM K^+, respectively. (*B*) Current-voltage relationships for channels from control and 20 : 4-enriched cells. Enrichment with 20 : 4 changed neither the conductance nor the reversal potential of the channel

tial. Representative data are shown in Fig. 6*a*. A linear relationship was observed over the range of voltages where channel activity could be observed. The slope conductance for the channel was 19.3 pS with a reversal potential of −80 mV. As would be expected for a K^+ channel, elevation of the K^+ concentration in the pipette to 15 mM decreased the reversal potential to the extent predicted by the Nernst equation but had little effect on the single-channel conductance. Enrichment with 20 : 4 had no detectable effect on the single-channel conductance. Representative *I/V* relationships are shown in Fig. 6*b*. The mean conductances for five patches each for control and 20 : 4-enriched cells were 18.2 ± 0.9 and 17.5 ± 1.7 pS, respectively. Thus, even a substantial change in the degree of unsaturation of membrane phospholipid did not alter the ion-conducting properties of the channel.

Table 1. Summary of overall kinetic parameters

Parameter	Control		20:4	
	Patch 1	Patch 2	Patch 1	Patch 2
Number of events	9,593	10,062	9,139	10,133
Mean latency $\langle t_{lat} \rangle$ (msec)	3.28	4.23	4.66	3.35
Mean cluster length (sec)	4.32	8.25	3.74	4.73
Mean open time m_o (msec)	5.4	7.8	7.3	7.9
Mean closed time m_c (msec)	14.9	26.6	8.20	10.8
Open probability p_o	0.27	0.23	0.47	0.40

The indicated parameters were determined for each of the four individual patches as described in Materials and Methods and the legend to Fig. 1.

Overall Characterization of Single-Channel Kinetics

An overall comparison of the kinetic properties of the channel in the control and 20:4-enriched cells is presented in Table 1. Activation is relatively rapid, all ensembles having $\langle t_{lat} \rangle$ of about 4 msec. Comparison of the values of $\langle t_{lat} \rangle$ showed that there was little variation between experiments, and that 20:4 enrichment did not alter the channel-activation kinetics. The mean open times also were similar for all four ensembles, suggesting that the open-state kinetics of the channel were not perturbed by 20:4 enrichment. The main difference caused by 20:4 enrichment lay in the closed channel kinetics. This is best revealed by comparing p_o (the probability of the channel being open within a cluster) for the four patches. The effect of 20:4 enrichment was an approximate doubling of p_o, from about 0.25 to 0.45, which resulted from the shorter mean closed time for the 20:4-enriched cells. The increase in p_o also can be readily seen by comparison of the records shown in Fig. 2.

Investigations of single channel properties of other ion channels [e.g., Na^+ channels (Horn, Vandenberg & Lang, 1984), cardiac Ca^{2+} channels (Cavalie, Pelzer & Trautwein, 1986)] have revealed slow gating behavior, seen as clustering of high and low activity sweeps. We have tested for such behavior using the one-sample runs test (Siegel, 1956), which allows one to determine if the degree of clustering deviates from what one would expect on a random basis. Sweeps were classified as high or low activity based on the number of events, and no unexplained sweep-to-sweep variability was found (*data not shown*). We are therefore confident that there was no slow gating behavior of the channel revealed by these experiments. Given the absence of any apparent slow gating behavior, all sweeps of an ensemble may justifiably be used during further analysis.

Latency Distributions

Analysis of the latency distribution provides information about the opening pathway of the channel (Aldrich, Corey & Stevens, 1983; Cavalie et al., 1986). In particular, the observation of a maximum in a latency pdf is evidence for there being more than a single closed state preceding the initial opening of the channel. The latency pdf for the control and 20:4-enriched patches are shown in Fig. 7. As can be seen, both pdf are biphasic, rising to a distinct maximum at about 2.5 msec. Fitting the equation;

$$f_L(t) = (\tau_2 - \tau_1)^{-1}[\exp(-t/\tau_2) - \exp(-t/\tau_1)]$$

to the latencies, using a maximum likelihood procedure (Colquhoun & Sigworth, 1983) gave time constants of $\tau_1 = 1.4$ msec for the rising phase and $\tau_2 = 3.1$ msec for the declining phase (control) compared to 2.0 and 2.8 msec for the 20:4-enriched patches. Such a form for the latency pdf would be expected if two closed states preceded the open state (Patlak & Horn, 1982)

$$C_1 \leftrightarrow C_2 \leftrightarrow O.$$

Thus, we conclude that at least two closed states precede the channel opening. Furthermore, as suggested by the mean latencies, it is apparent that 20:4-enrichment does not alter the activation kinetics of the channel. If more than two closed states were passed through prior to opening, one would expect a similar form for the first latency pdf, but with a multi-exponential declining phase. There are

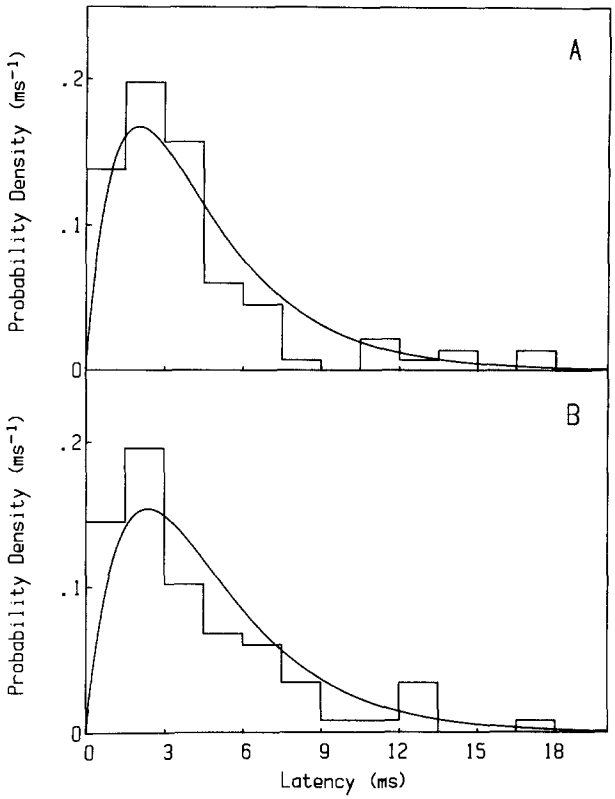

Fig. 7. Latency probability density functions for (*A*) the control and (*B*) the 20 : 4-enriched patches. In both cases the histograms represented the observed pdf, averaged over both patches for each condition, while the curve represents the biphasic function described in the text

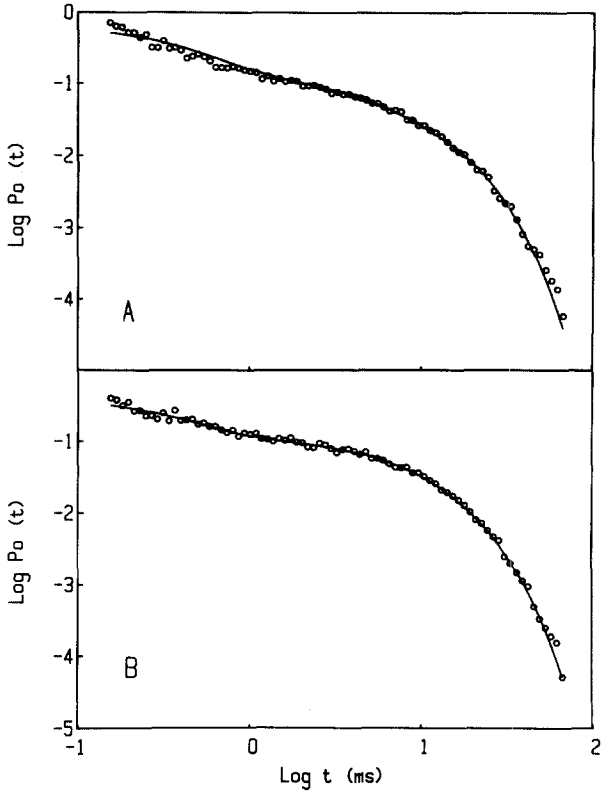

Fig. 8. Open time probability density functions for the control (*A*) and 20 : 4-enriched (*B*) patches. The distributions are the averages across both patches for each condition, and the curves represent the parameters given in Table 2. The data are displayed using a double logarithmic plot

suggestions that this may be the case, but more data will be required before this possibility can be examined.

Open and Closed Time Distributions

Analysis of open and closed time distributions provides information concerning the number of kinetically distinct open and closed states, respectively, of the channel (e.g., Kerry et al., 1986). An open or closed time pdf takes the form of the sum of N_o or N_c exponential components, where N_o and N_c is the number of kinetically distinct open and closed states, respectively.

The open time pdf was estimated from histograms of all openings of an ensemble. For both the control and the 20 : 4-enriched cells, the open time pdf corresponded to the sum of three exponential components, i.e., $N_o = 3$, as shown in Fig. 8. The relevant open time parameter estimates are given in Table 2. In fitting the pdfs events of duration less than 0.15 msec were not taken into account, as not all such events were detected.

The short openings (τ_1 0.2–0.3 msec) accounted for about 18% of the total channel openings in control patches. Enrichment with 20 : 4 appeared to decrease the proportion of short openings; additional experiments will be required to confirm or extend this observation. The relative proportion of longer openings did not appear to be altered by enrichment with 20 : 4, and the time constants for all three exponentials were unaffected within the reliability of the measurements. Thus, enrichment with 20 : 4 may have an effect to decrease the probability of the channel entering a short-lived open state.

Closed time histograms were derived in a similar manner, excluding the first and last closing of each sweep. In contrast to the open time pdf the closed time pdf ranged over nearly three orders of magnitude, from 0.15 msec to 5 sec. As may be seen by examination of Fig. 9, the closed time pdf was complex, requiring at least six components for a satisfactory fit to be obtained. The value of $N_c = 6$ implies that the voltage-sensitive K^+ channel may exist in at least six kinetically distinct closed states under the conditions of these experiments. At this

Table 2. Summary of open time distribution parameters

i	α_i		τ_i	
	Control	20 : 4	Control	20 : 4
			(msec)	
1	0.180	0.097	0.215	0.303
2	0.224	0.242	2.90	5.05
3	0.595	0.661	8.90	9.23

Open time pdfs were modelled by the equation

$$f_o(t) = \sum_{i=1}^{3} (\alpha_i/\tau_i) \exp(-t/\tau_i)$$

as described in the methods section. The values given are for the average distributions for each condition.

Table 3. Closed time distribution parameters

i	α_i		τ_i	
	Control	20 : 4	Control	20 : 4
			(msec)	
1	0.660	0.764	0.0950	0.0854
2	0.175	0.142	0.353	0.321
3	0.0679	0.0424	1.41	1.23
4	0.0576	0.0327	7.42	6.42
5	0.0269	0.0159	49.2	30.3
6	0.0127	0.0041	369	247

Closed time pdfs were modelled by the equation

$$f_c(t) = \sum_{i=1}^{6} (\alpha_i/\tau_i) \exp(-t/\tau_i)$$

as described in Materials and Methods. The values given are for the average distributions for each condition.

stage of the analysis it is not possible to explore the relationship between these closed states and those which contribute to the observed delay in the activation of the voltage-sensitive K^+ channel.

Regardless of the difficulties of fitting large numbers of exponential components (Landaw & DiStefano, 1984) it is clear from visual comparison of the pdfs that 20 : 4 enrichment results in shorter channel closings within the clusters. Examination of the parameter estimates (Table 3) reveals that the difference lies in a decrease in the proportion of the longer components (time constant >1 msec). Unfortunately, given the problems already alluded to, it is difficult to be more specific than this. However, it does appear that 20 : 4 enrichment brings about an overall shift in the closed channel kinetics within the clusters of channel openings. As pointed out earlier, this shortening of channel closings results in an increase in p_o.

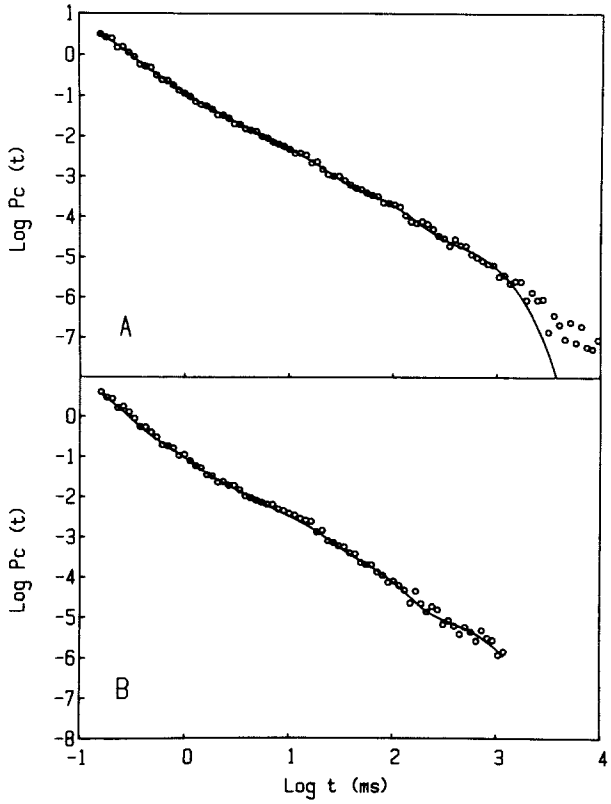

Fig. 9. Closed time probability density functions for the control (*A*) and 20 : 4-enriched (*B*) patches. As with the open time distributions, the average distributions are shown. The curves represent the 6-component functions described in Table 3. The data are displayed using a double logarithmic plot

Ensemble Average Analysis

Ensemble averaging can be used to give information on the probability of the channel being open as a function of the time elapsed since onset of depolarization ($P_o(t)$). This is the single channel equivalent of measuring the current flowing through many channels as a function of time (Aldrich & Yellen, 1983), and so provides an overview of the gating of the channel. Ensemble averages for the control and 20 : 4-enriched patches are shown in Fig. 10 plotted in the normal linear fashion. The data are replotted in a semi-logarithmic manner in Fig. 11 to more clearly represent both the rising and falling phases of the ensemble averages. As can be seen from Fig. 11, the ensemble averages consist of activation followed by a biphasic decay. The rising phases of both ensemble averages were satisfactorily fit by the cumulative latency distributions derived from the latency pdfs discussed above (Cavalie et al.,

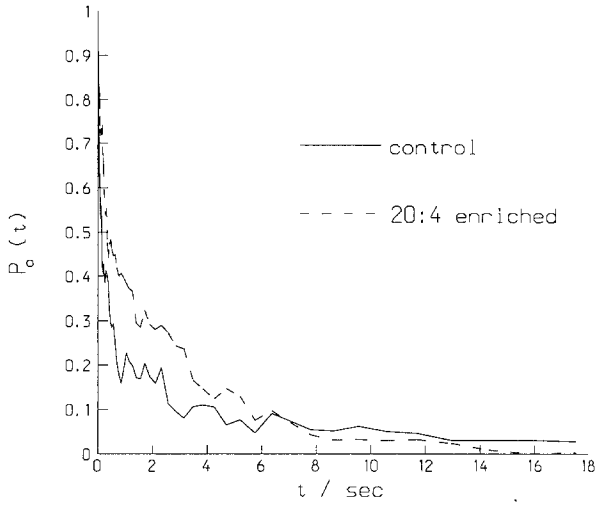

Fig. 10. Ensemble averages, derived from idealized single-channel records, for the control (solid lines) and 20:4-enriched (dashed lines) patches. The data are the averages for each condition and are displayed in normal linear form

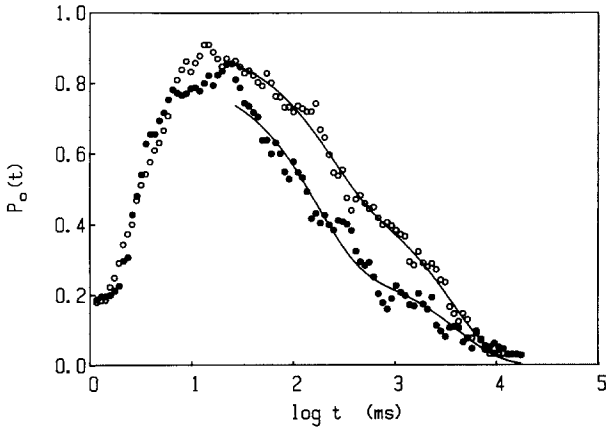

Fig. 11. Same data as Fig. 10 plotted in semi-logarithmic form for control (filled circles) and 20:4-enriched (open circles) patches. The lines represent the calculated bi-phasic decays defined in Table 4

1985). As can be seen, there were no differences between control and 20:4-enriched patches in this phase of the curves.

The most dramatic effect of enrichment with 20:4 can be seen in the substantial delay in the decay of the ensemble average. Channels in membranes from 20:4-treated cells appeared to remain open for a longer period. The data presented in Figures 10 and 11 are for the pooled data from each treatment. Analysis of individual patches showed that the two control patches agreed very closely with each other as did the 20:4-enriched patches; both patches from 20:4-enriched cells showed the

Table 4. Ensemble average decay parameters

i	α_i		τ_i	
	Control	20:4	Control	20:4
			(msec)	
1	0.551	0.421	170	214
2	0.268	0.484	4240	3700

The decay phases ($t > 25$ msec) of the ensemble average data were interpreted in terms of two component exponential functions

$$P_o(t) = \alpha_1 \exp(-t/\tau_1) + \alpha_2 \exp(-t/\tau_2).$$

The values given correspond to the averages over both patches for each condition.

same prolongation of opening. The decay phase of the ensemble averages was fit by the sum of two exponentials; the time constants and proportions of these exponentials are given in Table 4. The time constants that describe the decay were very similar between the two conditions, but the proportion of the curve corresponding to the slow time constant was approximately doubled in the 20:4-enriched patches. By calculating the areas under the decay phases of the two curves (beginning at 25 msec) it was determined that the differences would amount to a 1.5-fold increase in the total ionic charge passing through the channels of the 20:4-enriched patches. These results provide initial evidence suggesting that the gating properties of the voltage-sensitive K^+ channel may be sensitive to the lipid composition of the membrane.

Discussion

Relationship to other K^+ Channels

This paper describes the properties of single K^+ channels from NG108-15 cells and initial results suggesting possible effects of changes in membrane lipid composition on these properties. The single-channel conductance is 18 pS, with an occasional opening to a subconducting state. The channel inactivates slowly and exhibits complex bursting kinetics while open. To what extent do the properties of this channel resemble those of other voltage-activated channels that have been described?

Activation upon depolarization, followed by slow inactivation, suggests that the channel resembles the delayed rectifier. As discussed in the introduction, the classical delayed rectifier channel has a

conductance of about 15 to 20 pS (Conti & Neher, 1980; Coronado et al., 1984), which would correspond well with that of the NG108-15 channel. The complexities of the gating kinetics of the NG108-15 K^+ channel, in particular the existence of multiple closed states, also are consistent with current views of the gating of the delayed rectifier (Latorre et al., 1984; Bezanilla, 1985).

Perhaps the closest resemblance, at least in terms of gating kinetics, is to the 11-pS K^+ channel of PC12 cells (Hoshi & Aldrich, 1986). This channel inactivates slowly (time constant 300 msec at +40 mV), and has at least two open states and three closed states. It therefore has complex gating kinetics, as does the NG108-15 channel.

An interesting property of the PC12, 11-pS channel [and of several other voltage-gated channels (Horn et al., 1984; Cavalie et al., 1986)] is clustering of low activity sweeps—i.e., depolarizations during which few or no channel openings are detected. Such clustering is thought to be due to slow gating transitions—the 'hibernating' states of Horn et al (1984). We looked for clustering of low activity sweeps, using the one sample runs test (Siegel, 1956) but failed to detect them. This may reflect an important feature of the design of the experiments described in this paper, namely the use of particularly long duration depolarizations (20 sec) and repolarizations (10 sec). One reason for employing such a design was to permit direct investigation of the slow component(s) of channel inactivation. It is conceivable that the 300-msec time constant for inactivation of the 11-pS PC12 channel corresponds to the first (200 msec) component of inactivation of the NG108-15 channel. Furthermore, the use of extended sweep durations may well have removed the clustering of low activity sweeps. Indeed, it seems likely that one could vary sweep duration to estimate the time constants of the processes underlying clustering of activity.

Kinetics of the Channel in Control Cells

One aim of investigating the gating kinetics of the NG108-15 K^+ channel was to establish a model of gating which can be used to study the effects of altered membrane lipid composition on the dynamics of this membrane protein. The results reported here are not sufficient to permit proposal of a definitive model, but they do allow description of some of the constraints that such a model must satisfy.

Analysis of open time distributions was relatively straight forward. The open time pdf is made up of three exponential components. Attempts to fit two components did not fully account for the longest openings of the distribution. The time constants (0.21, 2.9 and 8.9 msec) are reasonably well separated, presenting no real problems with correlations of estimates. Consequently, one can be reasonably certain that there are at least three kinetically significant open states of the channel. It is unlikely that one of these open states would represent the subconductance state which was occasionally observed, because the frequency of observing the latter was much lower than the proportional contribution of any of the three open states.

Analysis of closed time distributions proved more difficult. Estimating the number of components in a mixture of exponential distributions is not straightforward (Everitt & Hand, 1981), particularly when the number is large. However, attempts to fit the observed closed time pdf with fewer than six components failed to account for the longest closed times, and so one is forced to conclude that the channel exhibits a minimum of six closed states. By comparison, closed time distributions with five components have been detected in studies of the nAChR of BC3H1 cells (Sine & Steinbach, 1984).

Only limited analysis of first latency times has been undertaken. First latency distributions provide information on the number of closed states experienced by the channel prior to opening following activation (Patlak & Horn, 1982; Aldrich, Corey & Stevens, 1983; Horn, 1984; Cavalie et al., 1986). A peak was present in the first latency pdf for the NG108-15 channel, indicative of there being more than a single such closed state. The data were interpreted in terms of just two such states, but it is possible that there are more awaiting detection. Indeed, as a result of their studies of gating currents, White and Bezanilla (1985) have proposed a model for the activation of the delayed rectifier involving ca. 15 closed states prior to activation.

Combining this information gives a minimal model made up of three open states and six closed states, with two closed states prior to channel activation. (The former six closed states may incorporate the latter two). One possible model accommodating these constraints is

$$C \leftrightarrow C \leftrightarrow O \leftrightarrow O \leftrightarrow O \leftrightarrow C \leftrightarrow C \leftrightarrow C \leftrightarrow C.$$

Clearly other models exist, and at this stage it would be premature to discuss their relative merits.

The kinetic complexity of the system makes it difficult to relate single channel properties to the macroscopic behavior of the channel, the latter being represented by the ensemble average $P_o(t)$. Until a full model of channel gating is developed, it is not possible to arrive at mechanistic conclusions

from analysis of changes in macroscopic properties. As will be seen, this has implications with respect to interpretation of the effects of changes in membrane lipid composition. At present, it is only possible to describe the form of $P_o(t)$, and any effects on it. The decay of $P_o(t)$ is biphasic, with a tail persisting for up to about 10 sec. The activation phase has been shown to be reasonably well accounted for by the cumulative form of the two component distribution fitted to the first latency data.

Effects of 20:4 Enrichment

Our initial studies provide suggestive evidence that enrichment of the cell membranes with 20:4 alters the gating of the K^+ channel. Because of the close agreement between the two control and two 20:4-enriched patches, we feel it is appropriate to discuss the observed differences between the two lipid compositions. It is important to keep in mind, however, that, due to the small number of patches containing only a single K^+ channel we have been able to obtain and study in detail, our interpretations of lipid effects must be taken as tentative.

Enrichment with 20:4 had no effect on the ion conducting properties of the open channel. Analysis of current-voltage relationships clearly demonstrated the absence of any effect on either slope conductance or reversal potential of the channel. The open time pdf did appear to be slightly altered, but at this point the decrease in the probability of short openings for 20:4-enriched patches may or may not be significant. The estimate of α_1 obtained from analysis of the data from the individual control patches did not agree as closely as most of the other parameters. Therefore, until more data are obtained, it is premature to speculate on the meaning of the change in open time pdf.

Turning to the closed time distributions, a difference between control and 20:4-enriched patches manifests itself. In view of the reservations expressed concerning the fitting of closed time pdfs, it would be unwise to over-interpret the details, but examination of Table 3 shows that enrichment with 20:4 results in a decrease in the relative number of longer closings within clusters of channel openings. This may also be seen in the decrease in mean closed time (from 20.8 to 9.5 msec) induced by 20:4 enrichment. Results obtained from individual control and 20:4-enriched patches agreed very closely for these parameters, which increases our confidence that the observed differences between treatments may be real.

A related way of expressing this change is as an increase in the value of p_o, i.e., the average probability of a channel being open during a cluster. Enrichment results in an approximate doubling of p_o (see Table 1). This is mirrored in the decrease in the rate of macroscopic inactivation of the channel in the 20:4-enriched patches. Inactivation of $P_o(t)$ remains biphasic, but the relative contribution of the faster (time constant ca. 200 msec) component is decreased. Thus, the overall effect of 20:4 enrichment is to cause the channel to remain open for a longer time after depolarization, both by shifting the equilibrium between open and closed states within the clusters of openings and by delaying the macroscopic inactivation of the channel. (These two effects are clearly interdependent to some extent.)

The changes in gating of the channel could either represent changes in the relative values of the rate constants of the gating mechanism or could result from an overall shift in the voltage dependency of gating. For example, exposure of NG108-15 cells to DMSO results in a shift in the voltage dependency of Na^+ channel inactivation (Jourdon, Berwald-Netter & Dubois, 1986). More complete modelling of K^+ channel gating using data obtained at several different membrane potentials should resolve this question.

From the results obtained thus far it is impossible to predict whether or not the changes in K^+ channel gating we have observed would have any effect on action potentials generated by the cells. In theory, increased current flow through the K^+ channels could decrease the action potential amplitude if the rate of K^+ channel activation was sufficiently fast. A more likely effect of increased conductance through K^+ channels would be a more rapid decay of the action potential and greater after hyperpolarization. Unfortunately, these two parameters were not measured in our previous studies so we cannot say if either change actually occurred. Also, since many different types of K^+ channels probably are present in the cells a change in the gating of one of these channels could have little overall effect. Thus, it remains to be determined if the changes in the K^+ channel gating produce a change in the macroscopic appearance of action potentials produced by the cells.

A major question concerning the change in channel kinetics is whether it reflects the altered lipid composition at the time the measurements are made, or rather reflects events which have occurred during the time the cells are exposed to 20:4. (It is very unlikely that the changes are due to high levels of free (unesterified) 20:4 accumulated in the plasma membranes of the cells; the binding of 20:4 to the plasma albumin present in the culture medium prevents any significant accumulation of free fatty acids in the cell membranes.) If the changes in channel kinetics are due to the altered phospholipid fatty acid composition it would mean that the

NG108-15 channel is inherently sensitive to relatively subtle changes in its lipid environment, as has been shown to be the case for the nicotinic acetylcholine receptor (Criado, Eibl & Barrantes, 1984; Zabrecky & Raftery, 1985; Fong & McNamee, 1986). Alternatively, exposure to 20:4 could cause a metabolic change, such as an alteration in the post-translational modification of the channel protein, which produces the change in gating. At present there is no evidence for such an effect of 20:4 on ion channels. We have shown previously that elevation of extracellular unsaturated free fatty acids can cause selective decreases in the number of opiate receptors, PGE_1 receptors and Na^+ channels in NG108-15 cells (McGee & Kenimer, 1982; Love et al., 1985) and nicotinic receptors in PC12 cells (Williams & McGee, 1982), but the properties of the remaining receptors and channels were not detectably altered. Exposure of the cells to a variety of fatty acids for different periods of time should allow separation of these two possible explanations for altered K^+ channel gating.

Ultimately, we would like to determine the physicochemical explanation for alterations of channel gating with changes in membrane lipid composition. In the studies presented here, the enrichment with 20:4 might be expected to produce a membrane of lower viscosity, although one could not be certain that such a change had occurred without direct measurements. If a decreased viscosity is responsible for the changes in gating it would suggest that transitions into one or more long-lived, closed states may be impaired by decreased viscosity. This hypothesis should be testable using control cells exposed acutely to chemicals known to fluidize membranes, such as long-chain alcohols and anesthetics.

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