Temperature dependence of drug blockade of a calcium-dependent potassium channel in cultured hippocampal neurons

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ABSTRACT The temperature dependence of drug blockade of a calcium-dependent potassium channel K(Ca) has been studied in cultured CA1 hippocampal neurons. Channel openings from a 70-pS K^+ channel were recorded when inside-out patches were exposed to a bath solution containing 140 mM K^+ and 0.2 mM Ca^{2+}. The mean open times of channel events were not significantly altered when the bath temperature was lowered from 24° to 14°C (Q_{10} = 1.2). Introduction of the drug RP-62719 into the bath solution (at 5 μM) resulted in the mean open time of the K(Ca) channel to be diminished by 85% (at 24°C) with no change in the amplitudes of the unitary currents. Over the same temperature range of 24° to 14°C, in the presence of RP-62719, the mean open times were significantly prolonged (Q_{10} = 2.2). A simple open channel block scheme was used to determine the temperature dependence of the onward- (blocking) and off- (unblocking) rate constants. Thermodynamic analysis, using transition rate theory, showed that the blocking rate constant was associated with a large increase in entropy. The relatively high temperature dependence for channel blockade is not consistent with a rate-limiting process established by simple diffusion of the agent to a channel blocking site. Channel block may involve conformational changes in the channel protein as a consequence of hydrophobic interactions between drug and channel sites.

INTRODUCTION

A number of drugs depress macroscopic currents through voltage- and chemically-gated ion channels in a manner qualitatively consistent with channel blockade. At present, however, very little is known concerning the molecular mechanisms underlying channel block. It is often assumed that blockade involves simple diffusion of the blocker to a site and that the blocking action represents occlusion of the channel. However, there are little data available to support this argument.

One useful approach in the determination of molecular mechanisms involved in channel block would be to examine the temperature dependence of the process. For example, the temperature dependence of channel mean open time could be used to determine the effects of temperature on specific rate constants, assuming a particular channel blocking model. A simple model for channel block is a sequential open channel scheme whereby an activated channel can undergo transitions to the usual closed state or to an additional blocked state. Measurement of the temperature dependence of the kinetic rate constants in the blocking interaction could then be used to better characterize the molecular nature of the interactions between the drug and the ion channel site. For example, if the mean open time and the onward (blocking) rate constant were relatively temperature independent, then the results would be in reasonable accord with a diffusion-limited process. If, on the other hand, the kinetics of channel block were strongly temperature dependent, then a nondiffusional process would be suggested. In this case, information regarding molecular mechanisms may be obtained by measurement of the temperature dependence of channel blocking rate constants in order to determine the thermodynamic parameters, such as enthalpy and entropy, which are associated with the blocking reaction.

METHODS

Preparation of cultured cells

The procedures which were used to isolate the CA1 hippocampal neurons from rat followed those described previously (Banker and Cowan, 1977). Briefly, laminin-coated coverslips, containing the cultured neurons, were treated with poly-D-lysine. The coverslips were then placed with the growth side downward and incubated in Dulbecco's Modified Eagle's Medium and 5% CO_2 at 37°C. After a period of 3–5 d, the cells were treated with 5-fluoro-deoxy-uridine to inhibit the growth of glial cells. The electrophysiological studies used cells from days 5–10 after isolation.

Electrophysiology

In these experiments unitary currents were recorded from inside-out patches excised from the cultured CA1 neurons. Before patch excision the bath solution contained (in mM): NaCl, 140; KCl, 5; $CaCl_2$, 0.2; $MgCl_2$, 1; Hepes, 10; pH was 7.3. The patch pipette solution had the same ionic composition as the bath solution with the exception that the Ca^{2+} concentration was 1 mM. After excision of the patch, the bath solution was exchanged for one in which the concentrations of K^+ and Na^+ were reversed (i.e., 140 mM KCl and 5 mM NaCl) with the other ion concentrations unchanged. This latter solution served as control

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and unitary currents were recorded from inside-out patches with the patch pipette held at a potential of 0 mV. The drug RP-62719 (a benzopyran compound) was added to the bath solution containing the high K^+. RP-62719 was obtained from Rhone-Poulenc (Vitry, France) and has been developed as a blocker of K^+ channels in cardiac cells with potential utility as an antiarrhythmic and antifibrillatory agent. In most experiments the concentration of RP-62719 was 5 μM, however, in several studies lower concentrations of the drug were used.

An axopatch amplifier (Axon Instruments, Foster City, CA) was used to record the single channel currents. The data were sampled at either 5 kHz (low pass filter set at 2 kHz) or 20 kHz (low pass filter set at 5 kHz). The distributions for the single channel properties of current amplitudes and open and closed times were found using the analysis routines included with pClamp (version 5.0 or 5.5; Axon Instruments). In most experiments a minimum of 200 events, obtained from a single level of opening, were used to define the distributions. In a few cases where the data was obtained at low temperature, the number of events was between 100 and 200.

A sequential, open channel block model (see Results) was used to determine the rate constants for channel block of K(Ca) by RP-62719. An analysis for the transition state thermodynamic parameters associated with the onward (blocking) and the off (unblocking) rate constants followed the methods outlined in Minneman et al. (1980) and Weiland and Molinoff (1981). A plot of ln (rate constant) vs. T^{-1} (Arrhenius plot) was first used to determine the transition state activation energy E_a. The enthalpy $\Delta H^\dagger$ and free energy of activation $\Delta G^\dagger$ were then found from $\Delta H^\dagger = E_a - RT$ and $\Delta G^\dagger = -RT$ ln (rate constant) + RT ln (kT/h), respectively, where R is the universal gas constant, k is Boltzmann's constant and h is Planck's constant. The entropy of activation $\Delta S^\dagger$ was then found from $\Delta G^\dagger = \Delta H^\dagger - T\Delta S^\dagger$. Values for the associated state thermodynamic parameters were also obtained by subtraction of the values for the channel unblocking transition state parameters from the magnitudes of the channel blocking transition state parameters (Minneman et al., 1980). The associated state parameters are equilibrium values if the channel block and channel unblock processes are governed by the same rate-limiting reaction.

The bath temperature of the recording chamber was varied from 24° to 14°C using a proportional temperature control system. This system, which used thermistors and Peltier devices, was similar in design to that described in Chabala et al. (1985). Data were initially recorded at T = 24°C and the set-point was then changed to T = 14°C. Data were then recorded at intervals of T = 2°C as the bath temperature was cooled to the low temperature set-point. Because the time required to change the bath temperature by 10°C was 4 min, then the time period for recording at an intermediate temperature (example 20 ± 0.2°C) was ~10 s. In most experiments, after data were obtained at the steady state temperature of T = 14°C, additional single channel currents were recorded at the intermediate temperatures by alternating the temperature control system between T = 14°C and a given intermediate temperature. No significant differences in the amplitudes or mean open times associated with the unitary currents were observed at a given temperature between the data recorded during the initial cooling run or that obtained in a step to that temperature from T = 14°C. Furthermore, no systematic changes in the properties of the unitary currents were found with prolonged exposures of the patches at either of the steady-state temperatures of 24° and 14°C. In a few experiments data were also recorded at T = 19°C in order to illustrate the changes in current amplitudes and channel open times at a temperature intermediate between the two extremes of set-point temperatures. A digital thermometer was used for calibration purposes to ensure accuracy in the readings from the temperature controller. As a measure for the temperature dependence of channel kinetics, Q_{10} values have been determined from the ratios of mean open times or rate constants at 24°C to those determined at 14°C. All errors are stated as ±SEM.

RESULTS

Activation of a 70 pS calcium-dependent K^+ channel (K[Ca]) in cultured CA1 neurons has previously been demonstrated when inside-out patches were exposed to a bath solution containing 140 mM K^+ and 0.2 mM Ca^{2+} (McLarnon, 1990; McLarnon and Wang, 1991). Both the K^+ selectivity (McLarnon, 1990) and the Ca^{2+}-dependence (McLarnon and Wang, 1991) for the channel have been documented. Typical channel events, recorded from an inside-out patch with V = 0 mV, are shown in Fig. 1 *A* (trace *a*) with the bath temperature held at 24°C. When the temperature was decreased to

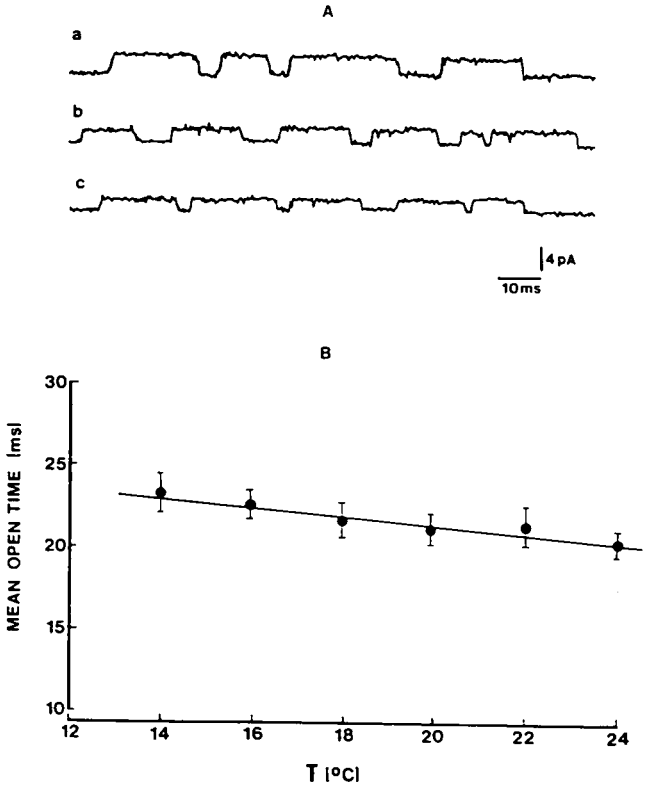

FIGURE 1 Temperature dependence of single channel currents and mean open times in control solution. (*A*) Channel events (*upward openings* from the baseline) recorded from an inside-out patch with V = 0 mV. The temperatures of the bath solution were: trace *a*, 24°C; trace *b*, 19°C; trace *c*, 14°C. The low-pass filter was set at 2 kHz and the sampling frequency was 5 kHz. (*B*) Plot of the dependence of mean open time on temperature. The mean open times are average values from n = 6 patches. The fit to the data was determined using linear regression analysis. The Q_{10} associated with the temperature dependence of mean open time was 1.2.

19°C (Fig. 1 *A*, trace *b*) and 14°C (Fig. 1 *A*, trace *c*), the amplitudes of the unitary currents were progressively diminished; however, the durations of open times remained relatively constant. In Fig. 1 *B*, a plot of the channel mean open time dependence on temperature is shown for six patches, including the patch with the channel openings illustrated in Fig. 1 *A*. The mean open times were not significantly changed over a temperature range from 24° to 14°C. A Q_{10} value of 1.2 ± 0.2 was determined for the temperature dependence of mean open time. At all temperatures the open time distributions were well fit with single exponential functions. Typical distributions for channel open times are shown in Fig. 2 *a* (*top*) with T = 24°C and in Fig. 2 *b* (*top*) with T = 14°C. In most instances the distributions for channel closed times required fitting with a two-component exponential function. Typical distributions for channel closed times are illustrated in Fig. 2 *a* (*bottom*) with T = 24°C and in Fig. 2 *b* (*bottom*) with T = 14°C. As noted previously (McLarnon and Wang, 1991), the K(Ca) channel can exhibit a complex pattern of bursting activity with different closed times between, and during, bursts of channel openings. Decreasing the temperature by 10°C had a small effect to prolong the slow phase with no obvious effect on the rapid time component of the closed time distribution.

As shown in Fig. 1, the amplitudes of the single channel currents were diminished with decreasing temperature. In n = 6 patches, the ratios of the channel height at T = 14°C to that at T = 24°C were determined. The Q_{10} value associated with the temperature dependence of current height, over the temperature range from 24° to 14°C, was 1.4 ± 0.1 (n = 6). Thus both the mean open time and the amplitude of single channel currents for K(Ca) were not strongly dependent on temperature.

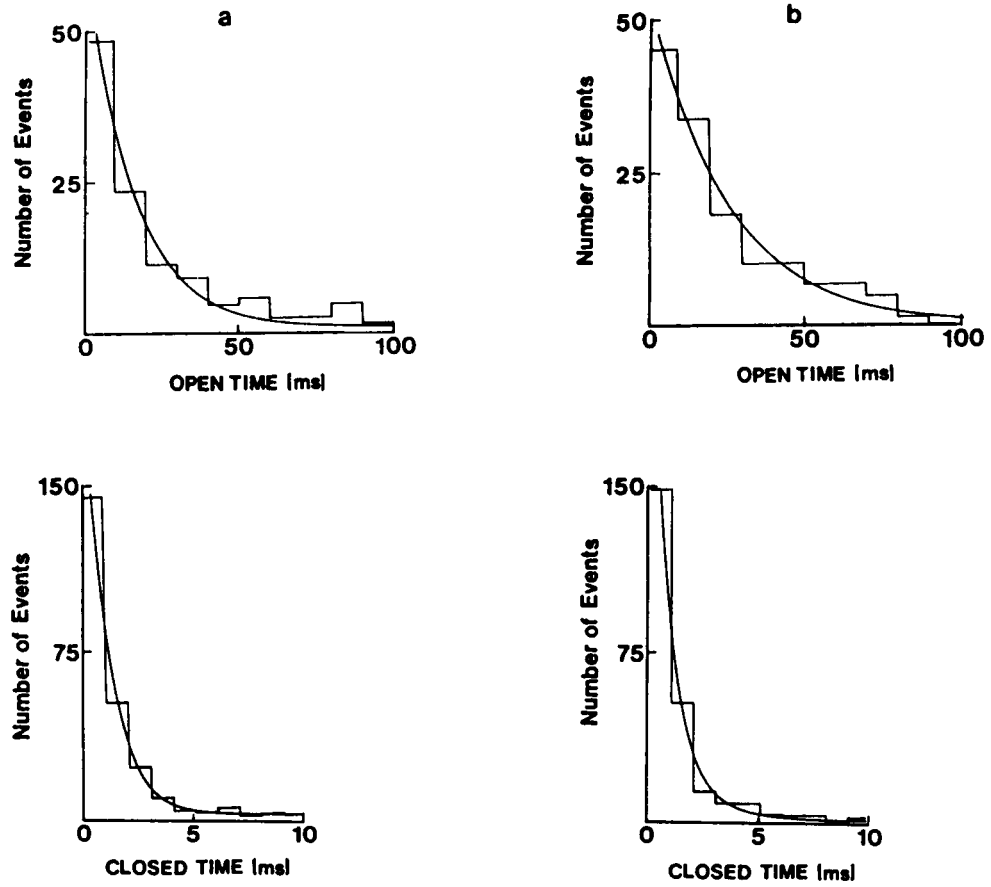

FIGURE 2 Distributions for channel open and closed times at T = 24°C and T = 14°C. (*a*, *top*) Distribution of open times at T = 24°C with mean 26.2 ± 1.3 ms, N = 310 events. (*b*, *top*) Distribution of open times at T = 14°C with mean 29.4 ± 1.8 ms, N = 360 events. (*a*, *bottom*) Distribution of closed times at T = 24°C with mean values for the two components of 0.9 ± 0.2 ms, and 4.1 ± 0.6 ms. N = 270 events. (*b*, *bottom*) Distribution of closed times at T = 14°C with mean values for the two components of 0.8 ± 0.2 and 6.3 ± 1.4 ms, N = 330 events.

When the compound RP-62719 (a benzopyran derivative) was added (at a concentration of 0.5 μM) to the solution bathing inside-out patches, the channel openings evinced flickering transitions to a nonconducting level (Fig. 3, *a* and *b*). With this concentration of drug, the mean open times of K(Ca) were decreased by 34% compared with the mean open times in control solution (from 19 ms to 12.5 ms, $n = 3$ patches). When the concentration of RP-62719 was increased to 5 μM, the frequency of transitions from the open level to the nonconducting state was increased (Fig. 3 *c*). The mean open time of the K(Ca) channel was decreased by 85% with 5 μM of RP-62719 (from 20.5 ms [$n = 6$] in control solution to 2.9 ms [$n = 7$] with drugs). The rapid transitions from the open state in the single channel records with RP-62719 are similar to those first described for local anesthetic actions to block end-plate channels (Neher and Steinbach, 1978) and also the block of K(Ca) by a number of cardiac drugs (McLarnon, 1990; McLarnon and Wang, 1991).

The dependence of drug actions on temperature was investigated by cooling the bath solution from $T = 24°C$. In Fig. 4, *a*, *b*, and *c*, unitary currents, in the presence of RP-62719 (5 μM), are shown for temperatures of 24°, 19°, and 14°C, respectively. The amplitudes of the currents and the channel open times were sensitive to temperature with decreasing temperature acting to diminish amplitude and to prolong open time. Typical distributions for channel open and closed times with RP-62719 are shown for temperatures of 24° and 14°C in Fig. 5. The open time distributions (Fig. 5, *a* and *b*) were well fit with single exponential functions at both temperatures. Thus, for both control and with drug, the kinetics of K(Ca) were consistent with a single open state. However, decreasing temperature by 10°C caused a considerable prolongation of mean open time in the presence of RP-62719 (Fig. 5, *a* and *b*). Distributions for closed times at both temperatures (Fig. 5, *a* and *b*), over the range from 0 to 15 ms, were also fit with single exponentials. The rapid time component seen for the closed time distributions with control solution (see Fig. 2, *a* and *b*) were not prominent in the presence of the drug for this patch. In general, however, closed time distributions with RP-62719 also exhibited two-time components. For the single time component fits and the slower time component of the double exponentials, the mean closed time was slightly increased as the bath temperature was decreased by 10°C.

The average values of mean open times from seven patches have been plotted as a function of temperature in Fig. 6. The Q_{10}, as determined from linear regression fitting to the data, was 2.2 ± 0.3. Thus, the channel blocking action of RP-62719 exhibited a considerable dependence on temperature, whereas in the absence of the drug, channel open times were not strongly sensitive to temperature (Q_{10} value of 1.2 ± 0.2). The mean open time dependence on temperature was determined at a single potential ($V = 0$ mV). In order to determine if the actions of RP-62719 were voltage dependent, several experiments were done with increased patch depolarization. Increasing the value of the patch depolarization by 20 or 40 mV increased the magnitudes of the unitary currents with no significant effect to alter the mean open time.

The actions of RP-62719 to increase channel flickering, in a concentration-dependent manner, are very similar to the effects on K(Ca) reported for a group of drugs which fit the general description of class III-antiarrhythmic agents (McLarnon and Wang, 1991). These drugs have been shown to block K(Ca) in a voltage-indepen-

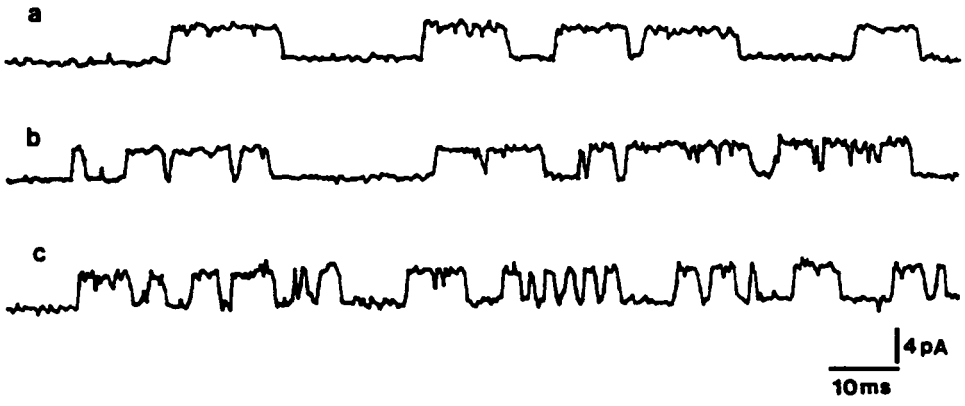

FIGURE 3 Single channel currents in control solution and with RP-62719. (*a*) Typical openings of K(Ca) in control solution. (*b*) Openings with RP-62719 (at 0.5 μM) added to the bath solution. (*c*) Openings with RP-62719 (5 μM). All events were from the same inside-out patch with $V = 0$ mV.

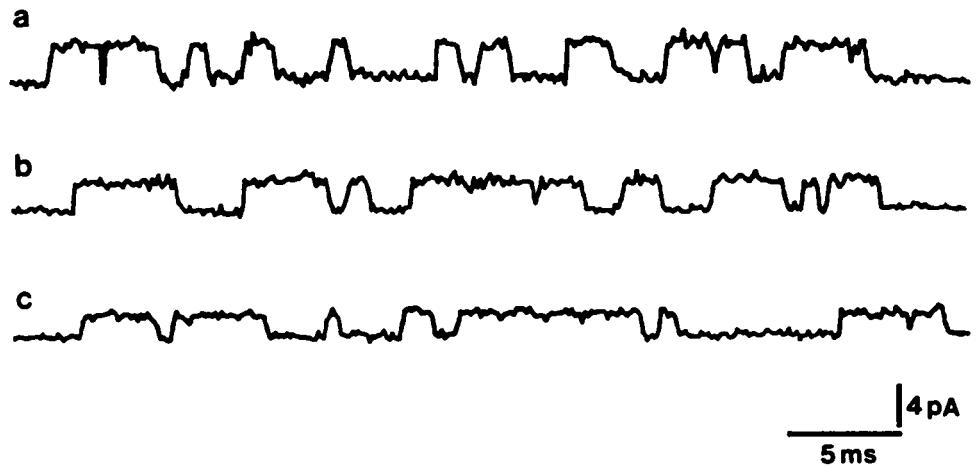

FIGURE 4 Unitary currents, with RP-62719 (5 μM), at different temperatures. The bath temperatures were (*a*) 24°C, (*b*) 19°C, and (*c*) 14°C.

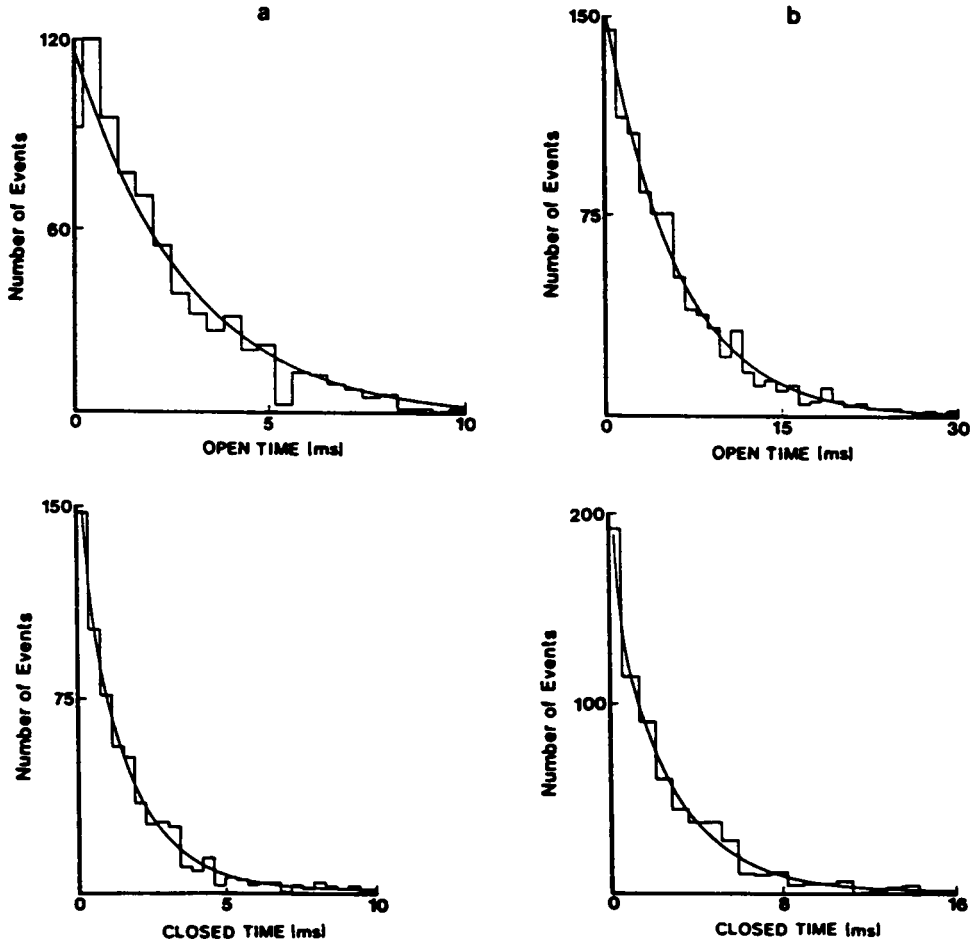

FIGURE 5 Distributions for channel open and closed times in the presence of drug. (*a*) Channel distributions for open time (*top*) and closed time (*bottom*) at $T = 24°C$. The time constants were 2.6 ± 0.3 ms for the open time and 1.7 ± 0.2 ms for the closed time; 760 events were recorded for the distributions. (*b*) Channel distributions for open time (*top*) and closed time (*bottom*) at $T = 14°C$. The time constants were 6.2 ± 0.2 ms for open time and 2.7 ± 0.3 ms for closed time; 970 events were recorded for the distributions.

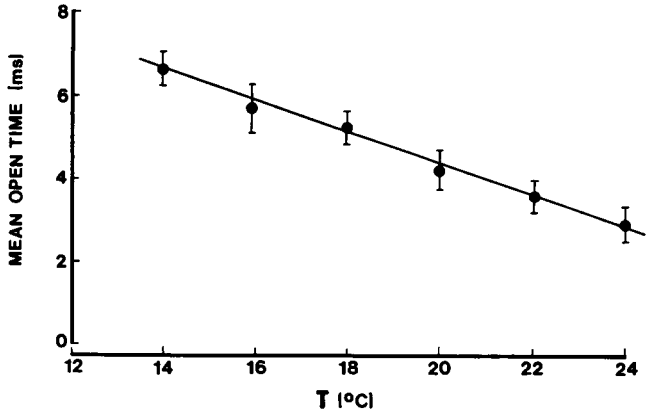

FIGURE 6 Plot of mean open time dependence on temperature with RP-62719 added to the bath solution. The values of mean open time are average values from $n = 7$ patches. The fit to the data was determined by linear regression analysis. A Q_{10} of 2.2 was found for the temperature dependence of open time.

dent manner when applied to both inside-out and outside-out patches (McLarnon and Wang, 1991). The actions of these drugs were consistent with a simple open channel block of the K(Ca) channel (Neher and Steinbach, 1978), whereby the amplitudes of the unitary currents were not changed and the channel mean open times were progressively decreased when the concentration of the drug was increased. In a similar fashion, increasing the concentration of RP-62719 tenfold (see Fig. 3) acted to decrease the mean open times with no change in the heights of the single channel currents.

The simple open channel blockade scheme has been used to determine the rate constants for channel block by RP-62719. This scheme can be represented as:

$$C \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} O \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} B,$$

where the states of the system are represented by C, closed; O, open; and B, blocked. The rate constants k_1 and k_{-1} represent the respective forward- and backward-rate constants for the transitions from the closed state to the open state. The blocking (onward) rate constant is k_2 and the unblocking (off) rate constant is k_{-2}. In this scheme the rate constant for the transition from open to blocked state is equal to the product of k_2 and drug concentration [D]. Thus, in a simple open channel block model, the inverse of the measured mean open time (with drug present) at a given temperature, is equal to the sum of k_{-1} and k_2 [D].

The blocking rate constant k_2 was determined at different temperatures using the data from the graphs of mean open time versus temperature for control (Fig. 2) and with RP-62719 (Fig. 6). The magnitudes of k_{-1}, as a function of T, were first found by inverting the mean open times found with control solution. Substitution for the values of k_{-1} and the values of mean open time from Fig. 6 (in the presence of the drug) into the channel block equation allowed solution for k_2. The results obtained at the extremes of the temperature range were:

$k_2(24°C)$; $5.9 \pm 0.2 \times 10^7\ M^{-1}s^{-1}$ and

$k_2(14°C)$; $2.2 \pm 0.2 \times 10^7\ M^{-1}s^{-1}$.

Using these values for the channel blocking rate constants, at the temperatures of 24° and 14°C, yields a Q_{10} of 2.7 for the blocking rate constant. Although the temperature dependence of k_2 was determined for a single concentration of RP-62719 (at 5 μM), a value for the onward rate constant was also found with RP-62719 at 0.5 μM ($T = 24°C$). At the lower concentration of the drug, the k_2 was $5.5 \pm 0.3 \times 10^7\ M^{-1}\ s^{-1}$ ($n = 3$). This value for k_2 was not significantly different compared with the magnitude of k_2 found with RP-62719 at 5 μM with $T = 24°C$.

The transition state thermodynamic parameters associated with k_2 were found using the procedures described in Methods (Minneman et al., 1980). An Arrhenius plot ($\ln k_2$ vs. T^{-1}) was first constructed and is shown in Fig. 7. The transition state activation energy E_a was found from the product of the slope of the linear relation in Fig. 7 and the universal gas constant R. This value of 17.3 kcal/mol, was then used (see Methods) to determine the enthalpy of activation, $\Delta H^\dagger$ and the free energy of activation, $\Delta G^\dagger$ (Table 1). From these results the entropy of activation was then calculated to be +33 entropy units (e.u.).

An estimate for the temperature dependence for the channel unblocking rate constant k_{-2} was also done from examination of the single channel records. Because K(Ca) exhibits bursting behavior in the absence of drug (McLarnon and Wang, 1991) it was necessary to differentiate between channel transitions to the nonconducting state during bursts (blocking events) and longer channel closing events between bursts. The values of k_{-2} were: (at $T = 24°C$) $k_{-2} = 550\ s^{-1}$; (at 14°C) $k_{-2} = 377\ s^{-1}$. These values were not dependent on the magnitude of the burst duration for durations in excess of ~2 ms. The ratio of the k_{-2} values yields a Q_{10} of 1.5.

An analysis, using the transition rate theory, was also applied to the channel unblocking rate constant k_{-2}. The parameters for free energy of activation, enthalpy, and entropy are included in Table 1. A value for the activation energy of 6.7 kcal/mol was considerably lower than the corresponding activation energy determined for the channel block rate constant k_2. The lower activation energy for k_{-2} suggests that the channel

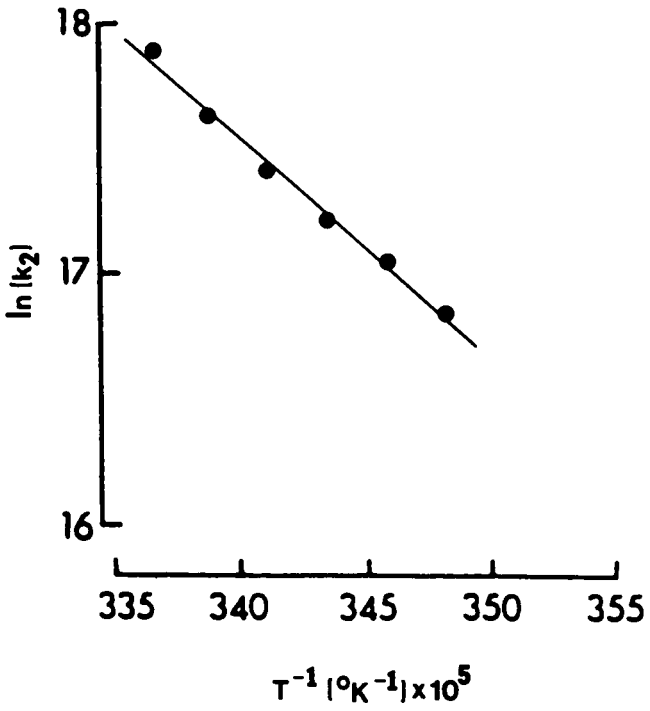

FIGURE 7 Arrhenius plot for k_2. The Arrhenius equation expresses the dependence of the rate constant k_2 on temperature T as follows $k_2 = Ae^{-Ea/RT}$ where A is a constant for the reaction and R is the gas constant. The fit to the data was done by linear regression.

unblocking process has a lower temperature sensitivity than the channel blocking step. The negative entropy associated with the unblocking rate constant (Table 1) indicates an increase in the order of the system. Thus, the entropy calculated for the channel unblocking rate constant is opposite in sign to that found for the entropy change determined for k_2.

TABLE 1 Transition State and Associated State Thermodynamic Parameters

Rate constant	Transition state parameters			
	E_a(kcal/mol)	$\Delta H^\dagger$(kcal/mol)	$\Delta G^\dagger$(kcal/mol)	$\Delta S^\dagger$(e.u.)
k_2($M^{-1}s^{-1}$) 5.9×10^7	17.3	16.7	6.9	+33.0
k_{-2}(s^{-1}) 550	6.7	6.1	13.7	−25.6
	Associated state parameters			
	—	10.6	−6.8	58.6

The values for associated state parameters were determined from the differences between the transition state parameters for channel blocking and unblocking. The rate constants are values with $V = 0$ mV and $T = 24$°C.

The thermodynamic parameters for the associated state of the system can be determined from the transition state parameters (see Methods). The data are presented in Table 1 and show positive values for both enthalpy and entropy. The overall free energy change in the blocking interaction, determined by subtracting the free energy value associated with k_{-2} from the corresponding value with k_2, was −6.8 kcal/mol. A negative value for the associated state free energy would be expected for a reaction that decreases the total energy of the system. The driving force for the blocking interaction is the large increase in the entropy.

DISCUSSION

We have measured the temperature dependence for the block, by the drug RP-62719, of a calcium-dependent potassium channel in hippocampal neurons. Previous work (McLarnon, 1990; McLarnon and Wang, 1991) has shown that a number of agents which modify the properties of K^+ channels in cardiac tissue (Colatsky and Follmer, 1989; Dukes et al., 1990) also act to block K(Ca) in neurons. The effects of RP-62719 on K(Ca) were qualitatively very similar to those found with the other cardiac drugs including actions to diminish the channel mean open times with bath applications to both inside-out and outside-out patches. Such actions were not dependent on patch potential. Several measurements suggest the data to be generally consistent with RP-62719 block of open K(Ca) channels (see below). For example, the amplitudes of the unitary currents were not altered by the drug. In addition, the mean channel open time was scaled with drug concentration such that the magnitude of the onward (blocking) rate constant was not significantly changed over a tenfold concentration range of RP-62719 (from 0.5 to 5 μM).

A sequential open channel block scheme (Adams, 1977; Neher and Steinbach, 1978) was used to determine the blocking (k_2) and unblocking (k_{-2}) rate constants for the interaction of RP-62719 with the K(Ca) channel. In the open channel block model the rate constant k_2[D] represents the binding of the drug to a channel site. This scheme, which is perhaps most relevant to charged ion block of ion channels from outside, assumes that the only pathway from the blocked state was via the open state. Such a simple model is only an approximation, however, because RP-62719, with a benzopyran structure, would be expected to exhibit a considerable degree of lipid solubility. Indeed, the results of studies using inside-out and outside-out patches from hippocampal neurons have suggested that a series of lipid-soluble drugs block K(Ca) at an internal site or sites (McLarnon, 1990; McLarnon and Wang, 1991). A similar

conclusion has been reached for the action of one of these agents, tedisamil, on delayed rectifier K^+ channels in ventricular myocytes (Dukes et al., 1990), and also for the actions of a series of drugs to block delayed rectifier K^+ channels in epithelial cells (Jacobs and Decoursey, 1990). It is possible that the binding of drug to a site associated with K(Ca) would be somewhat analogous to block of delayed rectifier K^+ channels in squid axons with internal tetraethylammonium (TEA) analogues (Armstrong and Hille, 1972; French and Shoukimas, 1981) or to block of Na^+ channels with hydrophobic agents (Hille, 1977).

In the presence of RP-62719, the mean open time of the channel was significantly prolonged with diminished bath temperature (Q_{10} of 2.2). In the absence of drug the kinetics of K(Ca) were essentially independent of temperature; in this case a Q_{10} value of 1.2 was found for the mean open time. The temperature dependence of the onward (blocking) rate constant k_2 was determined from measurements of the mean open times (with RP-62719 at 5 μM) and the channel closing rate constants (with no drug) for bath temperatures in the range 14° to 24°C. The rate constant k_2 was strongly dependent on temperature with a Q_{10} value of 2.7. The magnitude of k_2 (24°C) was $\sim 6 \times 10^7\ M^{-1}s^{-1}$, which is, respectively, about three times and double the values for the k_2 found with octanol and procaine block of the end-plate channel (McLarnon et al., 1984). Values of k_2 in the range from (1–25) $\times 10^7$ $M^{-1}s^{-1}$ were found at room temperature with other antiarrhythmic drugs (McLarnon and Wang, 1991). These values for the blocking rate constant are at least ten times the magnitudes of the blocking rate constants found for QX222 block of inactivating Na^+ channels (Starmer et al., 1986).

The relatively high Q_{10} values associated with the channel mean open time and with k_2 would not be consistent with simple diffusion of the blocking moiety to a channel site. If diffusion were rate limiting in the channel blocking step, then the process would be expected to be characterized by a low temperature sensitivity with a Q_{10} value in the range of 1–1.4. For example, a Q_{10} of 1.4 has been determined for the temperature dependence of the single channel conductance for the cation selective gramicidin channel in lipid bilayer membranes (Hladky and Haydon, 1972; see also Hille, 1984). It is possible, however, that the movement of a particular drug in an aqueous channel or through lipid may not be characteristic of simple diffusion, and a higher temperature sensitivity could reflect the drug pathway to a channel site.

In order to better clarify the high temperature sensitivity for the channel block rate constants, an analysis for the thermodynamic parameters of enthalpy, free energy, and entropy was carried out using the methods of transition rate theory (Minneman et al., 1980; Hitzemann, 1988; Raffa and Porreca, 1989). The results showed that in the formation of the transition state (activated complex of drug and channel site) the blocking rate constant is associated with large positive entropy and enthalpy changes. Because $\Delta G^\dagger = \Delta H^\dagger - T\Delta S^\dagger$, the blocking process is favored (decrease in free energy) by the change in entropy and hindered by the change in enthalpy. These results are similar to those found with block of end-plate channels with *n*-alkanols and local anaesthetics (McLarnon and Quastel, 1984), and with binding of ligands to adrenergic receptors of turkey membrane (Minneman et al., 1980). In both cases the large increases in entropy were suggested to be consistent with increased disorder in the systems through the formation of hydrophobic bonds (Kauzmann, 1959) between the interacting species. Within the series of *n*-alkanols, the potency for block of end-plate channels was correlated with the degree of hydrophobicity of the agent (McLarnon and Quastel, 1984; McLarnon et al., 1986). The magnitudes of the activation energies and entropy values for both block of the end-plate channel by alcohols and for block of K(Ca) by RP-62719 were very similar, strongly suggesting that hydrophobic binding could also account for the latter actions. Indeed, a significant hydrophobic component in the channel block of K(Ca) by several other drugs has previously been documented (McLarnon and Wang, 1991). The large positive value for enthalpy would suggest the contribution of nonhydrophobic interactions, e.g., in the formation of hydrogen bonds, to be relatively unimportant in channel blockade.

The large values of E_a (in excess of 10 kcal/mol) and entropy determined for k_2 are considerably higher than would be expected if the kinetics of channel block were determined by simple diffusion of the drug in an aqueous medium. The results suggest that channel block could be a consequence of the drug interaction with a hydrophobic portion of the channel protein. Such an interaction could then account for the substantial increase in entropy through a decrease in the structured water of hydration at the site and possible destabilization of the protein segment.

The Q_{10} associated with k_{-2} was 1.5. Thus, the channel unblocking step exhibited a considerably lower dependence on temperature compared with the blocking step. Another difference between the blocking and unblocking steps was the calculation of a negative entropy for the unblocking rate constant. A negative entropy signifies increasing order which is opposite to that found with k_2. The channel unblocking step could also indicate changes in conformation of the channel protein or perhaps in the structured water near the site of interaction. In this case the changes are opposite to those

associated with the blocking step. It seems reasonable to assume, on the basis of previous work (McLarnon and Wang, 1991), that the magnitude of k_{-2} is strongly dependent on the degree of lipid solubility of the drug. The k_{-2} for RP-62719 block of K(Ca) is very close to that found with block of the end-plate channel by the lipid soluble *n*-alkanol, octanol, at the mammalian neuromuscular junction (McLarnon and Quastel, 1984).

The associated state thermodynamic parameters can be found from the transition rate values (Minneman et al., 1980). For example, the associated state free energy $\Delta G°$ is $\Delta G^{\dagger}(k_2) - \Delta G^{\dagger}(k_{-2}) = -6.8$ kcal/mol. A negative free energy is to be expected for the spontaneous blocking reaction. The associated state entropy $\Delta S°$ is $\Delta S^{\dagger}(k_2) - \Delta S^{\dagger}(k_{-2}) = +58.6$ e.u. Thus, the blocking reaction appears to be consistent with a rate-limiting, entropy-driven, step through the formation of hydrophobic bonding between the drug and channel site. It should be noted that the associated state parameters are equilibrium values if channel block and channel unblock are established from the same rate-limiting process. If this was the case (as suggested from the different signs of the entropy terms for the rate constants), then the blocking and unblocking steps have a large difference in temperature dependence.

Although the interpretation of this data is necessarily speculative, the results suggest that channel block could be a complex process. In this case it is interesting that the thermodynamic parameters for block of K(Ca) by RP-62719 are close to those determined for *n*-alkanol block of the end-plate channel (McLarnon and Quastel, 1984). This point may reflect the general commonality in channel structures. The K(Ca) channel, like other ion channels, is an aqueous pore; it is likely that the channel also includes regions of hydrophobic interfacial pockets. In terms of energy considerations, a nonpolar region of a blocking molecule would preferentially interact with hydrophobic segments of the channel protein. The large positive magnitudes of entropy associated with the channel block rate constant k_2 could then be due to increased disorder in the system involving changes in structured water or changes in protein conformation.

It should be noted that alterations in the temperature of the bath solution could also have effects on other aspects of the system. For example, changes in the buffering capacity of the solution or on the degree of ionization of the drug, could occur. It would be expected, however, on the basis that the temperature variation in this study was over a range of only 10°C, that such changes would be relatively small.

Methods, other than changing ambient temperature, have been used in order to determine the biophysical properties of drug interactions with ion channel proteins. Patch clamp measurements have been combined with alterations in solution viscosity to study the interaction of the neurotoxin charybdotoxin with a calcium-dependent K^+ channel in planar lipid bilayers (Miller, 1990). The results were in accord with rate-limiting diffusion controlling binding of the agent to a site in the channel mouth. Measurements of conformational changes in membrane proteins, induced by a number of compounds, have recently been reported (McCarthy and Stroud, 1989*a*, *b*). The kinetics of tritium-hydrogen exchange were used to demonstrate that binding of competitive inhibitors to the acetylcholine receptor (AChR), such as D-tubocurarine, caused significant conformational changes in the receptor complex (McCarthy and Stroud, 1989*a*). In addition, a probe, specific for hydrophobic segments associated with ion channel proteins, was used to show conformation changes in the subunits of AChR with the agonist carbamylcholine (McCarthy and Stroud, 1989*b*). It is apparent that measurements with a diversity of techniques will be necessary in order to characterize the complex interactions between ion channel proteins and modulators of the properties of the proteins.

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