Thermodynamic Entropy of Two Conformational Transitions of Single Na^+ Channel Molecules

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ABSTRACT Single cardiac Na^+ channel currents were recorded with improved resolution (bandwidth up to 20 kHz) at two temperatures, 10 and 25°C. The mean open time was determined at voltages between −50 and 0 mV by evaluation of the distribution of the event-related gaps in the center of the baseline noise. Fit of the voltage-dependent reciprocal mean open times at both temperatures allowed even for a single channel molecule to separate an entropic from an enthalpic part of activation energy for both deactivation and inactivation. Both entropies are positive and the entropy of deactivation exceeds that of inactivation by more than twice.

INTRODUCTION

Macroscopic Na^+ currents have been studied in great detail over more than four decades and their kinetics were analyzed by fitting either Hodgkin-Huxley-type models (Hodgkin and Huxley, 1952; Chandler and Meves, 1970) or the more general Markovian state models (Bezanilla and Armstrong, 1977; Armstrong, 1981), consisting of sets of rate constants and states. From the temperature dependence of the rate constants of both opening from the last closed state (C→O) and the reverse reaction (O→C), the respective enthalpic and entropic parts of transitional free energy were calculated (Levitan and Palti, 1975). Also, entropy differences between the states O, C, and the inactivated state I have been quantified yielding lower limit values for the entropies between the respective states and transitional states (Conti, 1986; Jonas, 1989).

In microscopic Na^+ currents, as measured through single ionic channels with the patch clamp technique (Hamill et al., 1981), the reciprocal mean open time τ_o was found to be a U-shaped function of voltage (Yue et al., 1989). This allowed the measurement of rate constants of microscopic deactivation (O→C) and inactivation (O→I). In normally working Na^+ channels, however, the relatively large noise did not allow the determination of respective entropy changes until now because of bandwith limitations. Only in batrachotoxin-modified Na^+ channels, which lack fast inactivation and therefore open at low temperature sufficiently often for reasonable statistical analysis, transitional entropy associated with deactivation has been reported recently (Correa et al., 1992).

Here, a low noise patch clamp technique is used to measure open channel life times at 10°C and 25°C (bandwidth up to 20 kHz) in single untreated Na^+ channels. The quality of the measurements was suitable to determine the transitional entropy associated with microscopic deactivation (O→C) and inactivation (O→I). It is concluded that energetics of both transitions is regularly very homogeneous within the population of Na^+ channels and that the transition O→C proceeds with a conformational change more than twice as large as the transition O→I.

MATERIALS AND METHODS

Cell isolation

Single mouse ventricular myocytes were freshly isolated by enzymatic digestion (Benndorf et al., 1985). Cells were exposed to the bath solution at least 15 min before starting the experiments. The depolarizing bath solution contained (mmol/liter) KCl 250.0, CsCl 20.0, $MgCl_2$ 5.0, EGTA 10.0, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 5.0, pH 7.3 with KOH. In some experiments 20.0 mmol/liter KCl was replaced by equimolar tetraethylammoniumchloride (TEA-Cl). Two pipette solutions were used (mmol/liter); solution I: NaCl 287.0, $CaCl_2$ 2.5, $MgCl_2$ 1.0, KCl 4.0, HEPES 5.0, nitrendipine 2 μmol/liter, pH 7.3 with NaOH; solution II: NaCl 255.0, TEA-Cl 20.0, $BaCl_2$ 5.0, $CdCl_2$ 1.0, $MgCl_2$ 5.0, KCl 4.0, HEPES 5.0, pH 7.3 with NaOH). The solutions were hypertonic to increase unitary current amplitude (Yue et al., 1989). The choice of the solutions was without relevance for the channel properties. The temperature was accurate within ±0.3°C.

Recording technique

Measurements were performed with a patch clamp technique (Hamill et al., 1981) with improved resolution using very short pipettes (total length 8 mm) in combination with an especially tipped holder (Benndorf, 1993). The pipettes had a final resistance between 5 and 20 MΩ and were coated with Sylgard 184 (Dow Corning, 6198 Seneffe, Belgium). The pipette solution was sealed against the holder with paraffin oil. All recordings were obtained from cell-attached patches (seal resistance between 60 and 140 GΩ) with either an Axopatch 200 or 200A amplifier (Axon Instruments, Inc., Foster City, CA), which had an intrinsic noise of 0.091 and 0.068 pA rms (5 kHz), respectively. The signals were filtered with an eight-pole Bessel filter (Frequency Devices, Inc., Haverhill, MA) on line with the cut-off frequencies f_c (−3 dB) of either 5 or 10 kHz at 10°C and 13 or 20 kHz at 25°C and sampled at $5 \times f_c$ (Colquhoun and Sigworth, 1983). Total background noise was 0.145–0.175, 0.30–0.45, 0.39–0.57, and 0.75–0.83 pA rms at the respective f_c value. Recording and analysis was performed on a PC-80386 or -80486 with the ISO2 software developed in this laboratory (MFK Computer, Frankfurt, Germany) in combination with an optical disk system. Patches were pulsed at a rate of either 5 or 10 Hz (10°C) or 20 Hz (25°C). The holding potential was set to −120 or −130 mV. Capacitive transients were compensated for carefully by the use of four exponentials (Benndorf,

Received for publication 25 January 1993 and in final form 10 May 1993.
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0006-3495/93/10/1585/05 $2.00

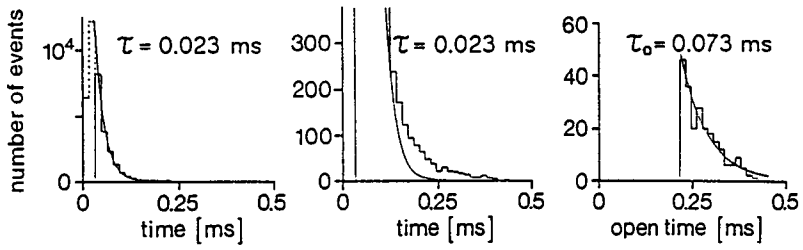

FIGURE 1 Baseline method for evaluation of the mean open time. Histograms were formed from an artificial file containing 1730 monoexponentially distributed openings with a τ_o of 70 μs. For further explanation see text.

1993). Individual traces were compensated for the remaining capacitive and leak currents by subtraction of a "sliding" averaged blank trace. This trace was formed from ten traces without channel activity which were recorded shortly before or after the actual trace. Single Na^+ channel currents were identified by the time course of the mean current and the high probability of observing the events. L-type Ca^{2+} channels were blocked by Cd^{2+} or nitrendipine.

Open time measured at the baseline

In the absence of background noise, a hypothetical rectangular opening of original width t_w is prolonged after filtering (eight-pole Bessel, cut-off frequency f_c) at the level of 10% of the full amplitude by approximately one rise time T_{10-90} which is given by $0.3321/f_c$ (Colquhoun and Sigworth, 1983). For open times longer than T_{10-90}, all pulses are equally prolonged by this amount and if the true open times are distributed with the time constant τ_o, the time distribution of the filtered openings measured at the 10% must obey the same τ_o. Superimposition with background noise, whose rms level is substantially larger than 10% of the opening level, may either shorten or prolong individual opening events depending on the phase of noise and the opening and closing transition. In general, in the presence of noise these 10%-level-open times depend also on the relation of the rise time and the amplitude of the openings. This error, however, decreases with increasing bandwidth. At multiple single channel currents, a 10% level can not be defined and it is therefore useful to set the threshold to the center of the baseline which introduces only negligible error at large noise levels. The total event distribution is then composed of two distributions, the distribution of a very large number of dwell times t_n of false noise events and the smeared distribution of dwell times of opening events. The mean dwell time of noise events τ_n is given by $\tau_n = 1/(2cf_c)$ (Papoulis, 1965) with c being a constant between 0.849 and 1.25 depending on the noise characteristic (Colquhoun and Sigworth, 1983). Clipping the first bins allowed, even in the case of oversampling (Nyquist theorem), perfect monoexponential fit of the t_n distribution with time constants shorter than τ_n. The left diagram in Fig. 1 shows a fitted histogram of an artificial file containing 1730 exponentially distributed openings (τ_o = 70 μs) of either 1.3 or 3.0 pA amplitude in the presence of typical noise at 13 kHz. Ordinate expansion of the histogram (*middle diagram* in Fig. 1) shows the deviation of the distribution of the comparatively small number of opening events from the curve fitted to the false events of the background noise. The final open time histogram (*right diagram* in Fig. 1) was obtained by clipping all bins from the left until the curve, fitting the background noise events, decreased to below the 8% value of the respective bin. Fitting the remaining bins yielded a precise estimate of τ_o, also in the presence of two levels. The same results were obtained at the other used bandwidths. In real patches, the mean open time determined with this method proved to be practically independent of the amplitude of the single channel events if only the open level was large enough to exclude that open channel noise peaks reach the zero level. Different levels were treated as energetically equivalent with respect to gating and were lumped in the histograms. In general, under the condition of a monoexponential distribution of open times (no evidence for the existence of a further exponential existed), the baseline method allows open time analysis with noise levels double as high as any analysis employing the conventional 50% threshold.

All fits were performed with a derivative free Levenberg-Marquardt routine (Brown and Dennis, 1972).

RESULTS

Statistical analysis showed that the availability of the channels in the patch was not constant during the run of an experiment. In 53 patches, only two tentative one-channel patches could be identified by the absence of any superimposition of openings, though in several patches it happened that more than 1000 consecutive depolarizations (−40 mV) were without any obvious superimposition. The following analysis includes only one- or two-channel patches. Patches with any indication for more than two active channels were excluded. In two-channel patches only less than 4% of obviously overlapping events were allowed (−40 mV). Inclusion and exclusion of tentative overlapping events in two-channel patches caused differences in the mean open time τ_o by 4 ± 2% (mean ± SD). Patches were excluded if τ_o of the

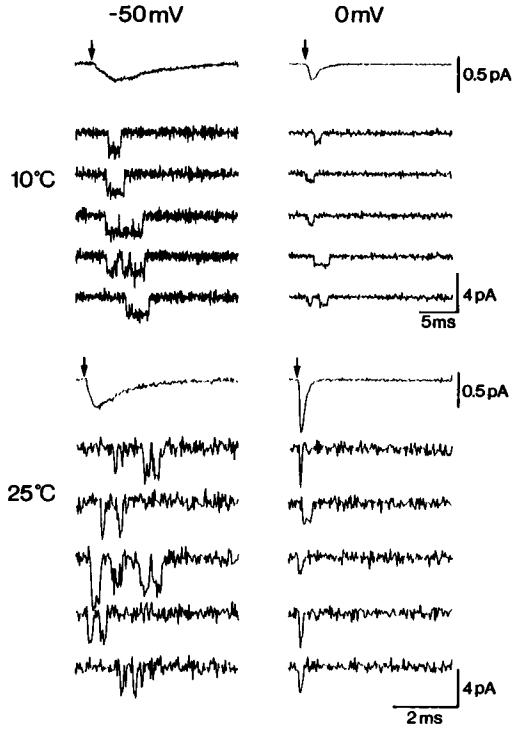

FIGURE 2 Gating behaviour of a cardiac sodium channel at two temperatures and voltages. Ensemble averaged currents (*top*) and representative single channel events are shown. At −50 mV, reopening appears at 25°C (filter 20 kHz) more frequent than 10°C (filter 10 kHz), whereas at 0 mV only one opening dominates the traces at both 25°C (filter 13 kHz) and 10°C (filter 5 kHz). Data at equal temperature were recorded from the same patch. Holding potential −130 mV.

first and the second half of traces differed by more than 10% (in the minutes before seal break, a continuous increase of τ_o up to 60% was observed eventually) or a switch to the bursting mode (Patlak and Ortiz, 1986, 1989) occurred which happened twice in 53 patches and could be easily identified by noninactivating mean currents and an increase of τ_o by a factor larger than 2.

Fig. 2 illustrates typical single Na^+ channel currents in cell-attached patches of mouse heart cells at test pulses of −50 and 0 mV and 10 and 25°C each. The temperature decrease of 15°C causes a dramatic prolongation of the open channel life time at both potentials. Recordings at −50 mV at the high temperature contain markedly more reopenings than at the same voltage at the low temperature whereas at 0 mV only one opening dominates at both temperatures, which implies that two processes with different temperature sensitivities contribute to the closing of the channels. The amplitudes of the single channel events are more homogeneous at the low temperature than at the high temperature, where multiple amplitudes were observed between 1 and 4.5 pA at −50 mV (Benndorf, 1993).

For sufficient statistics, either 500–1000 (10°C) or 1000–2000 (25°C) traces were recorded at each potential. The following analysis is independent of the contribution of one or two channels in the patch. It is based on 54,000 traces of 18 patches in which no evidence existed for the presence of more than two channels. After exclusion of long bursting openings, open time histograms, as obtained with the baseline method, could be fitted reasonably well with a single exponential at all voltages and both temperatures. Histograms in Fig. 3 illustrate that at 25°C τ_o is largest at −20 mV, whereas at 10°C τ_o only decreases toward more positive potentials. Therefore, the temperature coefficient Q_{10} at −50 mV must exceed that at 0 mV. Fig. 4 *A* illustrates data obtained in a single patch and Fig. 4 *B* summarizes from 54,000 traces the voltage dependence of $1/\tau_o$ at both temperatures. As suggested by the histograms in Fig. 3, between −50 and 0 mV $1/\tau_o$ is U-shaped only at 25°C but increases monotonically at the more positive potentials at 10°C. Q_{10} was found to be 4.8 at −50 mV and 3.6 at 0 mV. The surprisingly large value at −50 mV was confirmed by including as much as 20,000 traces at 25°C (10 patches) and 8,000 traces at 10°C (eight patches) in the analysis.

Assuming that exiting from the open state O can happen by deactivation to a closed state C (from which reopening is possible; rate constant β_C) or by inactivation to the absorbing inactivated state I (rate constant β_I; Aldrich et al., 1983; Horn and Vandenberg, 1984), $1/\tau_o$ is then given by

$$1/\tau_o = \beta_C + \beta_I \quad (1)$$

with β_C and β_I being voltage-dependent. Following Eyring rate theory, voltage dependence of both rate constants is (Hille, 1992)

$$\beta_C = kT/h \exp(-\Delta H_C^{\ddagger}/RT + \Delta S_C^{\ddagger}/R + Q_C^{\ddagger} V/RT) \quad (2)$$

$$\beta_I = kT/h \exp(-\Delta H_I^{\ddagger}/RT + \Delta S_C^{\ddagger}/R + Q_I^{\ddagger} V/RT) \quad (3)$$

where V is the voltage across the patch, R the molar gas constant, T the absolute temperature, k the Boltzmann constant, and h the Planck constant. $\Delta H_C^{\ddagger}$ and $\Delta S_C^{\ddagger}$ are the enthalpy and entropy, respectively, corresponding to the step from O to the transition state for deactivation. $\Delta H_I^{\ddagger}$ and $\Delta S_I^{\ddagger}$ are the respective values for inactivation. $Q_C^{\ddagger}$ and $Q_I^{\ddagger}$ are the equivalent charges which are given by the product of the

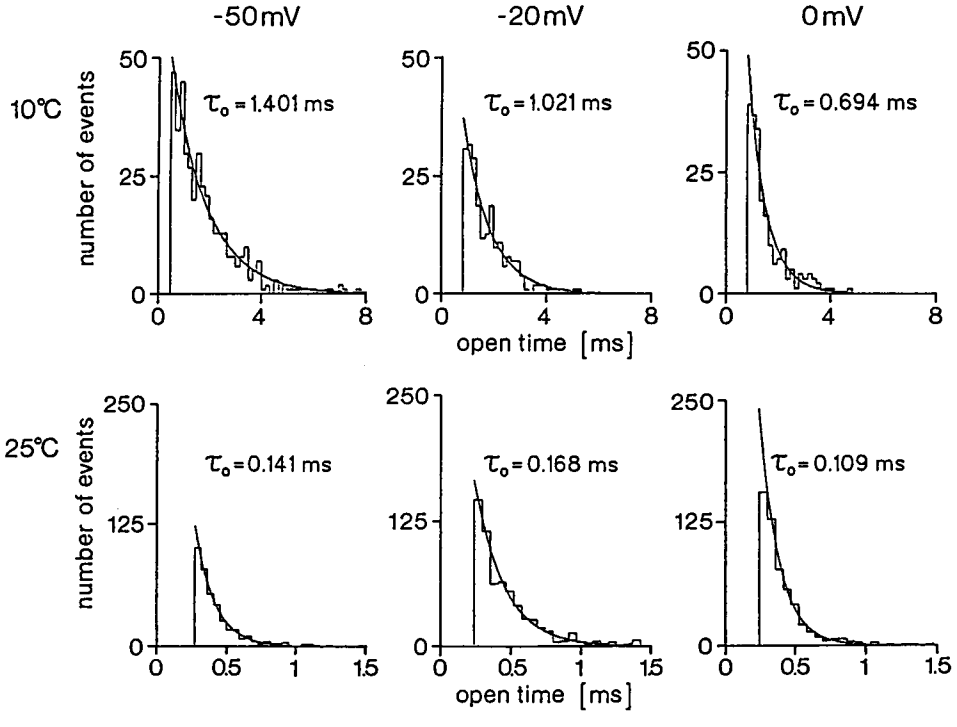

FIGURE 3 Histograms of the open channel life time as formed with the baseline method. (*A*) Histograms of two patches at two temperatures and three voltages. At 25°C (2000 traces each, filter 13 kHz, binwidth 45 μs), τ_o is smaller at both −50 and 0 mV than at −20 mV; at 10°C (1000 traces each, filter 5 kHz, binwidth 150 μs) τ_o decreases toward stronger depolarization.

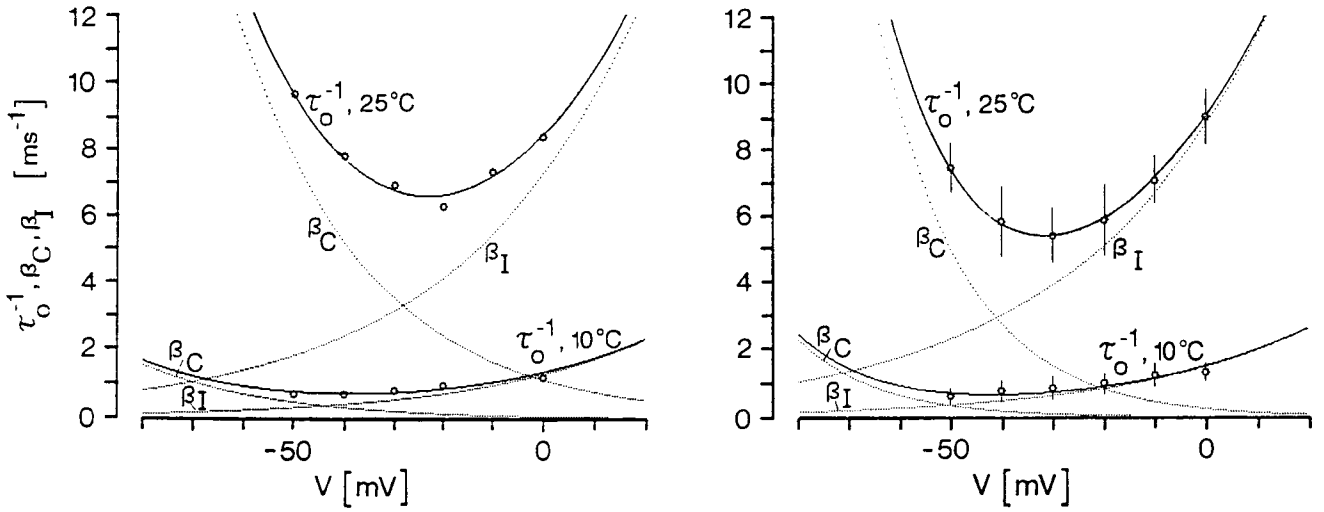

FIGURE 4 Energetics of deactivation and inactivation calculated from voltage and temperature dependence of τ_o. $1/\tau_o$ was plotted as function of voltage at 10 and 25°C. (*A*) Data were obtained from a tentative single channel patch with 1000 (25°C) and 500 (10°C) traces per patch and voltage. *Simultaneous* fit of the data at both temperatures with Eq. 1 yielded $\Delta H_C^\ddagger$ = 184 kJ mol^{-1} (0.35), $\Delta S_C^\ddagger$ = 0.42 kJ mol^{-1} K^{-1} (0.46), $Q_C^\ddagger$ = −1.59 equivalent charges (0.53), $\Delta H_I^\ddagger$ = 83 kJ mol^{-1} (0.08), $\Delta S_I^\ddagger$ = 0.11 kJ mol^{-1} K^{-1} (0.22), $Q_I^\ddagger$ = + 0.38 equivalent charges (0.37). The values in brackets indicate the normalized approximate standard deviation as obtained from the covariance matrix. The correlation matrix of the fit is as follows.

	$\Delta H_C^\ddagger$	$\Delta S_C^\ddagger$	$\Delta H_I^\ddagger$	$\Delta S_I^\ddagger$	$Q_C^\ddagger$	$Q_I^\ddagger$
$\Delta H_C^\ddagger$	-					
$\Delta S_C^\ddagger$	0.99	-				
$\Delta H_I^\ddagger$	0.43	0.39	-			
$\Delta S_I^\ddagger$	0.43	0.39	0.99	-		
$Q_C^\ddagger$	0.65	0.61	0.80	0.80	-	
$Q_I^\ddagger$	−0.87	−0.86	−0.61	−0.60	−0.69	-

(*B*) Lumped data from 18 patches. Data points at −50 mV are the mean of 10 (25°C) and eight (10°C) patches, all other points are the mean of three to five patches. The *simultaneous* fit with Eq. 1 yielded $\Delta H_C^\ddagger$ = 129 kJ mol^{-1}, $\Delta S_C^\ddagger$ = 0.23 kJ mol^{-1} K^{-1}, $Q_C^\ddagger$ = −1.54 equivalent charges, $\Delta H_I^\ddagger$ = 79 kJ mol^{-1}, $\Delta S_I^\ddagger$ = 0.10 kJ mol^{-1} K^{-1}, $Q_I^\ddagger$ = + 0.68 equivalent charges. Bars indicate SD. All curves were extrapolated to both sides of the measured data to demonstrate the calculated voltage dependence in the range of physiological interest.

Faraday constant, the gating charge displacement across the membrane, and the fraction of the total voltage drop across the membrane (Hille, 1992). Based on the characteristic bimodal voltage dependence of τ_o at 25°C and the decrease of reopening at more positive potentials, $Q_C^\ddagger < 0$ and $Q_C^\ddagger > 0$ must hold. No reasonable *simultaneous* fit of the data with Eq. 1 was obtained at both temperatures if only in one transition enthalpic and entropic part of activation energy were lumped. In other words, adequate *simultaneous* fit was obtained only if enthalpic and entropic part of energy were treated as independent parameters in both deactivation and inactivation, i.e., the constraints were obviously sufficient to separate both energy contributions in both transitions. The respective curves are illustrated for the tentative one-channel patch (Fig. 4 *A*) and the lumped data of 19 patches (Fig. 4 *B*). The relatively large error bars (SD) in Fig. 4 *B* correspond less to a scatter of the data *within* the individual patches but more to differences *among* the patches; i.e., the well-shaped voltage dependence of $1/\tau_o$ was much better defined than the error bars suggest. Therefore, the precision of the parameter estimates is only shown for the individual patch in Fig. 4 *A*. The legend of Fig. 4 *A* gives the normalized approximate standard deviation (square root of the normalized diagonal elements of the covariance matrix) and the correlation matrix (Colquhoun and Sigworth, 1983) of the fit. The high correlation between the entropic and enthalpic part of energy for both transitions indicates that, in the vicinity of the local minimum, fits with only slightly worse quality may be reached by a proportional change of $\Delta S^\ddagger$ and $\Delta H^\ddagger$. The approximate standard deviation shows that the data for inactivation are superior in precision above those of deactivation.

In spite of the influence of the heterogeneity among the patches, the energies and entropies in the individual patch and the lumped data are in the same range which shows consistency of the data among the patches. As a result from Fig. 4 *B*, 1) both transitional entropies are positive with deactivation entropy $\Delta S_C^\ddagger$ = 0.23 kJ mol^{-1} K^{-1} (27.6 *k*) being more than twice larger than inactivation entropy $\Delta S_I^\ddagger$ = 0.10 kJ mol^{-1} K^{-1} (11.6 *k*), 2) the transitional enthalpy of deactivation $\Delta H_C^\ddagger$ = 129 kJ mol^{-1} exceeds that of inactivation $\Delta H_I^\ddagger$ = 79 kJ mol^{-1}, and 3) the absolute value of the equivalent charge of deactivation $Q_C^\ddagger$ = −1.54 is larger than that of inactivation $Q_I^\ddagger$ = + 0.68.

DISCUSSION

The method of measuring open times τ_o of channels in the center of the baseline noise yielded more consistent results among the patches than setting the threshold to any other level and it was certainly most advantageous in those patches

with most pronounced heterogeneity of the single channel current amplitudes (Benndorf, 1993). Reliable estimates for the mean open time could be obtained if the τ_o exceeded the time constant approximating the false noise events by more than twice which was the case in all histograms. Theoretically, τ_o estimates determined with the baseline method must exceed those determined with the 50% threshold procedure because part of the closing events, with durations shorter than T_{10-90}, do not reach the baseline but cross the 50% threshold. The benefit, however, to measure open times at noise levels more than double as large as possible with the 50% threshold procedure, clearly dominates over this shortcoming. This becomes particularly obvious by the finding that at 25°C here open times in the range of 150 μs were found, whereas in a previous study, working with lower recording bandwidth and the 50% threshold technique, respective values around 400 μs were obtained (Benndorf, 1988). Major influence of short closures on the measured open times may also be excluded by the fact that data obtained from recordings filtered at 13 and 20 kHz were indistinguishable (Student's t test).

With respect to the determined entropies we conclude that conformational transition entropies may be determined even in individual channel molecules if a patch clamp technique with sufficiently low noise is used. In the cardiac sodium channel, deactivation proceeds with larger conformational change than inactivation. This fits nicely with the idea that deactivation goes along with a more complex arrangement in the channel core, whereas fast inactivation takes place at the cytosolic inactivation gate (Stühmer et al., 1989; Patlak, 1991). In comparison with previous findings in macroscopic sodium currents, transitional entropy of deactivation calculated here is more than three times larger than reported by Levitan and Palti (1975) and similar to (Conti, 1986; Kimura and Meves, 1979) or half as much (Jonas, 1989) as the estimated entropy differences between C and O, which may be considered as lower limit estimates of $\Delta S_C^{\ddagger}$. The reason for these differences is not clear. Compared to $\Delta S_C^{\ddagger} = 0.079$ kJ/mol^{-1} K^{-1} reported in single batrachotoxin-treated sodium channels by Correa et al. (1992), the value found here is three times larger. It is intriguing to speculate that batrachotoxin modifies the channel such that already smaller conformational changes are sufficient for closing an open channel. The amount of entropy associated with microscopic inactivation $\Delta S_I^{\ddagger}$ can not be related to entropy changes associated with macroscopic inactivation (Conti, 1986; Jonas, 1989), because the latter is predominantly a function of the first latency (Aldrich et al., 1983). Single channel data are not available at present.

Determination of transition entropies in single ionic channels with the present or improved resolution might help in the future to gain deeper insight into the microscopic heterogeneity of presumably identical channels or among families of closely related ionic channels. In conjunction with genetic techniques, knowledge of transitional entropy changes should also contribute to an improved understanding of structure-function relationships in ion channels.

We are indebted to B. Neumcke, H.-J. Apell, and V. Ganitkievich for critical comments on an earlier version of the manuscript and to D. Metzler and R. Kemkes for excellent technical assistance.

This work was supported by the Deutsche Forschungsgemeinschaft (Be 1250/1-4).

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