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Inactivation of sodium channels in isolated myocardial mouse cells

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Abstract. Inactivation of sodium currents is investigated in single myocardial mouse cells by the use of a one suction pipette voltage clamp technique. Semilogarithmic plots of the decay phase of sodium currents show two phases and analysis yields a fast (τ_{h1}) and a slow (τ_{h2}) time constant. At increased depolarization the contribution of τ_{h2} is decreased. Increasing the temperature from 22.0 ± 0.5 °C to 36.5 ± 0.5 °C influences τ_{h1} and τ_{h2} to the same extent. The Q_{10} values were 2.28 ± 0.32 for τ_{h1} and 2.46 ± 0.35 (mean ± SD) for τ_{h2}. Between −50 and −20 mV the time course of current decay is substantially faster than inactivation induced by prepulses. Onset of inactivation by the prepulse protocol needs two time constants (τ_{c1}, τ_{c2}) for a satisfactory description. Both decrease steeply with increasing depolarization. For small depolarizations, τ_{c2} values are in the range of several seconds. Recovery from inactivation by short prepulses (40 ms) could be described by two exponentials (τ_{r1}, τ_{r2}). For longer prepulses (1.000 ms) a very slow component, τ_{r3}, was observed indicating a history dependence of inactivation. Delay time constants for the onset of inactivation (τ_{dc}) are determined between −50 and −20 mV. The more depolarizing voltages generate smaller delay times (0.55 ± 0.10 ms at −20 mV, mean ± SD) but larger deviations from the exponential time course. Delay in recovery from inactivation (time constant τ_{dr}) has the value 2.0 ± 0.7 ms (mean ± SD) at −80 mV and decreases at more hyperpolarizing potentials. The remaining series resistance at maximum compensation was calculated as 80 kΩ. Its influence on the sensitive delay in the double pulse inactivation is discussed.

Key words: Myocardial mouse cells, voltage clamp, sodium channels, inactivation kinetics

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Introduction

There have been many more detailed investigations on macroscopic inactivation of sodium current in nerve than in heart membrane (for review see Armstrong 1981). The development of the voltage clamp technique for single heart cells (Lee et al. 1979; Brown et al. 1981a; Irisawa and Kokubun 1983; Fozzard et al. 1984) has permitted a more detailed analysis of ionic currents in heart. The heart cell membrane shows several properties that cannot be described by the Hodgkin-Huxley model. Brown et al. (1981b) reported biexponential time courses for Na current decay and for the onset of inactivation with the double step protocol. They also found a delay in the time course of recovery from inactivation. Clark and Giles (1984) found a delay in the onset of inactivation in bullfrog atrial myocytes. All these findings have their precedents in nerve preparations (Schauf 1974; Bezanilla and Armstrong 1977; Chiu 1977; Nonner 1980; Kniffki et al. 1981; Goldman and Kenyon 1982). The question as to whether there is identity of time constants of Na current decay and onset of inactivation by application of prepulses is the subject of dispute for the heart muscle (Ebihara and Johnson 1980; Brown et al. 1981b). For the description of findings which deviate from the Hodgkin-Huxley theory many types of models have been developed. These models imply more than two states of the h-gate (Goldman 1975; Oxford and Pooler 1975; Bezanilla and Armstrong 1977; Chiu 1977; Nonner 1980; Goldman and Kenyon 1982) and are of sequential or cyclic type with independent or coupled activation and inactivation. Since the introduction of single channel recording the analysis in terms of state models with multiple closed and one or two open states has become more and more important (Aldrich et al. 1983; Horn and Vandenberg 1984).

In this paper we present a detailed analysis of the time course of macroscopic inactivation in single heart cells. The time courses of Na current decay, double pulse inactivation, and recovery from inactivation can only be described by at least two exponentials. Furthermore, extremely slow inactivation components are quantified and the existence of lags in both double pulse inactivation and recovery from inactivation are demonstrated.

Methods

Isolated ventricular cells of adult albino mice were prepared by a technique similar to that described previously (Benndorf et al. 1985). In brief: By means of a Langendorff perfusion technique mouse hearts (200–300 mg each) were washed free from blood (3 min) with a solution composed of 150 m*M* Na, 5.4 m*M* KCl, 2.5 m*M* $CaCl_2$, 0.5 m*M* $MgSO_4$, 5 m*M* HEPES, 11.1 m*M* glucose.

The solution in the perfusion system was then changed (5 min) to a Ca free solution containing 140 m*M* NaCl, 5.8 m*M* KCl, 0.5 m*M* KH_2PO_4, 0.4 m*M* Na_2HPO_4, 0.9 m*M* $MgSO_4$, 11.1 m*M* glucose, 5 m*M* HEPES, 0.1 mg/ml fatty acid free albumin (FFA). The open perfusion system was then changed to a recirculating one for a period (15 min) of enzyme digestion with a solution composed of 120 m*M* NaCl, 5.8 m*M* KCl, 0.5 m*M* KH_2PO_4, 0.4 m*M* Na_2HPO_4, 0.9 m*M* $MgSO_4$, 11.1 m*M* glucose, 20 m*M* HEPES, 0.1 mg/ml FFA, 1 mg/ml collagenase.

The hearts were then washed (4 min) with a Ca free solution containing 52.5 m*M* NaCl, 4.8 m*M* KCl, 1.19 m*M* KH_2PO_4, 1.2 m*M* $MgSO_4$, 11.1 m*M* glucose, 145 m*M* sucrose, 10 m*M* HEPES, 1 mg/ml FFA. Then the hearts were removed from the apparatus and minced with scissors in a petri dish filled with the last washing solution. The suspension was immediately filtered through a 250–300 µm nylon sieve and stored for at least 3 hours in the same solution at room temperature. During the cell isolation procedure the solutions were gassed with oxygen and the temperature was adjusted to 37.0 ± 0.2 °C. The flow in the perfusion apparatus was kept constant at 1.5 ml/min by a roller pump.

For electrophysiological measurements 50 µl of cell suspension were added to the 1.2 ml external solution in the experimental chamber. The external solution hat the same composition as the last washing solution but with a physiological Ca level (2.5 m*M*); pH of all external solutions was adjusted to 7.4. Under visual control a brick-shaped cell was selected and sucked to the opening of a fire polished glass pipette which was perfused by an internal solution containing 150 m*M* KOH, 13 m*M* NaH_2PO_4, 1 m*M* EGTA, pH 7.1 ± 0.05 (H_3PO_4). Since we used physiological concentrations for intracellular sodium and potassium, experiments did not critically depend on an effective dialysis of the cell interior (cf. Benndorf et al. 1985). Non-linear outward currents appeared only at potentials positive to − 10 mV but never exceeded 2 nA at + 30 mV. For all properties of Na current inactivation described here the complete block of outward current by 1 m*M* 4-aminopyridine (Goldman and Kenyon 1982) had no influence. In general the drug was omitted because in most experiments all voltages were negative and the viability of the cell under investigation was better without the drug.

The voltage clamp amplifier was connected to the external and internal solution via Ag/AgCl electrodes and 3 *M* KCl agar bridges. The influence of series resistance (r_s) was reduced by *(i)* the exclusive use of pipettes with blunt shanks and wide (12 µm) and short (< 5 µm) channels at the tip, *(ii)* an external Na concentration of 52.5 m*M*, *(iii)* performing the experiments at room temperature (22 ± 0.6 °C) unless otherwise noted, and *(iv)* the use of a compensated feedback. As reported elsewhere (Benndorf et al. 1985) an r_s of only 80 kΩ was left as judged from time course of capacitive transients at maximum compensation. This value is more than one order of magnitude smaller than the membrane resistance during maximum sodium conductance. Using an additional microelectrode Benndorf et al. (1985) determined the settling time of the voltage clamp as 30 µs and a maximum difference at 3.5 mV between voltages measured with the suction pipette and the microelectrode. Later measurements showed that for the results presented here 2.5 mV is an upper limit.

All chemicals used were of analytical grade. Collagenase was obtained from Serva (puriss.), 4-aminopyridine from Merck. Essentially fatty acid free albumin was prepared from human albumin for clinical use (Serumwerk Dessau).

Currents were photographed from a storage oscilloscope (Messelektronik Berlin). Calculations and approximations were done on a MC 80 computer system (Elektronik Gera) and a Commodore 64 personal computer.

Results

Temperature dependence of the biexponential decay

In general, the decay phase of the Na current cannot be described with one time constant, as done by

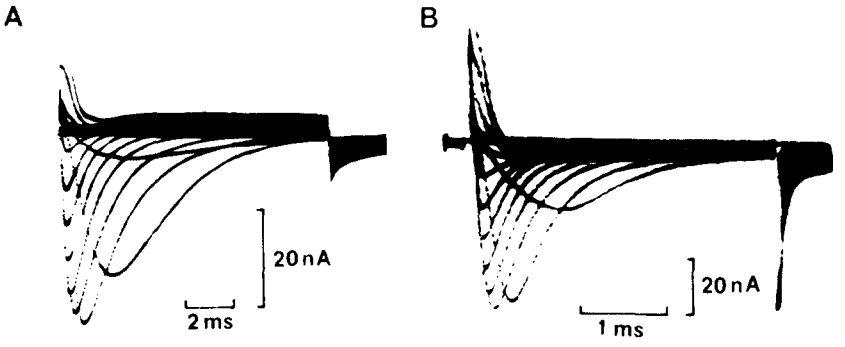

Fig. 1 A and B. Time course of Na current at two temperatures. **A** Current response to 16 depolarizing steps at 10 mV intervals in command potential at 22.3 °C. $V_h = -100$ mV, step duration 11 ms, cell 300484-9. **B** Same protocol as **A** at 36.7 °C, step duration 3.5 ms, 1 m*M* 4-aminopyridine, cell 160884-7

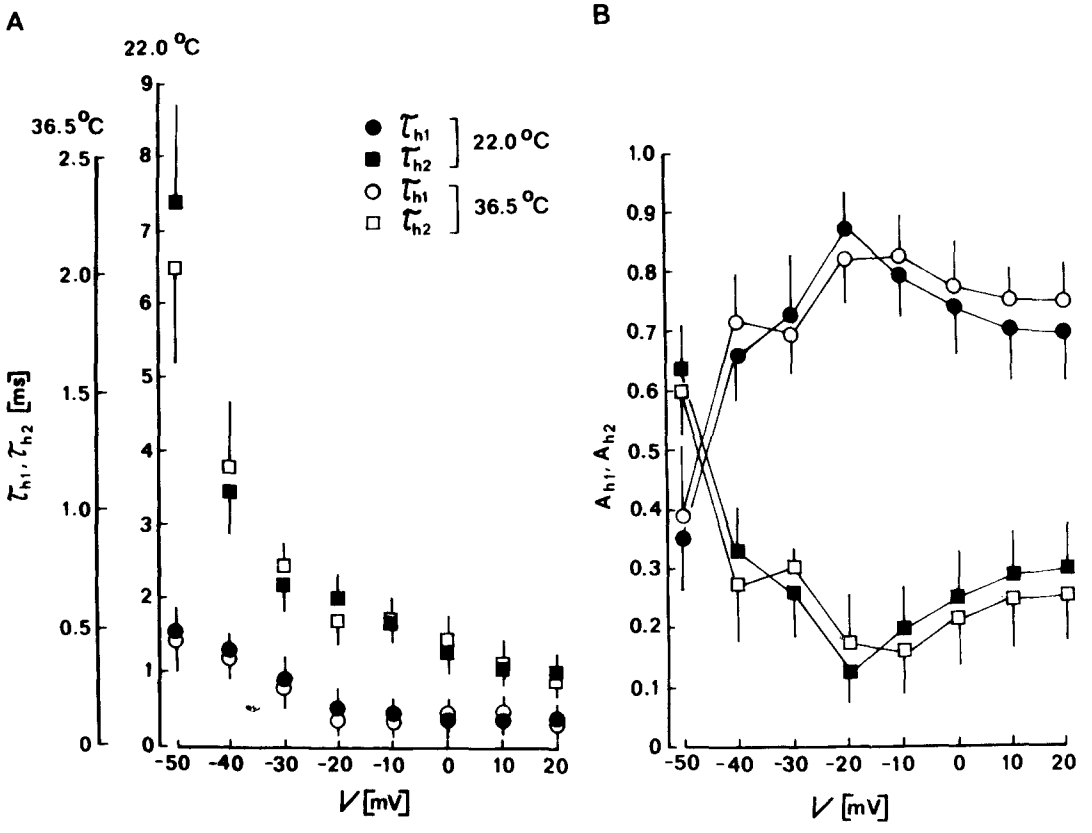

Fig. 2A and B. Voltage dependence of biexponential decay at two temperatures. The decay phase of the currents was plotted (40 points) semilogarithmically and could be described by two straight lines according to $I_{Na,dec} = A_{h1} \exp(-t/\tau_{h1}) + A_{h2} \exp(-t/\tau_{h2})$. **A** Voltage dependence of τ_{h1} and τ_{h2} at 22.0 ± 0.6 °C ($n = 4$) and 36.5 ± 0.6 °C ($n = 3$). *Bars* indicate SD. The temperature influences both time constants to the same extent. **B** Voltage dependence of the contribution A_{h1} and A_{h2} of both time constants. The symbols correspond to the time constants in **A**. There is no significant difference of the contribution of the time constants at both temperatures. *Bars* indicate SD

Hodgkin and Huxley (1952a, b) for the squid giant axon or Ebihara and Johnson (1980) for cardiac muscle. In myelinated nerve (Chiu 1977; Nonner 1980; Kniffki et al. 1981) or isolated rat ventricular cells (Brown et al. 1981 b) a better fit with two exponentials has been reported . Figure 1 shows two families of Na currents obtained from myocardial mouse cells at 22.3 °C and at 36.7 °C. From the semilogarithmic plot of the decay phases of such currents it is clear that the best fit is obtained with two exponentials. We described the decay by (cf. Chiu 1977)

$$I_{Na,ced} = A_{h1} \exp(-t/\tau_{h1}) + A_{h2} \exp(\alpha/\tau_{h2}) \quad (1)$$

A_{h1} and A_{h2} define the amplitudes of the fast and slow time constant τ_{h1} and τ_{h2}, respectively. In Fig. 2A both time constants of inactivation are plotted as a function of the test potential for 22.0 ± 0.6 °C ($n = 3$). The scaling of the ordinates has been chosen to demonstrate an equal accelerat-

ing effect of the increased temperature on both time constants. Q_{10} values have been calculated for τ_{h1} and τ_{h2} at every test potential by use of the equation for the conventional temperature coefficient

$$Q_{10} = [\tau_{hx}(T_1)/\tau_{hx}(T_2)]^{-10/(T_2-T_1)}, \quad (2)$$

where $\tau_{hx}(T_1)$ and $\tau_{hx}(T_2)$ are the fast ($x=1$) and slow ($x=2$) inactivation time constants at the higher (T_2) or lower (T_1) temperature, respectively. The Q_{10} values were voltage independent and have been determined as 2.28 ± 0.32 for τ_{h1} and 2.46 ± 0.39 for τ_{h2}. Panel B of Fig. 2 illustrates the voltage dependence of the normalized contributions of the time constants at both temperatures. There is no significant difference between the corresponding A_{h1} and A_{h2} values at the lower and the higher temperature. Thus, different behaviour of both components at different temperatures as reported for the squid giant axon by Matteson and Armstrong (1982) and for the nodal membrane by Benoit et al. (1985) could not be observed.

The decrease of A_{h1} and the increase of A_{h2} positive to -20 mV (Fig. 2B) may be explained by progressive uncertainties of the approximation procedure due to the smaller amplitude of the currents.

In order to exclude the possibility of an influence of outward currents on the later time course of the Na currents two families at each temperature were recorded in the presence of 1 m*M* 4-aminopyridine, which completely blocks the most prominent transient outward current (Benndorf and Nilius 1987). Negative to 0 mV the drug did not alter the decay and up to +20 mV unblocked outward currents had negligible effects.

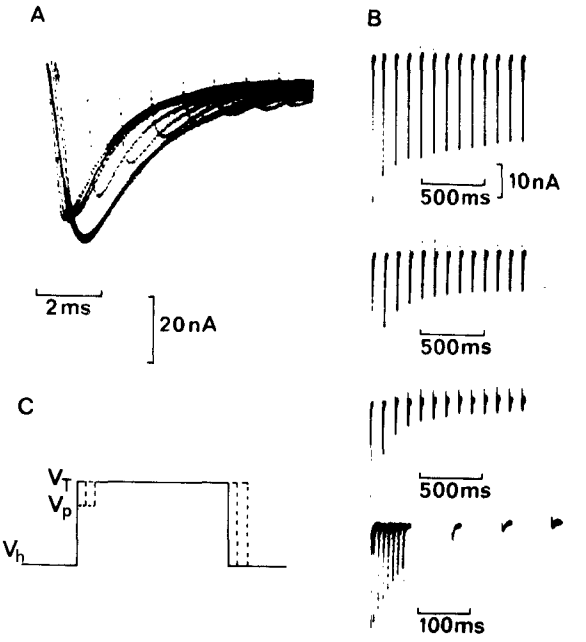

Fig. 3A – C. Onset of inactivation. **A** Current responses to a voltage protocol as shown in part **C**. $V_h = -90$ mV, $V_p = -30$ mV, $V_T = -10$ mV, test step duration 20 ms. Currents were elicited after prepulse durations of 0.2, 0.4, 0.6, 0.8, 1, 2, 3, 4, 5, 6, 7, and 8 ms. The lowest trace represents current at V_p. The time course of sodium current decay at V_p is faster ($\tau_{h1} = 2.5$ ms) than onset of inactivation at the same potential determined with the double pulse protocol ($\tau_{c1} = 4.4$ ms), cell 080584-22. **B** Four series of currents obeying the same protocol with less depolarizing V_p (-80; -70; -60; -50 mV; from the top), all recordings from the same cell over a period of 8 min. Calibration of 10 nA is valid for all recordings. $V_p = -80$ mV; $\tau_{c1} = 125$ ms; $\tau_{c2} = 10.000$ ms; $V_p = -70$ mV; $\tau_{c1} = 120$ ms. $\tau_{c2} = 5{,}200$ ms; $V_p = -60$ mV; $\tau_{c1} = 95$ ms. $\tau_{c2} = 1.950$ ms; $V_p = -50$ mV: $\tau_{c1} = 75$ ms. τ_{c2} not resolvable, cell 270384-13. **C** Pulse protocol for onset of inactivation, V_h holding potential, V_p prepulse potential, V_T test potential

Onset of inactivation measured with a double pulse protocol

The time course of inactivation of Na current by depolarizing prepulses of varying lengths was studied for comparison with the characteristics of Na current decay under maintained depolarization. From $V_h = -100$ mV the clamp potential was stepped to a prepulse potential (V_p) between -80 and -20 mV for various durations. The test potential was adjusted to -10 mV with a duration between 10 and 20 ms. The decline of peak Na currents during the test pulse was evaluated. Figure 3 shows such a series of currents at different time scales. The high time resolution in panel A of this figure reveals a less steep fall of currents compared to the decay of current at the beginning of the prepulse. A second finding is a delay in onset of inactivation during the first millisecond that will be examined later. Part B of Fig. 3 shows current traces for four less depolarizing prepulses of far longer duration. The existence of time constants in the range of seconds is evident. Alterations of current magnitude during recordings were excluded by comparison of a succeeding current without prepulse with the control current at the beginning. The onset of inactivation which was obtained by the peak inward current at test pulse potential was described by

$$I_{\mathrm{Na,on}} = A_{c1} \exp(-t/\tau_{c1}) + A_{c2} \exp(-t/\tau_{c2}) + C. \quad (3)$$

As for Eq. (2), τ_{c1} and τ_{c2} are the small and large time constants with a relative contribution A_{c1} and A_{c2}, respectively. C is a steady state value for incomplete inactivation at moderate depolarizing test pulses. In practice C was determined first, and the other parameters of (3) were then determined as described for (2).

Table 1 shows the results: numbers in parenthesis indicate numbers of investigated cells. Values of C

Table 1. Parameters for onset of inactivation with the double pulse method according to (3)

V_p [mV]	A_{c1}	τ_{c1} [ms]	A_{c2}	τ_{c2} [ms]	C
−20	0.98 ± 0.01 (2)	2.0 ± 0.3 (2)	0.02 (1)	32	0
−30	0.95 ± 0.03 (9)	5.0 ± 1.5 (9)	0.04 ± 0.02 (5)	51 ± 25 (5)	0
−40	0.95 ± 0.03 (7)	11.9 ± 4.2 (7)	0.05 ± 0.03 (3)	133 ± 70 (3)	0
−50	0.93 ± 0.04 (4)	53.2 ± 23.6 (4)	0.04 ± 0.03 (2)	960 ± 480 (2)	0.03 ± 0.2 (2)
−60	0.65 ± 0.09 (2)	91.0 ± 12.7 (2)	0.24 ± 0.08 (2)	1.640 ± 509 (2)	0.11 ± 0.04 (2)
−70	0.25 ± 0.13 (3)	120 ± 41 (3)	0.34 ± 0.16 (3)	3.250 ± 1.850 (3)	0.43 ± 0.10 (3)
−80	0.12 (1)	125 (1)	0.28 (1)	10.000 (1)	0.60 (1)

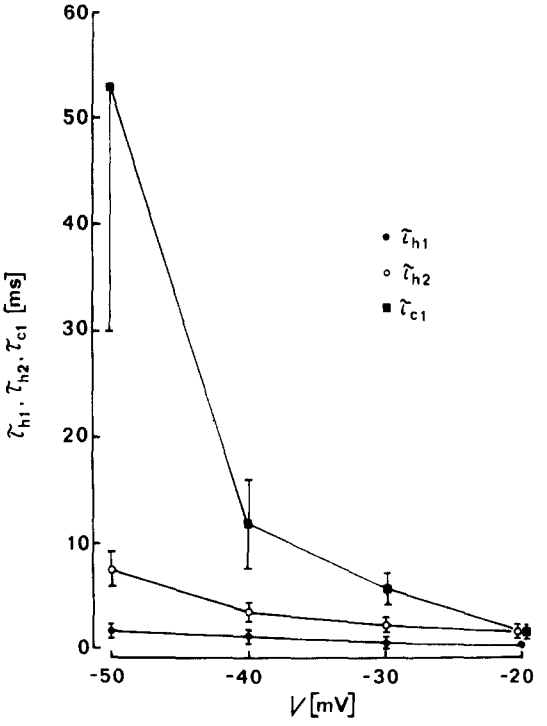

Fig. 4. Relation between time constants of Na current decay and onset of inactivation with double pulse protocol in dependence on membrane voltage. Data for τ_{h1} and τ_{h2} are equivalent to those obtained at room temperature in Fig. 2. Data of τ_{c1} were obtained from Table 1. Note the slow inactivation measured with the double pulse protocol in comparison to slow and fast part of Na current decay. *Bars* indicate SD

represent the curve of steady state inactivation h_∞. Between −20 and −50 mV, $I_{Na,on}$ can be described by τ_{c1} alone whereas for less depolarizing V_p its contribution is smaller. In spite of a considerable scatter between −50 and −70 mV τ_{c2} could reliably be identified and could easily be separated from the steady state component C. At −80 mV in one experiment a value larger than 10,000 ms was estimated for τ_{c2}, indicating very slow mechanisms of inactivation at very negative test potentials.

Inactivation determined with the double step method is far slower than Na current decay. Figure 4 shows the voltage dependence of τ_{h1}, τ_{h2}, and τ_{c1}, τ_{c2} was omitted due to the small values of A_{c2} in the voltage range considered. The larger time constant of decay τ_{h2} and the smaller one of onset τ_{c1} coincide at −20 mV whereas at more negative potentials τ_{c1} substantially exceeds τ_{h2}.

Recovery from inactivation

The previous section suggests that a complete inactivation of Na current occurs for a 40 ms pulse to −10 mV. Recovery from that inactivation was investigated using the pulse pattern of Fig. 5 C. The prepulse was followed by a testpulse also to −10 mV separated by an increasing time interval at different voltages (V_{ti}). Figures 5 A and B show two series of current records for recovery at $V_{ti} = -120$ mV and $V_{ti} = -80$ mV, respectively. The less negative V_{ti} causes a slower recovery. The large trace at the beginning of both recordings serves as control current for unaltered conditions during the period of recording and was therefore elicited last, without an inactivating prepulse. A very reproducible finding is the appearance of a transient outward current after sodium currents without a prepulse (cf. Fig. 6). Those outward currents which have been described as potassium currents (Benndorf and Nilius 1987) do not influence peak sodium currents because at this time they are still not activated. To a first approximation any delay was neglected and semilogarithmic plots of peak current versus recovery time were constructed (cf. Fig. 8 B). They revealed that two time constants were required for a satisfactory description of recovery phenomena. Table 2 shows the determined parameters according to

$$1 - I_{Na,rec} = A_{r1} \exp(-t/\tau_{r1}) + A_{r2} \exp(-t/\tau_{r2}), \quad (4)$$

where $I_{Na,rec}$ is the peak recovery current normalized to the peak of control current. A_{r1} and A_{r2} are the relative contribution of the small and large time constants τ_{r1} and τ_{r2}, respectively. At −80 mV recovery is dominated by τ_{r2} and at more negative potentials by τ_{r1}.

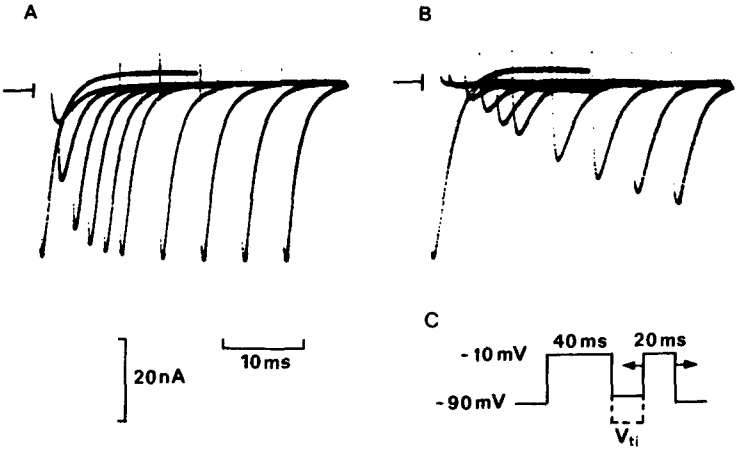

Fig. 5 A and B. Recovery from inactivation as a function of the voltage V_{ti} during the recovery interval. **A** Current records of a recovery time course at time interval voltage of −120 mV. With (4) τ_{r1} and τ_{r2} have the values 1.4 and 7.5 ms, respectively. The time course is dominated by the fast component, i.e. $A_{r1} = 0.77$. **B** The protocol is altered by adjusting V_{ti} at − 80 mV. Peak currents could be fitted by only one time constant (τ_{r2} = 28 ms). **C** Pulse protocol, for both series of currents values for time intervals were 1, 2, 4, 6, 8, 10, 15, 20, 25, 30 ms, cell 110484-13

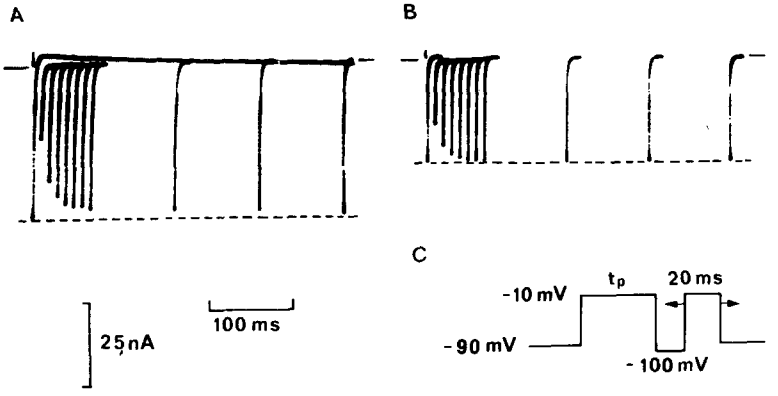

Fig. 6 A – C. Appearance of large time constants in recovery from inactivation after long lasting prepulses. **A** and **B** show series of recovering currents for prepulse times t_p of 1.000 ms and 40 ms, respectively. Using (4a) for τ_{r3} = 910 ms was calculated. No such slow process is detectable in **B**. The initial large current was elicited last without an inactivating prepulse for control of stable conditions. Both recordings are from the same cell. **C** Pulse protocol used, t_p prepulse time, currents were elicited after time intervals of 10, 20, 30, 40, 50, 60, 70, 170, 270, 370 ms. V_{ti} = − 100 mV, cell 110484-18

Table 2. Parameters for recovery from inactivation with 40 ms prepulse according to (4)

V_{ti} [mV]	A_{r1}	τ_{r1} [ms]	A_{r2}	τ_{r2} [ms]
− 80	0.09 (1)	3.9 (1)	0.96 ± 0.09 (3)	30.3 ± 9.3 (3)
− 120	0.74 ± 0.10 (3)	3.2 ± 0.9 (3)	0.24 ± 0.08 (3)	19.6 ± 6.8 (3)
− 120	0.89 ± 0.09 (3)	1.8 ± 0.6 (3)	0.12 ± 0.05 (3)	11.1 ± 4.5 (3)

The large time constants τ_{c2}, found between − 40 and − 80 mV for the onset of inactivation give rise to the question as to whether the completely inactivated states are the same in the cases of short and long prepulses. Therefore, in a second series of experiments 1,000ms was chosen for long lasting prepulses and the recovery program was repeated as already described. Figure 6A shows an example of such a series of currents, in comparison to the corresponding currents preceded by a 40 ms prepulse (B) recorded from the same cell. B was taken 7 min after A. That may explain the reduction of amplitude. The dotted line is intended as a help for detection of the very slow component in A. For quantification, Eq. (4) was expanded to

$$1 - I_{Na,rec} = A_{r1} \exp(-t/\tau_{r1}) + A_{r2} \exp(-t/\tau_{r2}) + A_{r3} \exp(-t/\tau_{r3}), \quad (4a)$$

where A_{r3} is the contribution of the time constant for very slow recovery from inactivation. Parameters again were obtained from semilogarithmic plots (Table 3). Adjusting V_{ti} at − 80 and − 100 mV a τ_{r3} clearly was separable, indicating a difference in inactivated states after short and long depolarizations. A second difference is demonstrated in Fig. 7. Open symbols result from experiments with 1,000 ms prepulses, filled symbols from experiments with 40 ms prepulses. For all three tested voltages, V_{ti}, there is a deceleration during the first 15 ms of recovery after long prepulses.

In general, recovery time constants seem to be not as strongly voltage-dependent as time constants for onset of inactivation. If the recovery time course is approximated by a bi- or triexponential equation as in (4) or (4a), the voltage dependence is influenced mainly by changes in the amplitude of the compo-

Table 3. Parameters for recovery from inactivation with 1,000 ms prepulse according to (4a)

V_{ti} [mV]	A_{r1}	τ_{r1} [ms]	A_{r2}	τ_{r2} [ms]	A_{r3}	τ_{r3} [ms]
− 80	0		0.95 ± 0.03 (3)	65.1 ± 16.2 (3)	0.06 ± 0.03 (3)	706 ± 258 (3)
− 100	0.31 ± 0.09 (3)	4.5 ± 0.9 (3)	0.70 ± 0.16 (3)	33.2 ± 11.3 (3)	0.09 ± 0.04 (3)	659 ± 311 (3)
− 120	0.29 ± 0.10 (3)	3.4 ± 0.8 (3)	0.70 ± 0.19 (3)	29.3 ± 10.4 (3)	0	

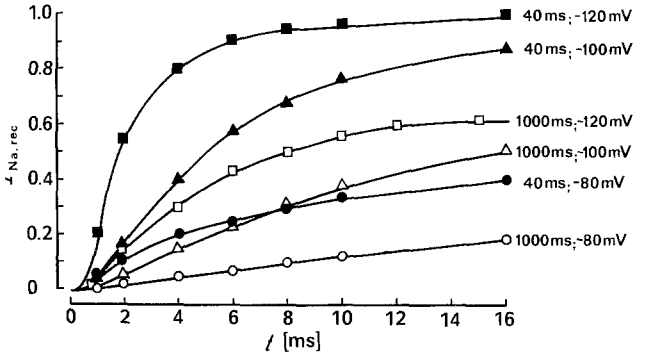

Fig. 7. Normalized time courses of early recovery after short (40 ms, filled symbols) and long (1,000 ms, open symbols) prepulse. Numbers beside the curves denote prepulse duration and potential of the time interval, respectively. Recordings at the same voltage are of the same cell. Curves are fitted by eye. Long prepulses decelerate time course of recovery at each potential

nents. Deceleration of recovery after long prepulses is caused by an increase of τ_{r2}, by appearance of a time constant of a very slow process τ_{r3}, but mainly by a shift in contribution to the component with larger time constants.

Delay in onset of and recovery from inactivation

As already mentioned both onset of (cf. Fig. 3A) and recovery from inactivation (cf. Fig. 5) show an initial delay. Time courses of both delays do not appear as a dead time but as an exponentially decreasing difference to the fastest component. For description of the delay two methods of quantification were used, which are described in Fig. 8.

Delay in onset of inactivation was tested for V_p between -50 and -20 mV using -10 mV as V_T. Table 4 summarizes the results. The delay time constant τ_{dc} shows the smallest value at the most depolarizing V_p and the deviation from an exponential time course is most clearly seen. Peak currents during this delay period may decrease, be constant, or even increase at such strong depolarizing prepulse potentials. At $V_p = -40$ mV only two of four cells showed a separable τ_{dc}. At more negative V_p, no delay could be detected.

To decide whether activation is responsible for the lag in inactivation the protocol was slightly modified. Between prepulse and test-pulse a gap of 0.5 ms duration at holding potential was included (Chandler et al. 1965; Goldman and Schauf 1972; Bean 1981). The time constant of deactivation was determined as 130 µs. Therefore, after 500 µs there is less than 2.5% activation left and a delay caused by activation is expected to disappear (Gillespie and Meves 1980; Bean 1981). In four cells investigated the delay after introduction of the gap did not disappear. Figure 9A clearly demonstrates the existence of the delay in such an experiment.

Kniffki et al. (1981) reported a protocol to avoid the influence of activation during the prepulse which allows more direct measurement of inactivation. The decay of test currents in a double pulse program is extrapolated to zero time of each test current. Thus $A_{h1}+A_{h2}$ for each current can be obtained from (1). These values are then plotted semilogarithmically as a function of prepulse duration. The authors found that the delay disappeared when applying this method. Similar plots were obtained from three cells, as shown in Fig. 10B. No disappearance of the lag was observed and the time constants proved to be fairly independent of prepulse duration. This finding may be taken as a further argument that the delay is a genuine property of inactivation, or, according to Goldman and Kenyon (1982) that inactivation is a process with kinetics similar to that of activation.

Delay in recovery from inactivation was tested for time interval voltages of -80, -100, and -120 mV. At -80 mV, because of the slow recovery process only in two out of four recordings was a delay time constant τ_{dr} resolvable (cf. Fig. 5B and Fig. 7). In the hyperpolarizing direction a decrease of τ_{dr} could be detected (Table 4). This is in accordance with the results in *Myxicola* (Schauf 1974) and myelinated nerve (Chiu 1977) but in contrast to the delay described in isolated rat ventricular myocytes (Brown et al. 1981 b). The τ_{dr} values reported in this paper agree with the values of Chiu (2–4 ms at 4.5 °C) quite well. The bell shaped dependence of τ_{dr} on voltage (Chiu 1977) could not be found because it was impossible to separate delay time constants from still slower recovery time courses at potentials positive to − 80 mV.

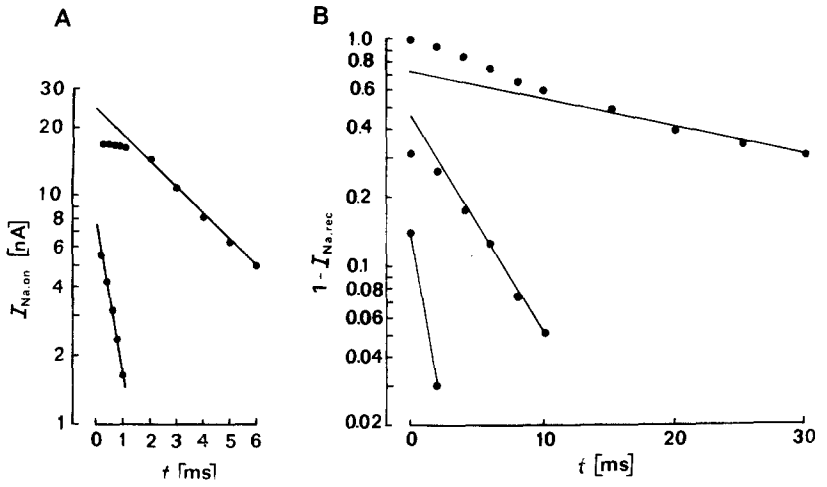

Fig. 8 A and B. Methods to determine delay time constants for onset of and recovery from inactivation. **A** Lag in onset of inactivation was determined by semilogarithmic plots of peak currents during the first 4 to 6 ms of this process against prepulse duration. After the delay at voltages positive to −50 mV one straight line was sufficient for description of the points (cf. Table 1). Delay time constant (τ_{dc}) was obtained from a logarithmic plot of the difference of the very early data points to the extrapolated line. $V_h = -90$ mV; $V_p = -30$ mV; $V_T = -10$ mV; $\tau_{dc} = 0.6$ ms; $\tau_{c1} = 3.8$ ms. **B** Peak currents of recovery time course were normalized to the current without prepulse and plotted semilogarithmically against time interval. Time constant for delay in recovery (τ_{dr}) was obtained from the semilogarithmic plot of the difference of data points to the regression line of the fast recovery phase. $V_h = -90$ mV; $V_{ti} = -100$ mV; $t_p = 40$ ms; $\tau_{dr} = 1.2$ ms; $\tau_{r1} = 4.4$ ms; $\tau_{r2} = 37.0$ ms

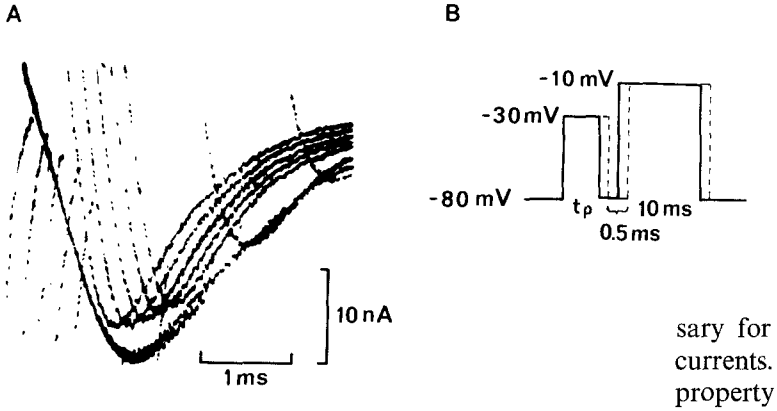

Fig. 9 A and B. Persistance of delay after introduction of a 0.5 ms gap between prepulse and test-pulse. **A** Current traces when applying the voltage protocol shown in **B**. Inspite of a 0.5 ms gap between prepulse and test-pulse the delay in onset of inactivation is clearly to be seen, $\tau_{dc} = 0.45$ ms. **B** Pulse protocol with 0.5 ms gap; prepulse time t_p had the values 0.2, 0.4, 0.6, 0.8, 1, 2, 3 ms; cell 080584-26

Table 4. Delay time constant for onset of inactivation (τ_{dc}) and recovery from inactivation (τ_{dr}). A_d denotes deviation of data from measured mono- or biexponential time course at zero time

V [mV]	A_d	τ_{dc} [ms]	τ_{dr}[ms]
−20	0.26 ± 0.11(2)	0.55 ± 0.10(2)	
−30	0.14 ± 0.07(7)	0.63 ± 0.21(7)	
−40	0.09 ± 0.03(7)	0.92 ± 0.26(7)	
−50	0.04 ± 0.02(2)	0.86 ± 0.32(2)	
−80	0.05 ± 0.02(2)		2.0 ± 0.7(2)
−100	0.14 ± 0.07(4)		1.3 ± 0.6(4)
−120	0.09 ± 0.04(4)		0.7 ± 0.3(4)

Discussion

The major finding of this report is that in the mouse myocardium macroscopic inactivation of Na currents is much more complex than can be described by a first order equation. Two time constants were necessary for the description of the decay phase of the currents. This finding is not new but seems to be a property of only a part of preparations used in electrophysiology. Even for heart muscle results differ. Ebihara and Johnson (1980) described a monoexponential decay in cultured clusters of heart cells whereas Brown et al. (1981 b), Fozzard et al. (1984), and Patlak (1984) needed two time constants. The analysis of the decay of both components at 36.5 °C and 22.0 °C provided no evidence for existence of two forms of Na channels with different temperature sensitivity as reported for the squid axon (Matteson and Armstrong 1982) and for myelinated nerve (Benois et al. 1985). However, their results have been obtained at temperatures below 20 °C. Patch clamp studies of the Na channel in our preparation (cf. Nilius et al. 1986) showed the existence of long lasting bursts in a small percentage of traces providing an easy explanation for the slow component. Patlak and Ortiz (1985) suggested from a detailed analysis of such bursts that Na channels can function in different modes each with a different inactivation rate. Thus, the slow component in the

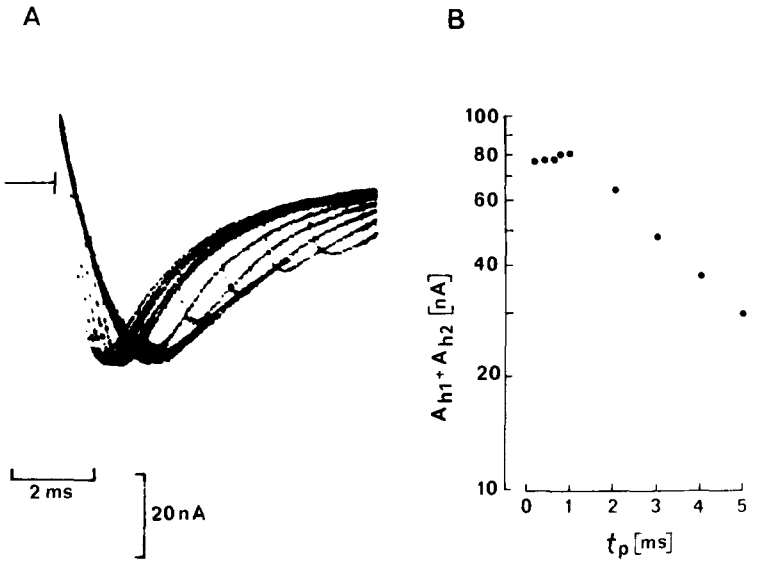

Fig. 10 A and B. $(A_{h1}+A_{h2})$ as function of prepulse duration t_p. $(A_{h1}+A_{h2})$ was obtained by extrapolating Na current decays of each test-pulse back to its zero time value by use of equation (1) and linear regression. $V_h = -90$ mV, $V_p = -30$ mV, $V_t = -20$ mV; t_p had the values 0.2, 0.4, 0.6, 0.8, 1, 2, 3, 4, 5, 6 ms. Currents corresponding to the pulse program described above; $\tau_{dc} = 0.7$ ms. **B** Semilogarithmic plot which reveals persistence of delay effect, cell 080584-16

decay phase of our currents could be caused by channels in such a bursting mode. As a further interpretation of the biexponential decay the existence of more than one open state of the Na channel could be assumed. Na channels with such opening characteristics have been reported by Nagy et al. (1983) in neuroblastoma cells. However, patch clamp experiments in our preparation (unpublished) provided no evidence for a major contribution of a second current level to the opening behaviour of Na channels.

The mean temperature coefficients Q_{10} of 2.28 and 2.46 for the decay time constants τ_{h1} and τ_{h2} are well in the range of data for nerve reported by Frankenhaeuser and Moore (1963), Schwarz (1979), Collins and Rojas (1982), Kukita (1982); for skeletal muscle reported by Adrian and Marshall (1977); and for cardiac Purkinje fibres reported by Colatsky (1980). The extrapolation of time constants of Na current decay from far lower temperatures to 37 °C (Dudel and Rüdel 1970; Colatsky 1980) agrees with our measured data indicating the validity of the Q_{10} value over a wider range of temperature than tested here.

Delays in onset of inactivation are reported from *Myxicola* axon (Goldman and Schauf 1972; Goldman and Kenyon 1972), squid axon (Bezanilla and Armstrong 1977), and crayfish giant axon (Bean 1981). There are two major objections against the existence of a genuine initial delay: An interference with properties of the *m*-gate and large r_s values. A decisive contribution of the first mechanism could be excluded by the persistence of the lag when inserting a sufficiently long gap between prepulse and test-pulse. Gillespie and Meves (1980) pointed out the importance of a low series resistance for the time course of inactivation. They showed that apparent lags appeared if $1/\bar{g}_{Na}$ was four times smaller than r_s but there was no effect if $1/\bar{g}_{Na}$ was 6.6 times larger than r_s. The latter is a ratio which is definitely reached in our case.

The delay described here does not have the character of a dead time but of an exponential. The conclusion of Gillespie and Meves (1980) that the shortest prepulse which leads to smaller test currents represents the upper limit of a possible true delay seems therefore not to be valid for the results presented here. The majority of peak currents during the delay showed a decrease from the beginning but τ_{dc} values could be separated easily. In conclusion there is considerable evidence for a true delay in the inactivation process. Therefore Na channels must pass at least a second closed state before inactivating.

The striking difference between time courses of decay and onset of inactivation (cf. Fig. 4) can hardly be reconciled with independent activation and inactivation. This finding is well known from reports in *Myxicola* axons (Schauf and Davis 1975; Goldman and Kenyon 1982), lobster axons (Oxford and Pooler 1975), and clusters of cultured heart cells (Ebihara and Johnson 1982). In contrast, in most nerve preparations the identity of the time courses both for the mono- and for the biexponential case has been stated (Bezanilla and Armstrong 1977; Chiu 1977; Gillespie and Meves 1980; Nonner 1980; Bean 1981). Brown et al. (1981 b) reported similar biexponential time courses for decay and double pulse inactivation in cardiac myocytes of the rat with even faster initial double pulse inactivation than the fast component. Even taking into account different methods (two suction pipettes in their case) this difference remains unexplained. In a series of seven experiments we repeated their protocol (cf. Fig. 11 A

in their report) with test steps to −30 mV and prepulses of −40 mV. But we always saw double pulse inactivation slower than current decay at the same potential as we also found in more than forty recordings using our protocol. We therefore evaluate our finding as a true property of our preparation giving evidence for a coupling between activation and inactivation (Goldman and Kenyon 1982).

Because of the small contribution of the component characterized by τ_{c2} to onset of inactivation between −50 and −20 mV excellent constant conditions over long times have to be obtained to investigate properties of such slow components. Nevertheless, the voltage dependence of τ_{c2} is a reliable finding. At prepulse potentials at which Na currents are still not present the inactivation time course is very slow and shows time constants in the range of seconds. As a further slow process recovery from inactivation showed a clear history dependence of inactivation giving evidence for a slow transition to another inactivated state at maintained depolarization. From Purkinje fibres (Gintant et al. 1984; Carmeliet 1984) and isolated rat ventricular cells (Brown et al. 1981 b) slow inactivation of currents and recovery time course, respectively, have been observed. Recently, a slow inactivation of sodium channels in ventricular myocytes from neonatal rats has been outlined in a patch clamp study by Kunze et al. (1985). In skeletal muscle slow inactivation has been reported in the range of seconds (Collins et al. 1982) or minutes (Almers et al. 1983). In squid axon a time constant of 30 s has been reported (Rudy 1978). Almers et al. (1983) gave evidence that such slow mechanisms are a potential dependent property of membrane and probably of sodium channels themselves.

For the fast phase of recovery we needed two time constants apart from the initial delay for a satisfactory description. This contrasts with the data of Colatsky (1980) and Brown et al. (1981 b). At a prepulse of similar duration (40 ms) and similar temperature, the recovery time course in myocardial mouse cells is very fast, but agrees fairly well with data (extrapolated to room temperature using a Q_{10} of 2.3) of myelinated nerve (Chiu 1977).

Delay in recovery from inactivation is reported from isolated rat heart cells (Brown et al. 1981 b), myelinated nerve (Chiu 1977), and *Myxocola* axon (Schauf 1974). Chiu found a bell shaped voltage dependence of delay. We could only confirm the decrease of τ_{dr} to more hyperpolarizing potentials. Extrapolating the data of Chiu to room temperature ($Q_{10} = 2.3$) reveals slightly shorter delays as in our case. Furthermore our data do not agree with the results of Brown et al. (1981 b) who found the largest delays at most negative potentials.

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