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Quantitative analysis of sodium and potassium activation delays in fresh axons of the squid: *Loligo forbesi*

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Abstract. Activation kinetics of the sodium and potassium conductances were re-examined in fresh axons of *Loligo forbesi* exhibiting very little if any potassium accumulation and a very small leak conductance, special attention being paid to the initial lag phase which precedes the turning-on of the conductances. The axons were kept intact and voltage-clamped at 2–3 °C.

In all cases, the rising phase of the currents could be fitted with very good accuracy using the Hodgkin-Huxley (1952) equations although, in most cases, the turning-on of the conductance did not coincide with the beginning of the depolarizing test pulse. The delay which separates the change in potential and the turning-on of current (the activation delay) was analyzed quantitatively for different prepulse and pulse potentials. The measured activation delay differed significantly from the delay predicted by the original HH equations. This difference (the 'non-HH delay') varied with prepulse and pulse potentials. For the potassium current, the relationship between the non-HH delay and pulse potential for a constant prepulse was bell shaped, the maximum value (0.7 ms for a prepulse to −80 mV) being reached for about 0 mV. For this same current, the relationship between the non-HH delay and the prepulse potential for a constant pulse potential was sigmoidal, starting from a minimum value of around 0.5 ms at −100 mV and rising to 5 ms at −15 mV. Essentially similar results were obtained for the sodium current although the non-HH delay was three to five times smaller and the dependency upon prepulse potential not significant. These results are in agreement with previous observations on squid axons and frog nodes of Ranvier and suggest that the opening of an ionic channel is preceded by a short but essential voltage-dependent conformational change of the channel protein.

Key words: Sodium current – Potassium current – Voltage-clamp – Squid axons – Kinetics

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Introduction

The mechanisms by which the axonal membrane becomes permeable successively to sodium and then to potassium ions during the action potential are known since the classical work of Hodgkin et al. (1952) on voltage-clamped giant axons of the squid. Despite numerous attempts, the "HH equations", proposed at that time by Hodgkin and Huxley (1952), which describe the changes in membrane conductance in terms of three independent processes named sodium activation (m), sodium inactivation (h) and potassium activation (n) remain practically unrivalled. One of the major discrepancies between the model and the experimental recordings is the existence, under most conditions, of a short delay between the beginning of the depolarizing voltage pulse and the turning-on of the ionic currents (Frankenhaeuser and Hodgkin 1957; Cole and Moore 1960). The observation that this delay is highly temperature sensitive (Keynes and Rojas 1976) and can be modulated pharmacologically, independently of the activation kinetics (Paternostre and Pichon 1987), has important implications concerning the biophysical phenomena underlying the opening of voltage-dependent channels following changes in the transmembrane electrical field.

The paucity of reliable data on this delay (the activation delay) which is observed for both sodium and potassium conductances reflects the difficulties in obtaining stable and noise free recordings of the ionic currents with no distortions due to insufficient membrane potential control, insufficient series resistance compensation or ion accumulation (Frankenhaeuser and Hodgkin 1956) or depletion. Compared to most preparations, intact giant axons of freshly caught specimens of *Loligo forbesi* are exceptionally stable, exhibit very little leak and very little, if any, potassium accumulation (Larmet and Pichon 1987; Pichon et al. 1987).

This paper deals with the effects of prepulse and pulse potentials on the activation delays for both ionic conductances and for membrane poentials ranging from −100 mV to +90 mV. As pointed out by Armstrong and

Bezanilla (1974), an increase in duration in the lag is predicted by the Hodgkin and Huxley equations following membrane hyperpolarization. Thus, significant negative delays are obtained if the initial value of the activation parameters are comparable to the final values (see also Neumcke et al. 1976). These predicted values were subtracted from the measured delays to obtain the non-HH delays. Preliminary accounts of this work has been published (Pichon et al. 1984; Larmet et al. 1985).

Methods

Large specimen of *Loligo forbesi* were caught at dawn in the coastal waters about one mile off the Marine Laboratory of Roscoff and kept alive in reformed rubber rafts filled with circulating and aerated sea water. The giant axons (axon diameters ranging from 550 to 800 μm) were mounted horizontally across a double air-gap nerve chamber filled with artificial sea water (ASW) maintained at a constant temperature of 1–3 °C. The axons were studied under voltage-clamp conditions as described by Kimura and Meves (1979) and Pichon (1981). Briefly, the membrane potential was measured between a micropipette of about 100 μm in diameter filled with 0.6 *M* KCl impaled into the axon and an external reference micropipette filled with ASW and placed as close as possible from the tip of the internal pipette in order to reduce the series resistance. Current was injected between an intracellular platinized platinum/iridium wire (100 μm in diameter) glued on the outside of the micropipette (piggy-back arrangement) and three pairs of platinized platinum plates placed on both sides of the axon. The voltage-clamp amplifier was positioned as close as possible to the preparation to reduce the response time and a floating 50 μm platinum wire placed inside the internal pipette up to the very tip to enhance high frequency response. The response time of the system with the usual 70% series resistance compensation was about 3 μs.

The voltage pulses were delivered to the preparation through a deglitched digital/analog converter driven by a computer. The currents were digitized on 12 bit and stored in blocks of 1024 words on digital cartridges for off-line analysis. The pulse protocol consisted of a conditioning prepulse of constant duration immediately followed by a 4 times shorter test pulse.

The ASW had the following composition (m*M*): Na^+ 470, K^+ 0 or 10, Ca^{2+} 11, Mg^{2+} 55, Cl^- 602 or 612. It was buffered at pH 7.8 using *Tris* buffer (Sigma). Sodium currents were recorded in the presence of 10 m*M* 3,4-diaminopyridine (Fluka). Potassium currents were recorded in the presence of 1 μ*M* tetrodotoxin (Sigma).

The sodium current records were fitted according to the following equations:

$$I_{Na} = I'_{Na} \cdot p\,(t, \delta_{Na}) \tag{1}$$

with

$$p\,(t, \delta_{Na}) = (1 - \exp - [(t - \delta_{Na})/\tau_m])^3 \cdot \exp - [(t - \delta_{Na})/\tau_h] \cdot \Phi\,(t - \delta_{Na}) \tag{2}$$

and

$$I_K = I'_K \cdot p\,(t, \delta_K) \tag{3}$$

with

$$p\,(t, \delta_K) = (1 - \exp - [(t - \delta_K)/\tau_n])^4 \cdot \Phi\,(t - \delta_K) \tag{4}$$

where τ_m, τ_h and τ_n are respectively the sodium activation, inactivation and the potassium activation time constants, δ_{Na} and δ_K the sodium and potassium delays, and $\Phi\,(t - \delta_{Na})$ and $\Phi\,(t - \delta_K)$ the Heaviside function.

The same basic procedure was used for the two currents. For the sodium current, inactivation was calculated first from the falling phase of the current and the total current divided by h.

The experimental values were compared with those predicted from the original HH equations and parameters (continuous lines in Figs. 2 and 3). The delays which originate from the non zero value of the activation parameters at the beginning of the test pulses (the HH delays, δ_{NaHH} and δ_{KHH}) were calculated using (5) and (6):

$$\delta_{NaHH} = \tau_m \cdot \ln \cdot [(m_\infty - m_0)/m_\infty] \tag{5}$$

$$\delta_{KHH} = \tau_n \cdot \ln \cdot [(n_\infty - n_0)/n_\infty] \tag{6}$$

where m_∞ and n_∞ are the steady state values of the activation parameters for the given voltage and m_0 and n_0 their values at the start of the test pulse (i.e. the value at the end of the prepulse).

Results

1 *Validity of the curve fitting procedure*

The validity of the curve fit was estimated from the value of the correlation coefficient of the linearized current trace and from the superposition of the computed traces on the experimental data. Example of such fits are shown in Fig. 1. In all cases the experimental data (dots) and the reconstructed current traces (dashed line) are superimposed using the original HH formalism and power functions and a variable delay.

The effects of conditioning pulse and test pulse potentials on the time constants of activation and activation delays were studied or membrane potentials ranging from −100 to +90 mV. A full analysis of either the potassium or the sodium current was performed for each of the 16 axons obeying the previous conditions and selected for this study.

2 *Potassium kinetics*

Effects of pulse potential. The time constants of activation and the activation delays were calculated for test pulses from −60 mV (holding potential) to +90 mV by 10 mV steps. In all cases, the test pulse was preceded by a conditioning pulse to −80 mV. In the experiments used in the present analysis, the potassium equilibrium potential measured at the end of the depolarizing pulse lay around

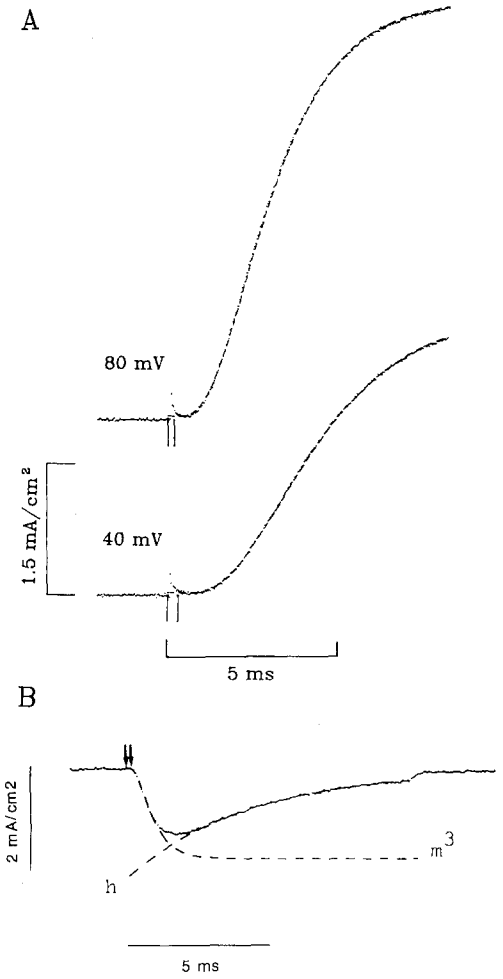

Fig. 1 A, B. Examples of fit of the ionic currents of intact squid axon with the Hodgkin and Huxley (1952) kinetics plus a positive delay. **A** Potassium currents: The membrane normally held at −60 mV was hyperpolarized to −80 mV during 80 ms and then depolarized to the indicated potentials for 20 ms. The dots are the (unretouched) experimental data and the dashed line the reconstructed currents. The potassium activation time constant (τ_n) and the delay (δ) (shown as the interval between two vertical bars) were 2.146 ms and 0.314 ms for 40 mV and 1.481 ms and 0.184 ms for 80 mV respectively. The corresponding correlation coefficients were larger than 0.999 for the 250 points of the recordings selected for the analysis. Temperature: 2 °C. **B** Sodium current: The membrane was hyperpolarized from its original level (−60 mV) to −80 mV during 40 ms and then depolarized to 0 mV for 10 ms. The dashed curve labelled *h* has a time constant of 4.4 ms, the dashed curve labelled m^3 a time constant of 1.23 ms and the delay (indicated by the two arrows) was 0.165 ms. The correlation coefficient for the two regressions was larger than 0.99. Temperature: 1 °C

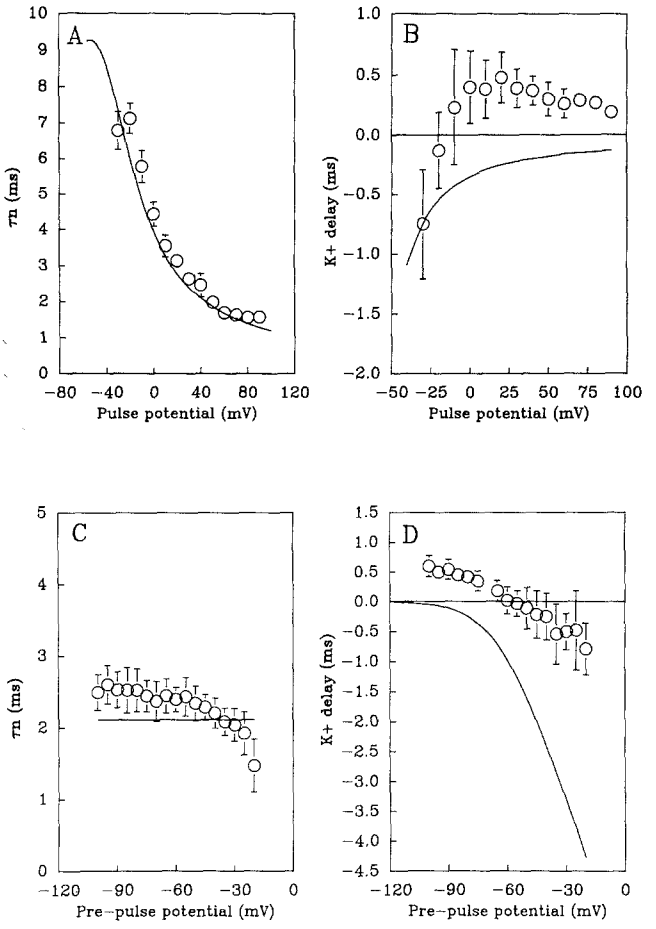

Fig. 2 A–D. Effects of pulse potential (**A** and **B**) or prepulse potential (**C** and **D**) on the time constant of activation (τ_n) of the potassium current (**A** and **C**) and the potassium delay (**B** and **D**). Holding potential: −60 mV. Test prepulse (**A** and **B**): −80 mV. Test pulse (**C** and **D**): +20 mV. Prepulse duration: 40 ms. Pulse duration: 10 ms. In **A**: Mean ± SD of 3 axons. Temperature: 2 °C. Continuous line: time constant of activation of the potassium current calculated from the HH equation for the same temperature but shifted by 20 mV towards more positive membrane potentials. In **B, C** and **D**: Mean ± SD of 4 axons. Temperature: 1–3 °C. Continuous line: values predicted from the HH equations at 2 °C

−85 mV and did not change with the membrane potential during the test pulse.

The potassium activation time constant was found to vary with membrane potential as predicted from the HH equations: it decreases almost exponentially between −30 mV and +90 mV from 6 ms to 1.5 ms. In all cases, there was a good agreement between the time constants calculated from the experimental data and the values calculated from the equations although the experimental time constants were usually shifted towards more positive potentials. This is illustrated in Fig. 2 A for three axons at 2 °C: the calculated time constants can be superimposed on the predicted HH activation curve at 2 °C shifted by 20 mV towards more positive membrane potentials.

The corresponding potassium activation delay was negative for small values of depolarization (around −0.5 ms at −30 mV), decreased with increasing membrane depolarization, inverted around −10 mV and reached a maximum of about 0.5 ms between 0 and +20 mV. It then slowly decreased when the membrane was further depolarized. This is illustrated in Fig. 2 B for 4 different axons studied at 1–3 °C. The comparatively large standard deviation is likely to be due to the temperature sensitivity of the delay.

Effects of prepulse potential. The time constants of activation and the activation delays were calculated for conditioning prepulses from −100 to −20 mV by 5 mV steps followed by a test pulse to 20 mV. For prepulse potentials more positive than −60 mV, current during the prepulse was not zero and the turning-on of the current during the

test pulse was not sigmoidal (as predicted from the theory). For prepulse potentials more negative than −60 mV (hyperpolarizing prepulses) the turning-on of the current was sigmoidal and shifted to the right but the time constant of activation did not change with prepulse potential. τ_n slightly decreased for more positive membrane potentials (depolarizing prepulses). For the 4 axons illustrated Fig. 2C, the mean time constant is almost equal to the predicted (unshifted) value of 2.05 ms at 2 °C.

The corresponding potassium activation delay was positive for hyperpolarizing prepulses, zero around −60 mV and negative for depolarizing prepulse potentials. In all cases the relationship between potassium activation delay and prepulse potential was almost linear from −100 to −60 mV and then slightly deviated from linearity for depolarizing prepulses. The main features of this relationship are illustrated in Fig. 2D for the same 4 axons as in Fig. 2C. The delay was found to change from 0.6±0.180 ms for −100 mV to 0.02±0.230 ms for −60 mV and to −0.790±0.430 ms for −20 mV. In all cases, the observed delay was significantly different from the HH delay.

3 *Sodium current*

Effects of pulse potential. The sodium activation time constants varied with membrane potential as first described by Hodgkin and Huxley (1952). It first increased with more positive membrane potentials, reached a maximum for −30 mV and then decreased. The relationship between pulse potential and τ_m is illustrated in Fig. 3A for 4 axons at 2 °C. Except for −40 and −30 mV for which the standard deviation is large, there is a close agreement between the time constants calculated from the experimental data and the values predicted by the HH equations (continuous line).

The corresponding sodium activation delays were negative for small values of depolarization (−0.04 ms at −30 mV), decreased rapidly with increasing membrane depolarization, reversed polarity around −20 mV and reached a peak positive value of about 0.15 ms between −10 and 0 mV. They then decreased almost linearly with membrane potential for more positive membrane potentials. This relationship between pulse potential and sodium activation delay is illustrated in Fig. 3B for 4 axons at 2 °C.

Effects of prepulse potential. As for potassium currents, the time constants of sodium activation and the activation delays were calculated for conditioning prepulses from −100 to −20 mV by 5 mV steps followed by a test pulse to 20 mV. For the entire range of prepulse potentials, the time constant of activation was constant. For the 4 axons illustrated Fig. 3C, the mean time constant (0.23 ms) is slightly smaller than the predicted value of τ_m at 2 °C (0.28 ms).

The corresponding sodium activation delays decreased with decreasing prepulse potentials. The relationship between the delay and membrane potential was linear between −100 mV and −40 mV and slightly deviated from linearity for more positive prepulse potentials. The delay reversed polarity around −40 mV. The main features of this relationship are illustrated in Fig. 3D for the same 4 axons as in Fig. 3C. The delay was found to change from 0.13±0.01 ms for −100 mV to 0.08±0.02 ms for −60 mV and to −0.040±0.03 ms for −30 mV. Here again, the measured delay was significantly different from the HH delay for the entire range of potentials.

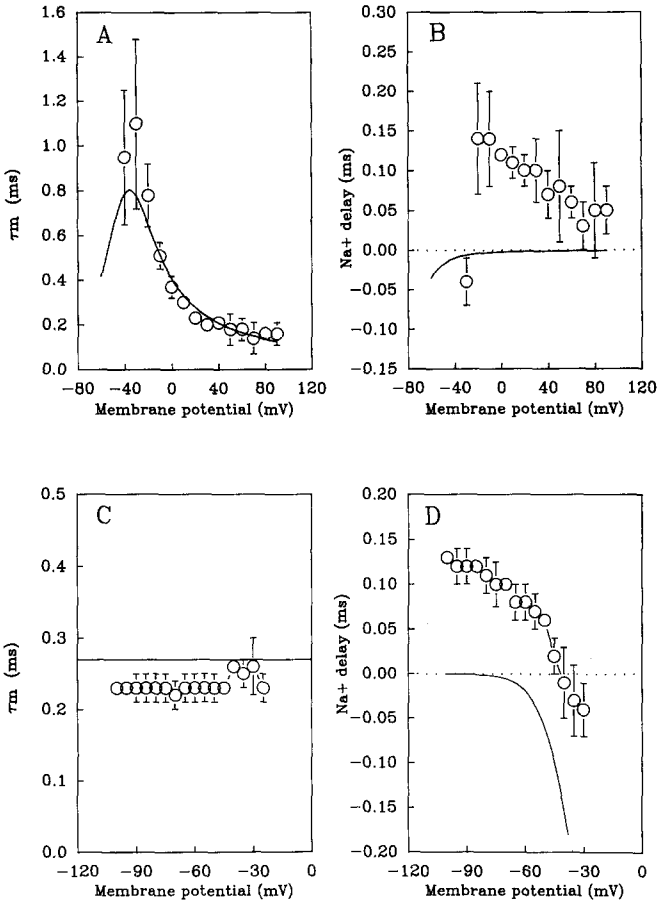

Fig. 3A–D. Effects of pulse potential (**A** and **B**) or prepulse potential (**C** and **D**) on the time constant of activation (τ_m) of the sodium current (**A** and **C**) and the sodium delay (**B** and **D**). Holding potential: −60 mV. Test prepulse (**A** and **B**): −80 mV. Test pulse (**C** and **D**): +20 mV. Prepulse duration: 20 ms. Pulse duration: 5 ms. Mean ±SD of 4 axons. Temperature: 2 °C. The continuous line represents the time constants calculated from HH at the same temperature

4 *Non HH delays*

The non-HH delays were obtained by subtracting the measured delays from those predicted from the HH formulation which are due to finite values of m_0 or n_0 at the beginning of the test depolarizations. Figure 4 illustrates the effects of prepulse potential (A and C) and test membrane potential (B and D) on the non-HH delay.

There were obvious similarities between the delays for the two conductances: with the exception of the sodium delay for very small depolarization, non-HH delays were always positive. The largest value of the delay was ob-

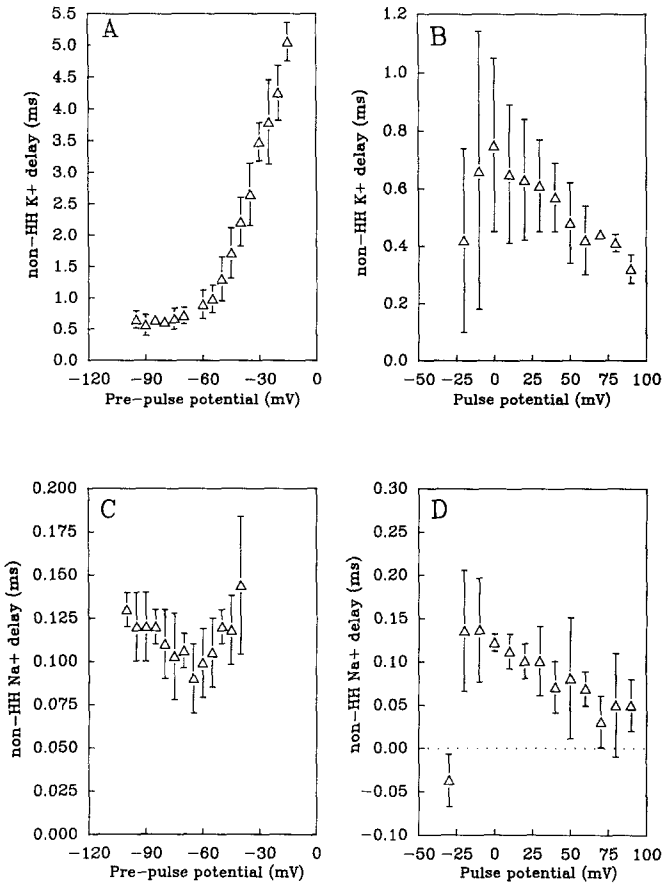

Fig. 4A–D. Non-HH delays for potassium (**A** and **B**) and sodium (**C** and **D**) for different prepulse (**A** and **C**) or pulse potentials (**B** and **D**). Data from Figs. 2 and 3. Temperature: 2 °C

served for moderate depolarizations (around −10 mV for the sodium and 0 mV for the potassium). It decreased almost linearly at more positive membrane potentials. There were also clear differences: the potassium delays were generally 3 to 5 times larger than the sodium delays. In the case of potassium, the non-HH delay slightly increased with decreasing prepulse potential from −100 to −60 mV, then dramatically increased with further depolarizations. This voltage dependence was not observed for sodium currents.

Discussion

General features

The data presented here are in good agreement with previously published figures on intact axons of *Loligo* (Hodgkin and Huxley 1952; Frankenhaeuser and Hodgkin 1957; Cole and Moore 1960; Paternostre and Pichon 1987).

Power functions and time constants

The sodium and potassium currents in fresh, non-accumulating, axons of *Loligo forbesi* can be described adequately using the Hodgkin and Huxley (1952) formulation plus a voltage-dependent delay. Our observation that the original 3rd power for m and 4th power for n are adequate to account for the time course of both ionic currents over the entire potential range (−50 to +90 mV) is at variance with several reports on squid axons (Keynes and Kimura 1983; Keynes 1983) or nodes of Ranvier (Neumcke et al. 1976; Neumcke and Stämpfli 1982). These differences may arise from differences in the experimental conditions (the experiments reported by Keynes and Kimura (1983), were performed on perfused axons) or from species differences. In the case of the potassium current, some differences might also arise from uncontrolled potassium accumulation in the vicinity of the axonal membrane. Differences in the time course of the ionic currents arising from incomplete compensation of the resistance in series with the membrane cannot be ruled out, especially when very large currents are involved.

There is also a good agreement between our values of the time constants and those predicted from the HH equations for similar temperatures. The potential for which the time constant of activation of the sodium system is maximum (around −35 mV) is almost identical to that reported by Keynes and Kimura (1983) for the same species but in perfused axons.

Effects of prepulses

The fact that hyperpolarizing prepulses induce a delay in the turning-on of the potassium current has been first reported by Frankenhaeuser and Hodgkin (1957) for intact squid axons. This delay can only be partly accounted for by the HH equations as shown by Cole and Moore (1960) also for squid axons (*Loligo pealei*). Similar effects were observed in a variety of nerve preparations including giant axons of *Myxicola* (Goldman and Schauf 1973; Schauf et al. 1976) giant axons of *Procambarus clarkii* (Shrager 1974; Young and Moore 1981), giant axons of *Periplaneta americana* (Pichon, unpublished) and frog nodes of Ranvier (Palti et al. 1976; Begenisich 1979; Ilyin et al. 1980) among others. A similar phenomenon was described for the sodium current in squid axons (Keynes and Rojas 1976; Keynes and Kimura 1980, 1983) and frog nodes of Ranvier (Neumcke et al. 1976). Whereas in the earlier experiments of Cole and Moore (1960) (see also Bezanilla et al. (1982)), the effects of the conditioning prepulses appeared as a shift of the current trace, more recent work on several preparations showed that the shift was accompanied by a change in the kinetics, so that superposition of the current traces was not always possible (Begenisich 1979; Young and Moore 1981, Clay and Shlesinger 1982; Gilly and Armstrong 1982). Our experiments are in perfect agreement with the first observations as well as with some more recent experiments on squid axons (Keynes and Rojas 1976; Moore and Young 1981). The small change in τ_n observed for depolarizing prepulses is hardly significant except for large prepulses in which *(1)* a precise measurement of the time constant is more difficult, *(2)* potassium can accumulate during

80 ms long prepulses. τ_m on the other hand remains unchanged for all values of the prepulse potential.

Voltage dependence of the activation delay

For both conductances, the activation delay changed with prepulse and pulse potentials in a characteristic way: at a given prepulse potential, δt decreased with more positive pulse potentials while for a constant pulse potential, the delay increased progressively as the prepulse was made more negative. The relationship was very similar for the two conductances although, at the same temperature, the sodium delay was 3 to 5 times shorter. Thus, in the experiments illustrated in Figs. 3 and 4, the maximum potassium delay (0.480 ± 0.210 ms) was over three times larger than the maximum sodium delay (0.140 ± 0.060 ms). This maxium value for the sodium delay is almost identical to that reported by Keynes and Rojas (1976) for perfused squid axons (for a similar temperature range) but three times larger than the equivalent delay measured on the node of Ranvier by Neumcke et al. (1976) (around 0.050 ms) but at 10 °C. If one assumes a Q_{10} of about 3, the two values are almost identical for the two species. This is not the only resemblance between our data and those of Neumcke et al. (1976): the voltage dependence of the delay is also similar for the two preparations. Thus, in their study, the delay decreased linearly for decreasing prepulse potentials between -60 mV and 0 mV (i.e. for hyperpolarizing potentials) and then deviated from linearity exactly as in squid axons. Similarly, the curve relating the delay and the test pulse potential for a constant prepulse was bell shaped, started from zero or negative values around 32 mV, (30 mV depolarization in our experiments), reached a maximum around 48 mV (against 40–50 mV depolarization in our experiments) and then slowly declined if the membrane was further depolarized. Such similarities can hardly result from pure coincidence but rather strongly suggest that this is a common feature to most axons. Unfortunately, most other studies have been performed under different experimental conditions and often with very large (and sometimes short) hyperpolarizing prepulses which are probably not equivalent to our moderate potential changes.

Non-HH delay

Our analysis clearly shows that, the delay predicted by the HH equations (see Frankenhaeuser and Hodgkin 1957; Armstrong and Bezanilla 1974; Keynes and Rojas 1976; Neumcke et al. 1976), is always negative and can be subtracted from the experimental values to obtain the "actual" (non-HH) delay which is (nearly) always positive. The relationship between this delay and the membrane potential of the test pulse is reminiscent of that relating the time constants of activation and membrane potential. It is basically similar for the two ionic conductances. Interestingly, depolarizing prepulse potentials strongly increase the non-HH potassium delay but have no effect on the non-HH sodium delay.

Molecular interpretation

The molecular mechanisms which underlie the non-HH delay are as yet unknown. As pointed-out in the introduction, the observation that this delay has a much higher temperature sensitivity that the sodium activation time constant and the gating current time constant (Keynes and Rojas 1976) and the fact that it can be modulated pharmacologically, independently of the activation kinetics (Paternostre and Pichon 1987 and unpublished) suggests a fundamental relation between the two processes.

Keynes and Rojas (1976) proposed that the delay could reflect a process operating in series rather that in parallel with the gating particles. High resolution recordings of the sodium gating currents in freshly mounted squid axons have, however, recently revealed the existence of a fast non-inactivating component with a relaxation time constant of the order of 5–25 μs (Bekkers et al. 1989) preceding one or two slower components. This component is qualitatively similar to that observed by Starkus et al. (1981) in crayfish axons. Interestingly, in the latter preparation, the fast component of gating current coincides with the delay between the turning on of the pulse and the rapid activation of the sodium conductance. It is reasonable to assume, under these conditions, that the delay could reflect hydrophobic interactions between the transmembrane α helices of the channel protein and the membrane lipids. These interactions would correspond to the transition of the channels from a 'preresting' state to an 'activable' state equivalent to the resting state of the Hodgkin and Huxley model. As recently suggested by Keynes (1989), the delay might reflect the independent transitions of the four S4 membrane-spanning voltage sensors of the sodium channel (Noda et al. 1986; Salkoff et al. 1987) operating in parallel.

The similarity of the relationship between test membrane potential and non-HH delay for the two ionic conductances most probably reflects the known homologies in the amino acid sequences of the voltage-sensitive sodium and potassium channels (Baumann et al. 1988). Conversely, the fact that a strong voltage dependence upon prepulse potential is seen only for the potassium conductance is suggestive of differences which should be investigated further. Such study would require an approach similar to that of Stühmer et al. (1989) in which site-directed mutagenesis and patch-clamp recording were combined to study the structure-function relationship of the sodium channel.

Activation delays and single channel data

Since it is now established that in many neuronal preparations (Christensen et al. 1988; Amar et al. 1989; Llano et al. 1988) the voltage-dependent ionic currents correspond to the contribution of several populations of ionic

channels, it would be of interest to analyze the timing between the turning-on of the depolarization and the opening of the single channels for each population. In the cut-open squid axon preparation, Llano et al. (1988) found that, under certain conditions, the large conductance (40 pS) potassium channel had a low probability of being open and had a pronounced lag in its activation time course. Unfortunately, important differences were seen from patch to patch. Similar observations have been made during the past years in our Laboratory for the potassium channels which are present on the soma of cultured insect neurones, but, for technical reasons (small amplitude of the single channel events and limited bandwidth of the patch-clamp system) no definite conclusion could be drawn.

Conclusion

In conclusion, our experiments on fresh squid axons confirm the original observations of Hodgkin and Huxley (1952) on the same species but clearly establish that, for all potential values, activation of the voltage-dependent conductances is preceded by a small but consistent positive delay which probably reflects a voltage-dependent transition from a 'preresting' state to an 'activable' state.

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