SLOW SODIUM INACTIVATION IN *MYXICOLA* AXONS

EVIDENCE FOR A SECOND INACTIVE STATE

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ABSTRACT Sodium inactivation and reactivation have been examined in voltage-clamped *Myxicola* axons after long-lasting membrane depolarizations produced either directly by changes in holding potential or indirectly by elevation of external K^+ concentration. The results suggest the existence of a second inactivated state of the sodium channel with associated voltage-dependent rate constants at least two orders of magnitude lower than those of the fast inactivation process commonly examined. No specific influence of external [K^+] on slow Na^+ inactivation could be detected.

INTRODUCTION

In *Myxicola* giant axons at membrane potentials between −60 mV and −45 mV the time constants for reactivation of the sodium conductance after complete inactivation by depolarizing prepulses are 30–50% larger than the time constants for sodium inactivation determined simultaneously at the same potential in the same axons (Schauf, 1976). Such a result could be explained if during a depolarizing prepulse of long duration a significant number of sodium channels were driven into another inactive state from which there is a slower recovery at hyperpolarized potentials.

A process of slow sodium inactivation, possibly reflecting the existence of such a distinct inactive state, was first described in detail some years ago in squid axons by Adelman and Palti (1969*a*,*b*). These workers demonstrated that axons bathed in high [K^+] solutions showed an increasing attenuation of peak sodium currents after large hyperpolarizations, accompanied by a marked decrease in the slope of the steady-state sodium inactivation relation. This attenuation could be removed after conditioning hyperpolarizations very much longer than those normally used. Adelman and Palti (1969*a*,*b*) ascribed these effects to a pharmacological inactivation of the Na^+ channel by external K^+, combined with depletion of external K^+ in the Frankenhauser-Hodgkin (1956) space during long hyperpolarizing pulses. However, in later experiments on voltage-clamped *R. temporaria* nodes, Peganov et al. (1973) postulated the existence of a second inactive state to explain similar observations.

Although previous experiments (Schauf, 1976) in 10 mM K^+ at recovery potentials near rest showed no evidence for a second, slow process, we were stimulated to carry

out a more complete investigation over a wider range of membrane potentials. The results support the existence of a process of slow Na^+ inactivation in *Myxicola* which depends solely on changes in membrane potential, and thus only indirectly on $[K^+]_o$. However this process does not seem likely to be the primary source of the longer reactivation time constants characteristic of this axon.

METHODS

Myxicola giant axons were voltage-clamped using compensated feedback by methods previously described (Binstock and Goldman, 1969; Goldman and Schauf, 1973; Schauf, 1974, 1976; Schauf and Davis, 1975). Early experiments were performed in artificial seawater (ASW) with 230 mM NaCl, 10 mM $CaCl_2$, 50 mM $MgCl_2$, and 200 mM tris(hydroxymethyl)aminomethane pH 7.8, or in a solution in which some of the Tris was replaced by K^+ at constant Na^+ concentration. In later experiments KCl was simply added to a standard ASW containing 430 mM NaCl, 10 mM $CaCl_2$, 50 mM $MgCl_2$, and 20 mM Tris, the resulting changes in osmolarity having no effect by themselves (Schauf, 1975). In all cases nonsodium currents were obtained in the presence of 10^{-6} M tetrodotoxin (TTX) and subtracted from total membrane currents to obtain sodium currents, using protcols identical to those used in the absence of TTX. Temperature was controlled to 5.0 ± 0.5°C. Because of the presence of slow processes we generally allowed 20 s between application of command pulse.

RESULTS

In the first series of experiments steady-state Na^+ inactivation curves were obtained using prepulses of 100 ms duration and large test pulses (Goldman and Schauf, 1972) in solutions in which $[K^+]$ was varied from 0 to 100 mM at a constant $[Na^+]$ of 230 mM. The axons were held at their natural resting potentials in each solution and the data normalized to the maximum inward sodium current during the test pulse produced after a 100 ms prepulse to −180 mV in K^+-free ASW. Fig. 1 shows the typical result of such a procedure.

The solid symbols were obtained in K^+-free ASW before and after exposure of the axon to high K^+. They are well described by the relation (Goldman and Schauf, 1972).

$$I_{Na}^{V}/I_{Na}^{-180} = 1/(1 + e^{(V - V_h)/K_h}), \tag{1}$$

where $V_h = -45$ mV and $K_h = 5.0$ mV. On exposure to 50 mM K^+ with the axon held at its new resting potential (open symbols), two effects are apparent. There is a small but significant decrease in the maximum Na^+ current following a prepulse to −180 mV, and a much more noticeable change in the shape of the steady-state Na^+ inactivation relation. The half-maximum of the curve is shifted in the hyperpolarizing direction and its steepness is reduced. The resulting data are only poorly described by Eq. 1, but for purposes of comparison we may estimate $V_h = -72$ mV and $K_h = 23$ mV (dashed curve in Fig. 1). If instead of holding the axon at the new resting potential in 50 mM K^+ −ASW, it is held at the original resting potential in K^+-free ASW, the resulting steady-state inactivation curve still shows a slight decrease for hyperpolarized prepulses, but no change in shape ($V_h = -46$ mV; $K_h = 5.0$ mV).

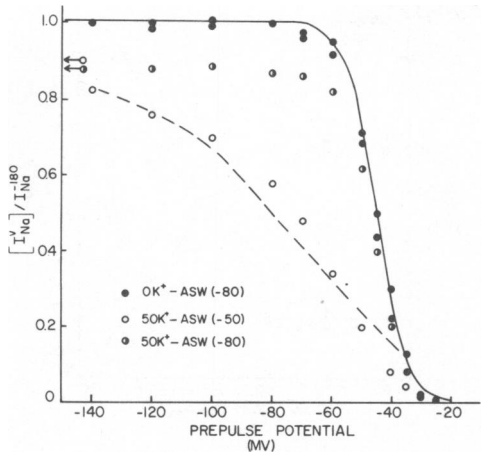

FIGURE 1

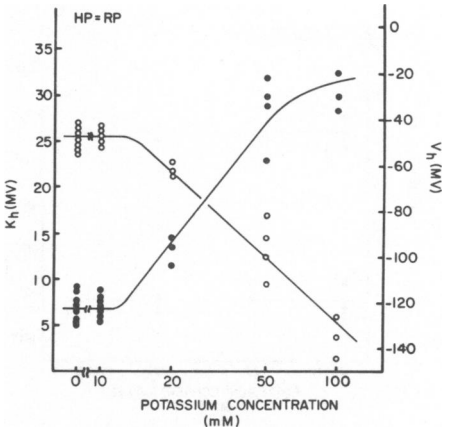

FIGURE 2

FIGURE 1 Steady-state inactivation curves measured in K^+-free ASW (●), and 50 mM K^+ −ASW (○, ◑) using holding potentials of −80 mV (●, ◑) and −50 mV (○). The peak sodium current during a test pulse to 0 mV after a 100 ms prepulse to the indicated potentials (I^{V}_{Na}), divided by that obtained in K^+-free ASW after a 100 ms prepulse to 180 mV (I^{-180}_{Na}), is plotted as a function of prepulse potential. Arrows at the left of the figure indicated the values obtained for −180 mV prepulses in 50 K^+ −ASW at the two holding potentials used. Solid and dashed lines were calculated using text Eq. 1. Points in K^+-free ASW were obtained before and after exposure of the axon to 50 mM K^+. Temperature 5°C.

FIGURE 2 Values for the shape parameters K_h (solid symbols) and V_h (open symbols) of text Eq. 1 as a function $[K^+]$ in axons held at their natural resting potential in each solution. Solid lines are drawn by eye. Temperature 5°C.

The data thus resemble those obtained by Adelman and Palti (1969*a,b*) in squid axons except that the inhibition of the maximum sodium current observed after large hyperpolarizing prepulses is much less in *Myxicola* at a given K^+ concentration (compare Fig. 3 of Adelman and Palti, 1969*a*). In fact the data in Fig. 1 represent the maximum inhibition observed at 50 mM K^+. Even at $[K^+]$ as high as 100 mM, the inhibition was never more than 15%, and in some cases no effect at all was seen with high $[K^+]$.

In order to compare results from axon to axon at various $[K^+]$, values for V_h and K_h were estimated despite the fact that Eq. 1 is not a totally satisfactory description of the steady-state inactivation relation in high $[K^+]$. The complete set of data are shown in Figs. 2 and 3 where the values of K_h and V_h are plotted as a function of $[K^+]$ with the axon held either at its new resting potential (Fig. 2) or at the resting potential initially observed in K^+-free ASW (Fig. 3). With increasing $[K^+]$ in axons held at progressively less negative potentials, K_h increases five-fold over the range 0–100 mM K^+, with some suggestion of saturation at high $[K^+]$. At the same time V_h becomes up to 100 mV more negative. No effects of $[K^+]$ on V_h or K_h are seen if the axons are held at their original resting potentials of −70 to −80 mV.

In order to determine whether these effects are directly $[K^+]$ dependent, or only in-

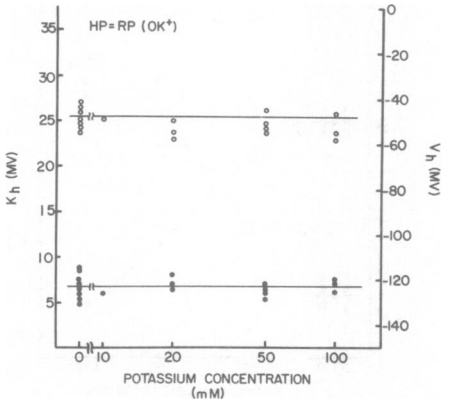

FIGURE 3

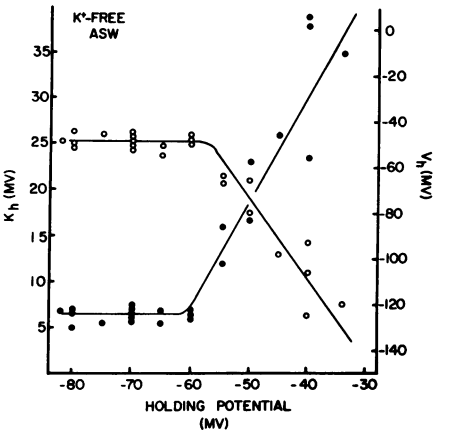

FIGURE 4

FIGURE 3 Values for the shape parameters K_h (solid symbols) and V_h (open symbols) of text Eq. 1 as a function of $[K^+]$ in axons held at their K^+-free ASW resting potential of −70 to −80 mV. Solid lines drawn by eye. Temperature 5°C.

FIGURE 4 Values for the shape parameters K_h (solid symbols) and V_h (open symbols) of text Eq. 1 as a function of holding potential in axons exposed to K^+-free ASW. Axons were held for 1 min at the indicated potentials prior to measurement of the steady-state inactivation relation and 20 s was allowed for recovery between each clamp pulse. Solid lines are drawn by eye. Temperature 5°C.

directly dependent on $[K^+]$ via the resulting depolarization, we carried out a set of experiments in which steady-state inactivation curves were measured using 100-ms prepulses in axons exposed to K^+-free ASW but held up to 35 mV depolarized from their resting state. At these potentials there is measurable activation of the K^+ conductance, but the Frankenhauser-Hodgkin space in *Myxicola* is sufficiently difficult to load (Binstock and Goldman, 1971) so that we could detect no significant increase in external $[K^+]$ using this procedure. The results are shown in Fig. 4. Holding *Myxicola* axons depolarized in K^+-free ASW produces increases in K_h and shifts of V_h comparable to those produced by elevation of $[K^+]$ by amounts causing equivalent reductions in resting potential. For example, the resting potential in 50 mM K^+ averages −40 mV. The range of K_h and V_h values in 50 mM K^+ are 23–32 mV and −82 to −112 mV, respectively, compared with ranges of 24–39 mV and −93 to −126 mV for K_h and V_h in axons exposed to 0 K^+ solutions but held at −40 mV. Thus it appears that the changes in steady-state inactivation curves produced by elevation of $[K^+]$ are primarily voltage dependent and thus only indirectly dependent on $[K^+]$, an interpretation significantly different from that used by Adelman and Palti (1969*a*,*b*) to explain similar effects in voltage-clamped squid axons.

These results are consistent with the existence of a second inactive state for the Na^+ channel which becomes occupied at potentials more depolarized than −60 mV and from which recovery is slow compared with the 100-ms prepulses used to define steady-

state Na^+ inactivation curves, at least at moderately hyperpolarized prepulse potentials. In order to test this hypothesis a series of experiments were done in which the time course of reactivation was determined after very long lasting depolarizations produced either by changes in holding potential in K^+-free ASW or by increases in $[K^+]$. In these experiments $[Na^+]$ was kept at 430 mM in order to provide larger sodium currents, the potential at which reactivation was determined being varied from −180 to −50 mV.

Fig. 5 shows the result of a typical experiment. The differences in the magnitudes of the peak sodium current during a test pulse to 0 mV after a 10 s prepulse to −160 mV (I_{Na}^{∞}) and following shorter recovery times (I_{Na}) are plotted semilogarithmically as a function of recovery time. (Note that these differences have been normalized by dividing by I_{Na}^{∞}.) In the left-hand portion the axon was in K^+-free ASW, while in the right it was exposed to 50 mM K^+. In both cases the membrane was held at −40 mV. Clearly, recovery from inactivation shows two distinct rate constants under these conditions. The faster process has kinetics similar to those previously determined by using relatively brief depolarizing prepulses to inactivate I_{Na} (Schauf, 1976) or simply by measuring removal of resting inactivation (Goldman and Schauf, 1972). This was the case at all recovery potentials examined. For example, when the recovery occurred at −80 mV the faster process had a time constant of 15 ms, comparable to previous data.

At 160 mV the slow process had a time constant ranging from 75 to 150 ms. Although there is considerable scatter from one axon to another, Fig. 5 shows that in any particular axon the time constants are dependent on recovery potential and are insensitive to the presence of K^+ in the bathing solution, thus reinforcing our earlier conclusion. In addition to being $[K^+]$ insensitive, the rate constant for slow reactivation is also not affected by the magnitude of the holding potential originally used to load the slow inactive state.

Fig. 6 shows the voltage dependence of the slow reactivation process obtained using 12 axons with a variety of values for holding potential and $[K^+]$. The rate constant declines monotonically with more positive recovery potentials, reaching values of the order of 0.1 s^{-1} for potentials slightly hyperpolarized to the normal resting potential of intact axons in ASW, where the faster reactivation process has a rate constant of the order of 40 s^{-1} (Schauf, 1976).

In contrast to reactivation, inactivation kinetics cannot be determined by direct measurement. However several indirect approaches may be used over limited ranges of membrane potential. It is, for example, possible to determine the fraction of channels in slow inactive state(s) as a function of holding potential by comparing the zero time intercept of the slow reactivation process (normalized) to the maximum peak I_{Na} after very long (20–50 s) recovery times at large hyperpolarized potentials. Although the scatter is quite large two conclusions are evident. First, the zero time intercept shows no systematic dependence on the potential at which reactivation is determined. In one experiment, for example, reactivation was measured at −80, −100, −130, and −160 mV with the fraction of channels showing slow recovery from the holding potential of −40 mV being 0.68, 0.70, 0.70, and 0.58, respectively. Secondly, the fraction of chan-

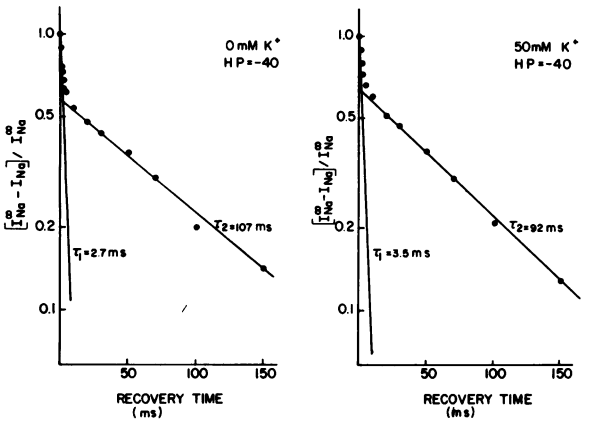

FIGURE 5

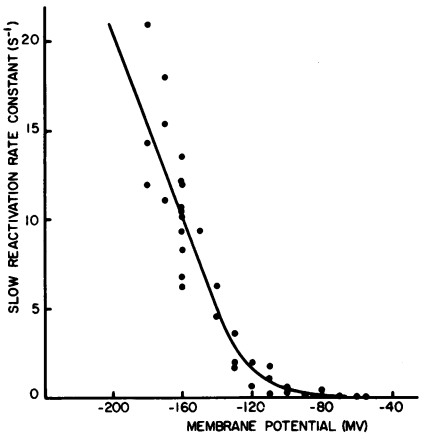

FIGURE 6

FIGURE 5 Time course of sodium activation at −160 mV from a holding potential of −40 mV in K^+-free ASW (left) and 50 K^+-ASW (right). The difference between the peak sodium current during a test pulse to 0 mV after a 10 s hyperpolarization to −160 mV (I_{Na}^{∞}) and the peak sodium current after a shorter hyperpolarization to −160 mV (I_{Na}) is divided by I_{Na}^{∞} and plotted as a function of recovery time at −160 mV. The axon was held at −40 mV for 2 min prior to the start of the reactivation experiment, and 20 s at −40 mV was allowed between each measurement. Solid lines represent the least squares fits of two exponentials to each set of data. Temperature 5°C.

FIGURE 6 Rate constants for the slow reactivation process as a function of membrane potential. Axons were held at potentials between −50 and −30 mV in solutions with $[K^+]$ ranging from 0 to 100 mM for 2 min, after which measurements with various recovery times followed at 20-s intervals. The solid line is drawn by eye. Temperature 5°C.

nels showing slow sodium reactivation is near zero at holding potentials of −60 mV, and increases to an average of 0.30 at −50 mV, 0.70 at −40 mV, and 0.82 at −30 mV. Even at −20 mV there is still evidence of an initial rapid reactivation of at least 10% of the available channels.

If one assumes that the slow inactivation process is rate limiting and exhibits simple first-order kinetics with purely voltage-dependent rate constants, then it is possible to estimate the rate constants for transitions into the slow inactive state from the reactivation rate constants of Fig. 6 and our estimates of the fraction of channels in this state. This argument suggests that over the range of −60 to −40 mV inactivation rate constants vary approximately from 0.1 to 0.5 s^{-1}, with negligibly small values at potentials more hyperpolarized than −60 mV.

In another series of experiments measurements of slow inactivation kinetics were made at depolarized potentials in the following way. The axon were exposed to a high $[K^+]$ solution and held at its natural resting potential. A long (1 s) hyperpolarizing pulse to −160 mV served to completely remove slow inactivation. The membrane was then allowed to return to the holding potential for a variable period of time (5 ms to 10 s), after which a brief (10 ms) pulse to −160 mV was used to reactivate mainly those channels which had not passed into the slow inactive state. Finally a constant

test pulse to +20 mV was used to determine I_{Na}. Plots of I_{Na} as a function of time at the holding potential yielded slow inactivation rate constants of 0.4–1.2 s^{-1} at potentials ranging from −20 to 0 mV.

From these data it is possible to conclude that the 30–50 mV depolarizations 100 ms in duration used in previous work to completely inactivate G_{Na} (Schauf, 1976) seem unlikely to have caused any significant loading of the slow inactive state.

DISCUSSION

Slow sodium inactivation in *Myxicola* has also been observed by Rudy (1975). In the single experiment shown the rate of recovery from the slow inactive state at a holding potential of −76 mV seems to agree very well with the present data.

In a previous study (Schauf, 1976) time constants for reactivation of G_{Na} after 100 ms depolarizations of 30–50 mV were found to be 30–50% larger than inactivation time constants determined in the same axon at the same potential. No evidence for a second slow process was found either during inactivation or with recovery times up to 200 ms, and it was concluded that a slow inactive state was unlikely to be the cause of the observed difference in time constants. This conclusion is strengthened by the present study. Slow reactivation has a time constant of 2–10 s at potentials of −60 to −40 mV, while time constants of slow inactivation are estimated to be in the range of 0.8–2.5 s at potentials of −20 to 0 mV. Moreover the slow inactive state is less than 10% loaded at holding potentials more negative than −60 mV such as are typical of the axon in 10 mM K^+ −ASW. Thus reactivation following a moderate 100 ms depolarization should not be detectibly affected by the slow processes examined here, and therefore differences in inactivation and reactivation kinetics presumably represent an inherent property of the fast gating machinery which must be dealt with in any model of channel gating.

Myxicola axons do not exhibit the marked decrease in maximum test pulse I_{Na} after moderate duration hyperpolarizing prepulses characteristic of squid axons in high $[K^+]_o$ (Adelman and Palti, 1969*a*). On the other hand, the effects on the shape parameters of the steady-state inactivation curve seem significantly larger. Unlike squid, neither exposure to high $[K^+]$ or changes in holding potential exert much effect on the time constant of fast sodium inactivation (Fig. 4 of Adelman and Palti, 1969*a*), and in *Myxicola* axons subjected to unusually long prepulses up to 50 s, only a single additional time constant is seen. In squid there seem to be two slow processes (Adelman and Palti, 1969*b*). Because of these differences and the lack of data on squid axons held for long times at depolarized potentials in K^+-free ASW, it is not possible to determine whether comparable processes are being examined in the two systems. Adelman and Palti (1969*b*) argued that the slow inactivation process in squid occurring over the 50–500 ms range was due to K^+ depletion during hyperpolarizing prepulses. Clearly this is not the case in *Myxicola*, but it would be premature to rule out this interpretation in squid axons because of the strong pharmacological effect of $[K^+]$ and the greater extent of loading of the Frankenhauser-Hodgkin space (Binstock and Goldman, 1971).

There are also marked differences in the slow inactivation process observed in *Myxicola* and in frog nodes (Peganov et al., 1973). In node there is no slow inactivation in K^+-free solution. However, in K^+-containing solutions the process of slow inactivation in node does not seem related to K^+ depletion. At potentials more positive than -50 mV, the fraction of channels in the slow inactive state becomes independent of potential in node, but the occupancy of this state depends on $[K^+]$. In *Myxicola,* loading of the slow inactive state seems independent of $[K^+]$ and only saturates at potentials above -20 mV where approximately 90% of the channels seemingly enter this state.

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REFERENCES

ADELMAN, W. J., JR., and Y. PALTI. 1969*a*. The influence of external potassium on the inactivation of sodium currents in the giant axon of the squid *Loligo pealei*. *J. Gen. Physiol.* **53**:685.

ADELMAN, W. J., JR., and Y. PALTI. 1969*b*. The effects of external potassium and long duration voltage conditioning on the amplitude of sodium currents in the giant axon of the squid *Loligo pealei*. *J. Gen. Physiol.* **54**:589.

BINSTOCK, L., and L. GOLDMAN. 1969. Current and voltage-clamped studies on *Myxicola* giant axons: effect of tetrodotoxin. *J. Gen. Physiol.* **54**:730.

BINSTOCK, L., and L. GOLDMAN. 1971. Rectification in instantaneous potassium current-voltage relations in *Myxicola* giant axons. *J. Physiol.* **217**:517.

FRANKENHAUSER, B., and A. L. HODGKIN. 1956. The after-effects of impulses in the giant nerve fibers of *Loligo*. *J. Physiol.* **131**:341.

GOLDMAN, L., and C. L. SCHAUF. 1972. Inactivation of the sodium current in *Myxicola* axons: evidence for coupling to the activation process. *J. Gen. Physiol.* **59**:659.

GOLDMAN, L., and C. L. SCHAUF. 1973. Quantitative description of sodium and potassium currents and computed action potentials in *Myxicola* giant axons. *J. Gen. Physiol.* **61**:361.

PEGANOV, E. M., B. I. KHODOROV, and L. D. SHISHKOVA. 1973. Slow sodium inactivation in the Ranvier node membrane: role of external potassium. *Bull. Exp. Biol. Med.* **76**:15.

RUDY, B. 1975. Slow recovery of the inactivation of sodium conductance in *Myxicola* giant axons. *J. Physiol.* **249**:22P.

SCHAUF, C. L. 1974. Sodium currents in *Myxicola* axons: nonexponential recovery from the inactive state. *Biophys. J.* **14**:151.

SCHAUF, C. L. 1975. The interactions of calcium with *Myxicola* giant axons and a description in terms of a simple surface charge model. *J. Physiol.* **248**:613.

SCHAUF, C. L. 1976. Comparison of two-pulse sodium inactivation with reactivation in *Myxicola* giant axons. *Biophys. J.* **16**:245.

SCHAUF, C. L., and F. A. DAVIS. 1975. Further studies of activation-inactivation coupling in *Myxicola* axons: insensitivity to changes in calcium concentration. *Biophys. J.* **15**:1111.