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H. Meves

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Asymmetry currents in intracellularly perfused squid giant axons

By H. Meves
Laboratory of the Marine Biological Association, Plymouth

Asymmetry currents were recorded from intracellularly perfused squid axons subjected to exactly equal positive and negative voltage clamp pulses at a temperature close to 0 °C. The voltage and time dependence of the asymmetry currents was studied at a holding potential of −80 to −100 mV. The effect of varying the holding potential was investigated. The latter experiments showed that the voltage dependence of the asymmetrical charge movement is different from the voltage dependence of the *m* system.

Introduction

Squid axons which are subjected alternately to exactly equal positive and negative voltage-clamp pulses show asymmetry currents which have been interpreted as sodium gating currents (Armstrong & Bezanilla 1973; Bezanilla & Armstrong 1974; Keynes & Rojas 1973, 1974; Keynes, Rojas & Rudy 1974). The asymmetry currents usually consist of a transient outward current at the beginning of the pulses and a transient inward current at the end of the pulses, reflecting presumably the charge movement associated with the opening and the closing of the sodium gates. Keynes & Rojas (1974) in a quantitative analysis of the asymmetry currents showed that the time constant of the asymmetrical charge movement is similar to the time constants of the *m* system and of the sodium conductance. They assumed a fixed number of mobile charges (thought to be sodium gating particles) within the membrane which are distributed between the inside and outside of the membrane according to Boltzmann's principle. The evidence for associating the asymmetrical charge displacement with the sodium gates is strengthened by the observations of Bezanilla & Armstrong (1974). They found that both the asymmetry current and the sodium current are blocked by internal perfusion with zinc ions or by holding the membrane potential at +56 mV for 2 min.

The experiments described here were started in collaboration with Professor T. I. Shaw and Dr W. Vogel and have been briefly reported elsewhere (Meves, Shaw & Vogel 1974). They are mainly concerned with the effect of pulse height and holding potential on the asymmetry currents and lead to the conclusion that the voltage dependence of the asymmetrical charge movement is different from the voltage dependence of the *m* system.

Methods

Giant axons of 600–1000 μm diameter were dissected from mantles of *Loligo forbesi* and occasionally *Loligo vulgaris*. The uncleaned axon was extruded and perfused by the method of Baker, Hodgkin & Shaw (1962). The voltage clamp method was the same as used by Chandler & Meves (1965) and Meves & Vogel (1973). The membrane currents were recorded with a double C electrode and amplified with a Grass P 18 differential amplifier (3–10 μs rise time) and a Tektronix 3A3 differential amplifier. The output signal of the 3A3 amplifier was fed

to the d.c. input of a Biomac 1000 Signal Analyzer. Overloading of the Biomac by the peaks of the capacitative currents was avoided by means of an f.e.t. series/shunt switch which automatically disconnected and grounded the Biomac input for a short period (30–50 μs) at the beginning and end of each clamp pulse. Thirty-two positive and 32 negative clamp pulses of equal size were applied alternately with a pulse interval of 0.7 s. The pulses were carefully checked for equal size before each experiment. The membrane currents associated with the 32 positive and 32 negative clamp pulses were algebraically summed by the Biomac. The averaged signal was recorded on an oscilloscope. All resting potential measurements were corrected for junction potentials determined at the beginning and end of each experiment.

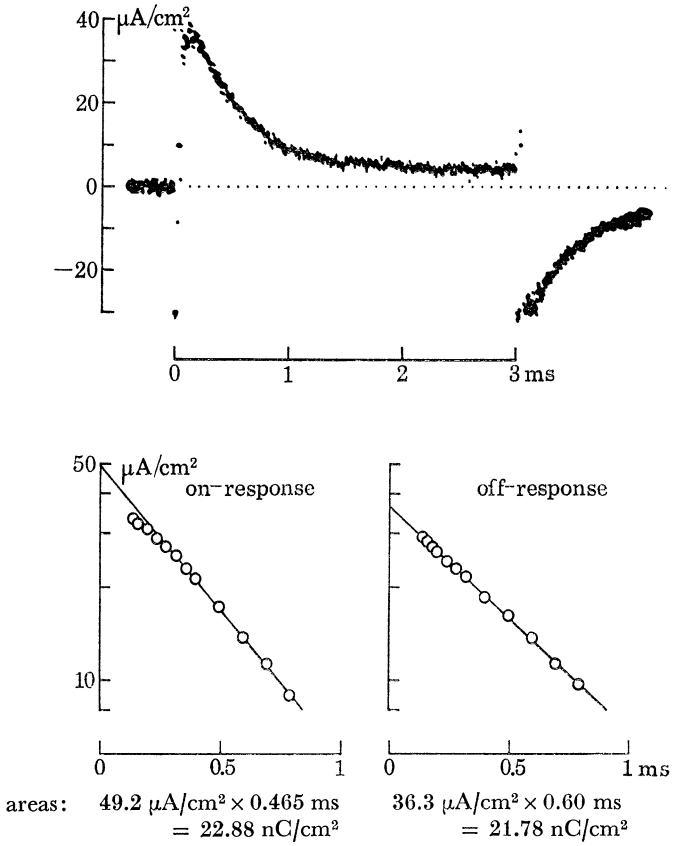

FIGURE 1. Asymmetry current obtained by averaging the membrane currents associated with 32 positive and 32 negative pulses of 110 mV amplitude. Internal solution: 275 mM RbF + 50 mM tetraethylammonium chloride + sucrose. Resting potential −68 mV, holding potential −88 mV. 30 μs blanking pulses. Axon diameter 680 μm, temperature +1 °C. The transient outward current at the beginning of the pulses (on-response) and the inward current at the end of the pulses (off-response) are plotted against time on a logarithmic scale. The area under the on- and off-response is calculated from the current at zero time and the time constant.

The external solution was Na-free tris seawater with 11 mM $CaCl_2$, 55 mM $MgCl_2$ and 524 mM tris (Trizma base from Sigma); HCl was added to give a pH of 7.7. The external solution contained 2 μM tetrodotoxin. The internal solution was 300 mM CsF + sucrose or 275 mM RbF + 50 mM tetraethylammonium chloride + sucrose; the pH was adjusted to 7.2–7.4 with 0.1 mM tris-HCl buffer. All experiments were done at a temperature around 0 °C.

Results

The effect of pulse height

Figure 1 shows a typical record of the asymmetry current obtained with ±110 mV pulses at a holding potential of −88 mV. The asymmetry current consists of a transient outward current at the beginning of the pulses (on-response) and a transient inward current at the end of the pulses (off-response). The on-response decays to a small sustained outward current which represents the asymmetrical part of the leakage current. In the lower part of figure 1 the on-response (after subtracting the sustained current) and the off-response are plotted on a logarithmic scale against time. Both responses follow an exponential time course, apart from a deviation at the beginning of the on-response. The areas under the on- and off-response were obtained from the extrapolated current at zero time and the time constant and represent the amount of charge movement. The figures given in figure 1 show that the charge movement during the on-response is almost the same as that during the off-response.

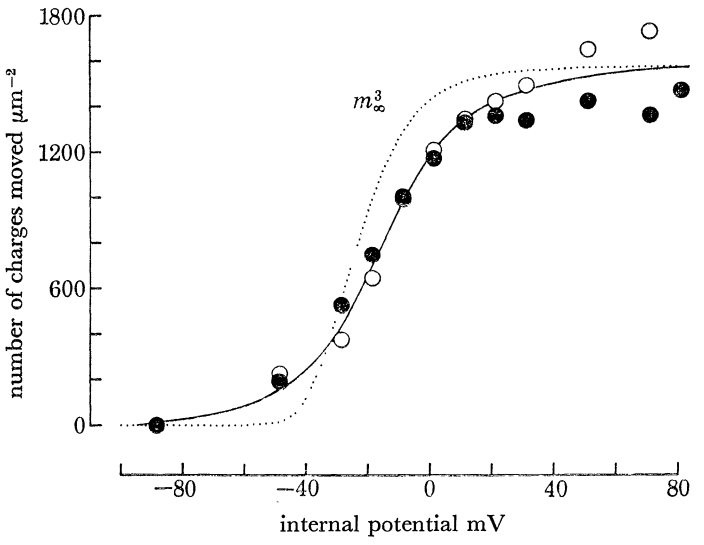

Figure 2. Charge movement during the on-response (○) and during the off-response (●) plotted against the internal potential during the depolarizing pulses. Same experiment as in figure 1. The charge movement (in electronic charges/μm^2 of membrane) was determined from semilogarithmic plots of the on- and off-responses (see figure 1). The continuous curve was obtained from the Langevin–Debye function which is given by $f(x) = \coth x - 1/x$; $x = \mu E/kT$; where μ is the dipole moment (assumed as 840 Debye units), E the potential gradient across membrane (calculated for a 7 nm thick membrane), k the Boltzmann constant, and T the absolute temperature. The dotted curve represents m_∞^3 and was calculated from Hodgkin & Huxley (1952).

The measurements were repeated with pulse heights varying between ±40 and ±170 mV. For each pulse height the areas of the on- and off-response were determined from semilogarithmic plots. They were plotted against the internal potential during the depolarizing pulses (figure 2), assuming that the asymmetrical charge movement occurs during and after the depolarizing pulses (see below). The on-responses (empty circles) and the off-responses (filled circles) increase along a sigmoid curve as the potential during the depolarizing pulses becomes more positive. At positive internal potentials the off-responses are consistently smaller than the on-responses whereas at negative potentials no systematic deviation is seen. The averages from

on- and off-response were used to draw the continuous curve. It was calculated from the Langevin–Debye function (see legend of figure 2) which describes the orientation of permanent dipoles in an electric field and has been applied to the nerve membrane by previous authors (e.g. Johnson, Eyring & Polissar 1954; Hamel & Zimmerman 1970). The best fit was obtained with a dipole moment $\mu = 840$ Debye units† and $f(x) = 0$ at -16 mV. The curve approaches a maximum of 1600 electronic charges/μm^2 of membrane at strong positive potentials. Its steepest part is in the potential range between -21 and -11 mV with a slope equivalent to an e-fold change in charge for 19 mV potential change. For comparison the dotted curve shows the voltage dependence of the sodium conductance variable m_∞^3 as calculated from the equations of Hodgkin & Huxley (1952), assuming a resting potential of -62 mV in their experiments. The m_∞^3 curve is clearly steeper than the continuous curve and its half potential is at -23.5 mV as opposed to -16 mV for the continuous curve; the half potential of the m_∞ curve (not shown) would be at -37 mV.

The analysis of the experiment in figures 1 and 2 gave numerical values for the time constants of the on- and off-response, τ_{on} and τ_{off}. Plotting τ_{on} against the internal potential during the depolarizing pulses showed a maximum of the time constant at an internal potential of -10 to -15 mV. The voltage dependence of τ_{on} resembled the bell-shaped curve for τ_m as calculated from the equations of Hodgkin & Huxley (1952) and the numerical values for τ_{on} were similar to those for τ_m at the same temperature; the only major difference was that the maximum of τ_{on} occurred at a 20–25 mV more positive potential than that of τ_m. The time constant τ_{off} was found to depend strongly on the height of the pulses. The value 0.60 ms given in figure 1 was obtained with ± 110 mV pulses; τ_{off} was the same for larger pulses (up to ± 170 mV), but decreased markedly for smaller pulses, dropping to a value of 0.172 ms for ± 40 mV pulses.

The results so far reported are in agreement with the quantitative results of Keynes & Rojas (1974). The limiting slope of the sigmoid curve in figure 2 (19 mV for an e-fold change) is identical with the average value given by Keynes & Rojas. The resemblance between τ_{on} and τ_m and the strong dependence of τ_{off} on pulse height are consistent with the observations of Keynes & Rojas. The maximum charge displacement of 1600 electronic charges/μm^2 (see figure 2) is somewhat smaller than their average value of 1882 electronic charges/μm^2, probably because this average includes measurements at a holding potential more negative than that used in the experiment of figures 1 and 2. The half potential of -16 mV for the sigmoid curve in figure 2 is in reasonable agreement with the average value of -21.5 mV which Keynes & Rojas found for four intact axons. Both values are clearly smaller than the potential for the midpoint of the theoretical m_∞^3 or m_∞ curve ($V = -23.5$ or -37 mV). A possible explanation would be that the m_∞ curve is shifted towards more positive potentials under our experimental conditions. To settle the question it would be necessary to measure the voltage and time dependence of the m system and of the asymmetry current under exactly the same experimental conditions, preferably on the same fibre.

The effect of holding potential

In the experiment of figures 1 and 2 the membrane was held at a potential of -88 mV. Further experiments were done with more negative and more positive holding potentials. The experiments showed that the effect of holding potential develops slowly. This is illustrated by the following two examples. A fibre with a resting potential of -33.5 mV was held at a

† 1 Debye unit $= 10^{-18}$ e.s.u. $\approx 3.3 \times 10^{-30}$ C m.

potential of −94 mV for 3 min, for 20 s and again for 3 min; the charge displacement during the asymmetry current (measured with ±90 mV pulses) was 8.23, 6.36 and 9.02 nC/cm², respectively. Another fibre with a resting potential of −45.5 mV was held at a potential of −81.5 mV for 3 min and for 25 min; the charge displacement during the asymmetry current (measured with ±90 mV pulses) was 7.33 and 14.40 nC/cm², respectively. These observations suggest a slow change in the distribution of the mobile charges in the membrane. The phenomenon may be particularly pronounced at low temperatures. To assess the effect of holding potential on the asymmetry currents it is essential to hold the membrane at the respective potential for a defined period of time. A period of 3 min was chosen for most experiments.

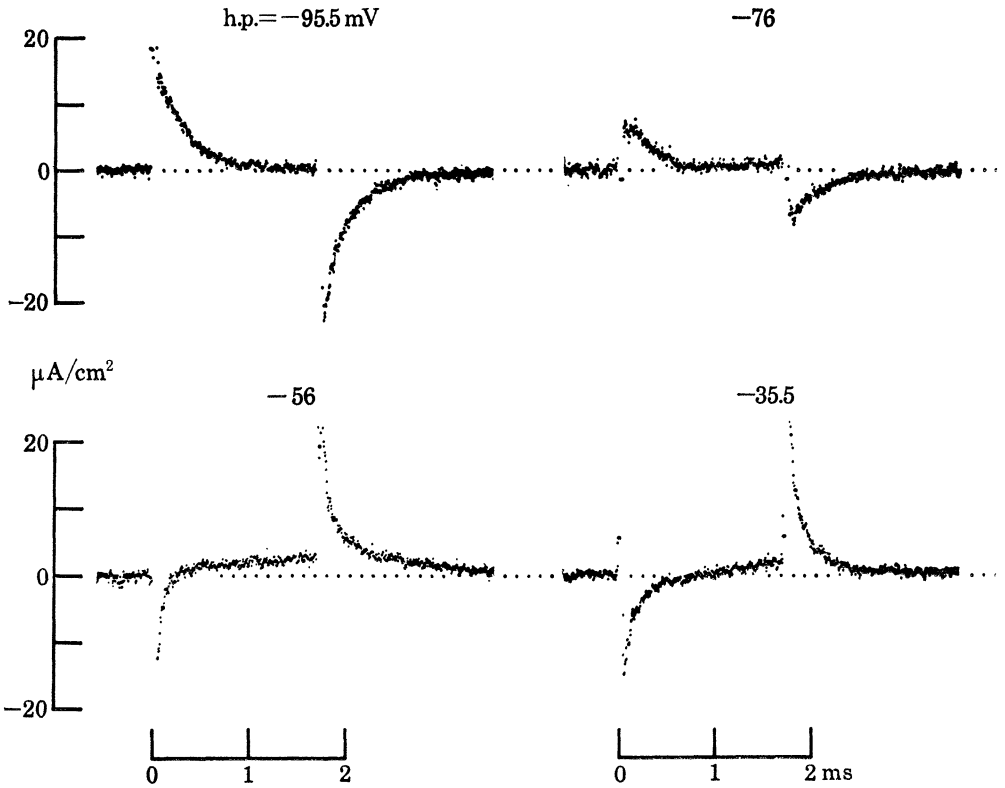

FIGURE 3. Effect of holding potential on asymmetry current. The records were obtained by averaging the membrane currents associated with 32 positive and 32 negative pulses of 90 mV amplitude. Internal solution: 300 mM CsF + sucrose. Resting potential −36 mV. Holding potentials as indicated; the membrane was held at the respective holding potential for 3 min before recording the asymmetry current. 30 μs blanking pulses. Axon diameter 1110 μm, temperature + 2.5 °C.

Changing the holding potential from −80 or −90 mV to −100 or −110 mV produced a small increase in the size of the asymmetry current. The charge displacement was determined from the areas of the on- and off-response and plotted against the internal potential during the depolarizing pulses as in figure 2. The charge displacements measured with the more negative holding potential were found to be larger by a factor of 1.4.

Changing the holding potential from −80 or −100 mV to a less negative potential caused a decrease of the asymmetry current. At holding potentials less negative than −65 or −60 mV the asymmetry current reversed its sign: the transient current at the beginning of the pulses turned into an inward current and the transient current at the end of the pulses became

outward. A sequence of records taken with ± 90 mV pulses at four different holding potentials is illustrated in figure 3. The records show the reversal of the asymmetry current at a holding potential between −76 and −56 mV. In ten other experiments the reversal took place in the same potential range and the reversal potential was estimated as −65 to −60 mV. The most positive potential used in these experiments was −3 mV; no inactivation of the asymmetry current was seen in the potential range studied.

The most likely interpretation is that at a holding potential of −65 to −60 mV the mobile charges are evenly distributed in the membrane and the charge displacement during the positive and negative pulses is symmetrical. This interpretation was confirmed by recording the membrane currents associated with single depolarizing and single hyperpolarizing pulses at a holding potential of −60 mV. At a more negative holding potential the charge displacement produced by the depolarizing pulse became larger and that produced by the hyperpolarizing pulse smaller; at a more positive holding potential the changes were in the opposite sense.

The observation that the asymmetry current reverses at low holding potentials is consistent with the findings of Keynes *et al.* (1974). Our reversal potential of −65 to −60 mV is more negative than the midpoint potential of the theoretical m_∞^3 or m_∞ curve ($V = -23.5$ or −37 mV); the difference would be even larger if the actual m_∞ curve were to some extent shifted to more positive potentials under our experimental conditions (see p. 496). The reversal potential −65 to −60 mV is also more negative than the half potential of the sigmoid distribution curve in figure 2 ($V = -16$ mV). If our findings are correct, it would seem that there is not a single distribution curve but that the distribution curve changes its shape and position according to such factors as holding potential and holding time. It remains to be seen whether a simple relation between the different distribution curves and the voltage dependence of the sodium conductance parameters m_∞ or m_∞^3 or $m_\infty^3 h_\infty$ exists.

To test our interpretation it would be necessary to repeat the experiment of figure 2 at different holding potentials, i.e. to measure the asymmetry current with pulses of varying amplitude at different holding potentials. In a single experiment of this kind the pulse amplitude was varied between 0 and ± 120 mV at two different holding potentials, −85 and −28.5 mV. The pulses were not large enough to reach the maximum charge displacement so that the half potentials could not be accurately determined. The measurements indicated a half potential of about −11 mV for the −85 mV holding potential (in approximate agreement with the experiment in figure 2 which gave a half potential of −16 mV for a holding potential of −88 mV) and a half potential more negative than −96 mV for the −28.5 mV holding potential. These findings support the idea that the position of the distribution curve depends on the holding potential, but more experiments are needed to establish this point clearly.

Discussion

Most of the results are in agreement with the observations of Keynes & Rojas (1974) and Keynes *et al.* (1974). At a holding potential of −80 to −100 mV the magnitude of the asymmetrical charge displacement increased with increasing pulse height along a sigmoid curve. As the holding potential was made less negative, the asymmetry current became smaller and finally reversed its sign. The experiments with varied holding potential led to two main conclusions: (1) the asymmetry current is not inactivated at low holding potentials in the

potential range studied (more negative than -3 mV); it seems that positive holding potentials are required to achieve the almost complete inactivation which Bezanilla & Armstrong (1974) saw at a holding potential of $+56$ mV. (2) The potential at which the mobile charges are evenly distributed is not identical with the potential at which m_∞^3 or $m_\infty = 0.5$ and is also not identical with the half potential of the sigmoid distribution curve measured with short pulses at a high holding potential. Our results seem to indicate that the distribution of the mobile charges that are responsible for the asymmetry current changes slowly when the membrane is held at different holding potentials. Consequently, a different distribution curve and a different half potential is found depending on whether short clamp pulses or long-lasting changes in holding potential are used for measuring it.

It seems possible that the slow effect of holding potential which was found in our experiments is in some way related to the slow change in sodium permeability reported by Narahashi (1964) and Adelman & Palti (1969). The latter authors discuss the possibility that this slow change in sodium permeability is caused by a slow change in membrane dipole orientation and quote examples from the literature which support this view. A further example is the recent finding that spin-labelled phospholipid molecules incorporated in membrane vesicle preparations may undergo a slow inside–outside transition ('flip-flop') with a half time of 3.8–7 min at 15 °C (McNamee & McConnel 1973).

Finally, it should be emphasized that the findings reported here do not invalidate the idea that the asymmetry currents are in some way related to the opening and closing of the sodium gates. They suggest, however, that the asymmetrical charge movement does not simply reflect the voltage dependence of the m system, but that the relation between asymmetrical charge movement and sodium conductance is more complicated. The complication could be due to the fact that (*a*) the voltage dependence of the asymmetrical charge movement reflects changes both in m and in h, (*b*) secondary reactions are interposed between the orientation change of the membrane dipoles and the sodium conductance change or (*c*) only part of the asymmetrical charge movement is related to the changes in sodium conductance.

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