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PRESYNAPTIC CURRENTS IN MOUSE MOTOR ENDINGS

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SUMMARY

1. We used external electrodes placed under precise visual control on motor endings of the mouse to record electrical activity promoted by nerve stimulation.

2. Three types of wave form have been observed in relation to well-defined electrode emplacements: (i) at the transition between myelinated and non-myelinated parts of the axon, the wave form consists of two negative deflexions preceded by a small positivity (preterminal response), (ii) at the main part of the terminal branches, we obtained a two component positive wave form (terminal response) and (iii) electrode positions in a narrow area between the former and the latter yielded triphasic (positive–negative–positive) wave forms (intermediate responses).

3. Since these responses could not be readily interpreted in terms of classical description of membrane currents associated with propagating action potentials, we used specific channel blocking agents to identify wave form components.

4. Bath application of tetraethylammonium or aminopyridines, or, better, a combination of both, suppressed delayed positive deflexions of terminal and intermediate responses and the late negative component of preterminal responses. Local inophoretic drug application showed that K channels are present only at the terminal part of the endings. K^+ outflux promotes a local circuit whose sink is located at the preterminal part where it generates the late negative deflexion of the preterminal response.

5. Local application of tetrodotoxin suppressed the first negative component of preterminal responses but failed to affect electrical activity at the terminal part of the endings. This indicates that Na channels, and, therefore, action potential generation, are restricted to the preterminal part.

6. Suppression of K conductance revealed a slow inward current at the terminal part of the endings which could be identified as a Ca current. Ba^{2+} and Sr^{2+} could substitute for Ca^{2+} as inward current carriers.

7. Activation of spatially separated Na channels, on one side, and of K and Ca channels, on the other, generated ionic currents and separated local circuit currents which flow between preterminal and terminal parts (and vice versa). Thus, the signals recorded at each point of motor endings correspond to the sum of ionic and passive currents entering (or leaving) the membrane at that point.

8. The present results represent a further example of heterogeneity of axonal membrane.

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INTRODUCTION

In the classical description of action potential electrogenesis membrane repolarization is mediated to a large extent by an increase in K conductance (Hodgkin & Huxley, 1952). However, recent reports on data obtained from mammalian nodes of Ranvier have shown that the repolarizing phase of the action potential depends mainly on Na inactivation and leakage conductance, the role of K conductance being negligible (Nonner & Stämpfli, 1969; Chiu, Ritchie, Rogart & Stagg, 1979; Brismar, 1980; but see Binah & Palti, 1981). Similarly, only large diameter fibres from amphibian nerves seem to display a nodal delayed K conductance, while small ones appear devoid of it (Smith & Schauf, 1981). Furthermore, the lack of effect of typical K channel blockers such as tetraethylammonium (TEA) and 4-aminopyridine (4-AP) on the action potential revealed the absence of K conductance in central myelinated axons of rat (Kocsis & Waxman, 1980) and goldfish (Kaars & Faber, 1981). On the other hand, analysis of membrane currents in demyelinated nerve fibres from alloxan diabetic rats (Brismar, 1979), in diphtheria toxin-treated nerves (Bostock, Sears & Sherratt, 1981; Brismar, 1981) or after acute myelin loosening treatments (Chiu & Ritchie, 1981, 1982) showed that K, but not Na channels are present under the internodal myelin of mammalian or amphibian nerve fibres. Interestingly, Na channels which are restricted to the nodal membrane under normal conditions (Ritchie & Rogart, 1977) appear at the internodes as a consequence of demyelination by diphtheria toxin (Bostock & Sears, 1978; Brismar, 1981; see Waxman & Foster, 1980, for review).

In mammalian nerves, Na and K channels appear, thus, segregated from each other along myelinated axons, although they are evenly distributed in non-myelinated ones (Bostock *et al.* 1981). This raises the important question of channel distribution in mammalian motor endings. Do Na and K channels show a separate distribution as in the parent axon, or are they randomly distributed as in non-myelinated fibres? A subsidiary question is the problem of active *vs.* passive impulse propagation in presynaptic terminals. Katz & Miledi (1965) and Braun & Schmidt (1966) demonstrated active propagation up to or very close to the extreme end of frog motor endings. This does not seem to be the case in crayfish motor terminals (Dudel, 1965) or in squid giant synapse (Takeuchi & Takeuchi, 1962). The observations of Hubbard & Schmidt (1963), which favour active invasion of rat motor endings, suffer from lack of precise optical control of electrode positioning.

We re-examined the problem of presynaptic membrane excitability in mammalian motor endings by recording external currents. This investigation was made easy by using a very thin and flat mouse muscle which permits precise electrode positioning under high power optical control (McArdle, Angaut-Petit, Mallart, Bournaud, Faille & Brigant, 1981). The use of specific channel blocking agents allowed us to identify wave form components related to Na, K and Ca currents.

METHODS

Adult Swiss mice were used. The thoracic cage was removed from the animal and the *triangularis sterni* muscle with one of its nerves was dissected under continuous perfusion with oxygenated Krebs solution. The neuromuscular preparation was pinned on the bottom of the experimental chamber

made of a glass slide covered with a 3 mm layer of a silicon elastomere (Rhodorsil, Rhone-Poulenc). Since the muscle consists of only two to three layers of fibres, most of the morphological details of the superficial motor endings can be visualized at a $\times 500$ magnification using a water immersion $\times 40$ Zeiss objective (working distance 1·6 mm) in a microscope fitted with interference contrast (Nomarski) equipment. The nerve was stimulated through a suction electrode (McArdle *et al.* 1981) at 1 Hz.

Solutions. Standard saline solution contained (mM); NaCl, 154; KCl, 5; $CaCl_2$, 2; glucose, 11 and Hepes 5 to buffer at pH 7·3. The preparation was perfused with the oxygenated solution at a rate of 15 ml/min and maintained at 22 °C. Unless otherwise stated, d-tubocurarine was added at a concentration sufficient to block all post-synaptic activity (range 20–100 μM).

Focal current recording. The technique was basically the same as that used previously by del Castillo & Katz (1956) except that we used thick-walled, heat-polished electrodes of 2–4 μm i.d. filled with standard Krebs solution (Neher, Sakmann & Steinbach, 1978; Dudel, 1981). These were gently pressed against visible branches of the terminal arborization. The reference was a large Ag–AgCl distant bath electrode. Extracellular current that enters (or leaves as the case may be) the membrane under the electrode opening was recorded as a potential drop across the sealing resistance. Positive signals indicated outward current; negative signals, inward current. Since the sealing was far from perfect due to conjunctive tissue and Schwann cell processes that partially cover the endings, signal amplitude was about 0·2 mV. To improve signal to noise ratio, twenty to thirty sweeps were sampled in 20 μs steps, averaged by means of a computer (Didac 800, Intertechnique) and displayed on a pen recorder. Amplitude calibrations were omitted in most of the present recordings because signal amplitude depended on many factors unrelated to membrane current.

Drug ionophoresis. Micropipettes of 100–200 MΩ filled with tetrodotoxin (TTX) ($1{\cdot}5 \times 10^{-4}$ M) or TEA (1 M) dissolved in Krebs solution were used for local drug application.

RESULTS

Electrical activity at different points of nerve termination

Typical records obtained from motor endings of the mouse are shown in Fig. 1. Wave form configuration varied with electrode position. At the transition between myelinated and non-myelinated portions of the axon we invariably observed a predominantly negative signal which was frequently preceded by a positive deflexion of variable size. The negative phase appears composed of two elements whose relative amplitudes varied on small readjustment of electrode position. The segment that generates this wave form lies above the end-plate and does not contact the post-synaptic membrane (Couteaux, 1958); it is, therefore, unlikely that it releases transmitter. The recordings obtained from this electrode position have been called preterminal responses. By moving the electrode slightly in the distal direction, the wave form becomes triphasic (positive–negative–positive). Wave form configuration was critically dependent on electrode position: negativity decreased and positivity increased in amplitude as the electrode was moved within narrow limits from proximal to distal positions. It became apparent after careful exploration of this segment that positive deflexions of the triphasic wave form corresponded to gradual reversal of the negative components of the preterminal response. Recordings obtained from the transition between initial and main parts of the endings have been called intermediate responses. Finally, from this electrode position, up to the tip of terminal branches we could record only positive signals composed of two elements whose relative amplitudes did not change appreciably with electrode position. We called these terminal responses. In a sense, the terminal response may be regarded as the inverted replica of the preterminal response. Averaged records of preterminal,

intermediate and terminal wave forms are shown in Fig. 1 *A–C*. To give an example of the relative length of the segment that generates each wave form, in an end-plate of 25 μm in diameter, the preterminal response was recorded from a stretch of 5 μm starting at the myelin end, the intermediate response from the next 3 μm and the terminal response in the remaining 17 μm.

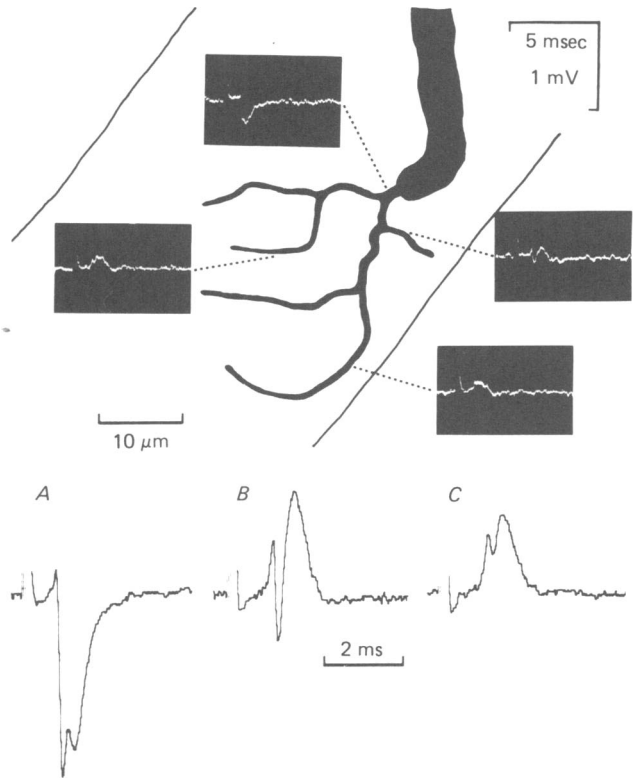

Fig. 1. Mouse motor ending drawn from a silver impregnated preparation showing the myelinated part of the axon and non-myelinated terminal branches where the location of typical wave forms is indicated. *A*, *B* and *C* show averaged recordings of preterminal, intermediate and terminal responses, respectively.

Of the three types of response recorded from mouse motor endings, only the intermediate response resembles the classical triphasic wave form observed in motor terminals of frogs (Katz & Miledi, 1965; Braun & Schmidt, 1966; Benoit & Mambrini, 1970) and rats (Datyner & Gage, 1980) which corresponds to membrane current related to a propagating action potential (Tasaki, 1959; Katz & Miledi, 1965; Bostock *et al.* 1981). The triphasic wave form corresponds thus, successively, to outward passive current, active Na current and delayed outward K current. The terminal response from mouse endings is positive like corresponding responses from rat (Hubbard & Schmidt, 1963), crayfish (Dudel, 1965, 1981; Zucker, 1974) and squid (Takeuchi & Takeuchi, 1962) but, unlike these, it displays two positive components. Positive responses have been taken to indicate passive depolarization beyond a

conduction block (see Eccles, 1964) and correspond to current flow which discharges membrane capacity. However, predominantly positive wave forms recorded at the tip of the presynaptic terminal do not necessarily signal conduction block (see Katz & Miledi, 1965). Since the wave forms we recorded from the initial and terminal parts of mouse motor endings do not resemble those classically described in linear conductors or closed ends, respectively, we did not attempt further interpretation based on theoretical considerations. We turned, rather, to a more direct approach and used specific channel blockers to describe the recorded wave forms in terms of specific (capacitive and ionic) membrane currents.

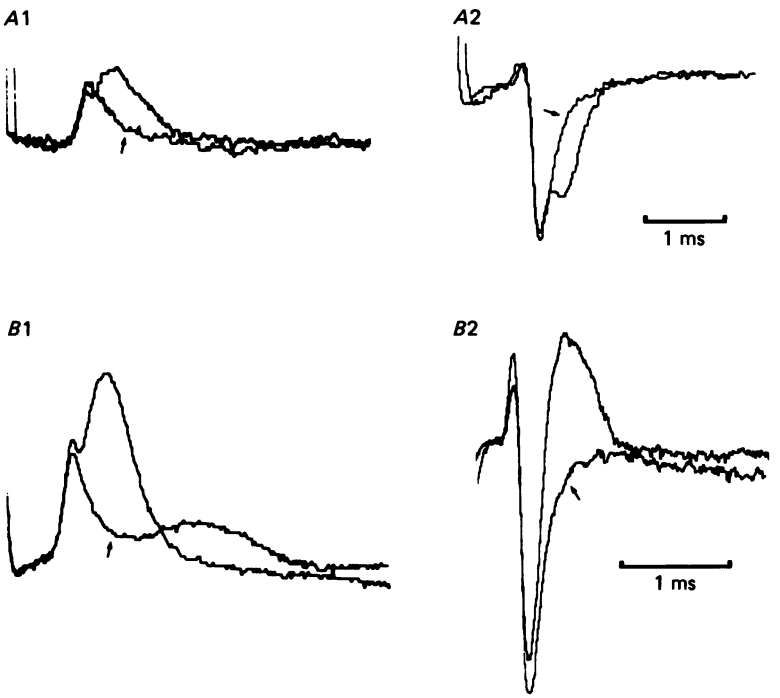

Fig. 2. Actions of TEA (10 mM) and 4-AP (2 mM) on electrical activity of motor endings. *A*1, terminal wave form; arrow indicates what remains after 5 min exposure to TEA. *A*2, preterminal wave form after brief exposure to TEA (arrow); second negative component shows incomplete recovery after 20 min wash. *B*1, 4-AP delays and depresses second component of terminal wave form. *B*2, 4-AP suppressed late positivity of triphasic intermediate wave form. Same time calibration for *A*1, *A*2 and *B*1.

Effect of K channel blockers on presynaptic currents

Substances which selectively inhibit delayed K conductance in frog nodes such as TEA (Hille, 1967) and 4-AP (Ulbricht & Wagner, 1976) are without effect on mammalian nodes (Bostock *et al.* 1981). Nevertheless, they affect K conductance in amyelinic and experimentally demyelinated mammalian fibres (Bostock *et al.* 1981); moreover, they potentiate transmitter release in mammalian endings (Lundh, 1978; Molgó, Lundh & Thesleff, 1980). One would, then, expect an action of these drugs on currents recorded from mouse motor terminals.

TEA. At concentrations of 5–10 mM, TEA increased, in a dose-dependent fashion, presynaptic response latency. We observed also (A. Mallart & J. L. Brigant, unpublished) a lengthening of the relative refractory period of conduction. This might be due to small action potential amplitude in TEA (see Bostock *et al.* 1981). The main action of this drug was, however, on late deflexions of presynaptic wave forms. Fig. 2*A*1, *A*2 shows the suppression of late positive and negative components of terminal and preterminal responses respectively within a few minutes of perfusion with the

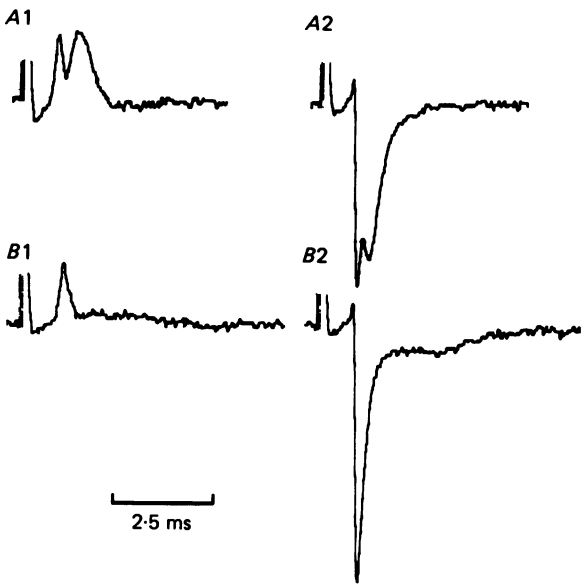

Fig. 3. Terminal (1) and preterminal (2) responses before (*A*) and during (*B*) perfusion of 3,4-DAP (100 μM) containing solution. Note incomplete suppression of late components of terminal and preterminal wave forms.

TEA-containing solution. The late positive deflexion of intermediate responses was also rapidly suppressed by TEA. The unaffected portion of the wave form decayed, then, monotonously to zero. This effect is comparable to that observed on triphasic wave forms of frog endings (Benoit & Mambrini, 1970) and rat demyelinated nerve fibres (Bostock *et al.* 1981).

Aminopyridines. The effect of 4-AP (1–2 mM) on delayed currents was essentially similar to that of TEA except that it failed to suppress them completely. Higher doses were somewhat more effective. The effect of 2 mM-4-AP on the terminal response is illustrated in Fig. 2*B*1; it can be seen that the second component is depressed and delayed. 3,4-diaminopyridine (3,4-DAP) which is a more potent K channel blocker than 4-AP (Kirsch & Narahashi, 1978), produced at doses of 100 μM a more pronounced depression of late components of both preterminal and terminal responses and a similar prolongation of their time course as compared to 4-AP (Fig. 3). The absence of total block with 4-AP or 3,4-DAP may be due both to the presence of a fraction of aminopyridine-resistant K channels (Dubois, 1981) and to unbinding of these drugs from K channels at depolarized levels of the membrane potential

(Ulbricht & Wagner, 1976; Meves & Pichon, 1977; Kirsch & Narahashi, 1978). It is thus likely that lengthening of late outward current corresponds to maintained membrane depolarization in the presence of aminopyridines.

Uranyl ions. UO_2 is not, properly speaking, a K channel blocker, yet it prolongs, as many other divalent cations, nerve and muscle action potentials (Mambrini &

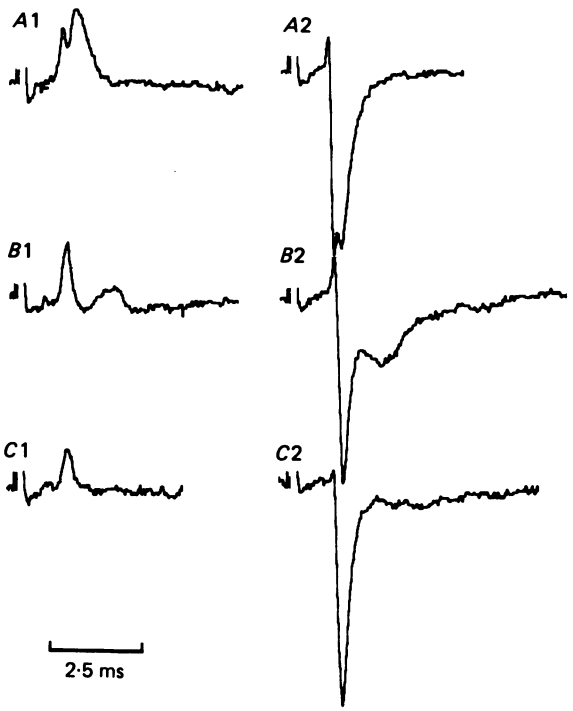

Fig. 4. Terminal (1) and preterminal (2) responses. *A*, control recordings; *B*, perfusion of 100 μM-UO_2 which delays the development of late components; *C*, addition of 5 mM-TEA.

Benoit, 1968) probably by affecting voltage-dependent rate constants of K permeability (Fitzhugh, 1960; Schwindt & Crill, 1981). Fig. 4*B*1, *B*2 shows the increased delay of the second component of both terminal and preterminal responses in 100 μM-UO_2. Delayed current amplitude was probably unchanged. As expected, the late components were blocked by TEA (Fig. 4*C*1, *C*2). Benoit & Mambrini (1970) reported a similar effect of UO_2 on the late component of the triphasic wave form of frog endings.

The preceding results clearly indicate the presence of a delayed increase in K conductance in mouse motor endings which generates outward current at terminal and intermediate segments and inward current at the preterminal portion. The explanation for the polarity of the latter is not obvious since inward K current requires a membrane potential more negative than the potassium equilibrium potential, E_K. A possible explanation is a second peak of inward current when the membrane is repolarized by K current, as predicted by the model proposed by Hodgkin & Huxley (1952) (see also Frankenhauser & Huxley, 1964). Another possibility is that

K current is present only at terminal and intermediate portions where it repolarizes rapidly the membrane. This would create a potential difference between proximal and distal parts of the presynaptic terminal which generates a local circuit to restore cell electroneutrality – the source being spread along the main part of the terminals, the sink focused near the myelin end. The late component of the preterminal wave form would, then, correspond to a sink of passive current.

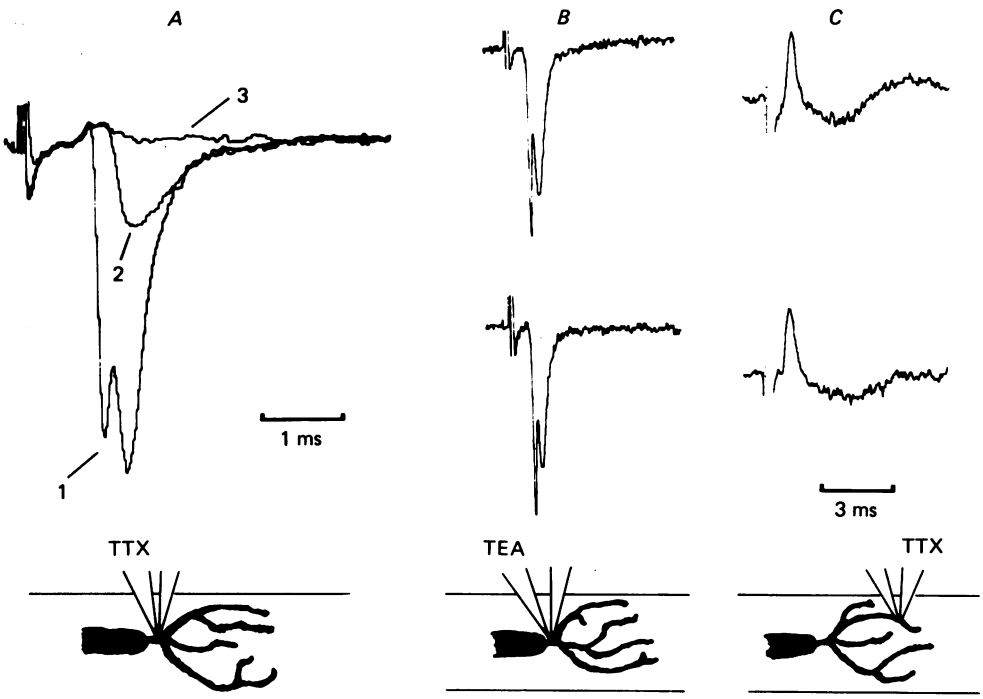

Fig. 5. *A*, effect of ionophoretic TTX application on preterminal wave form. 1, control; 2, suppression of first negative component leaves only second component; 3, suppression of the latter by bath perfusion of TEA. *B*, absence of action of ionophoretic TEA on preterminal responses. Upper trace is the control; lower trace, during drug application. *C*, terminal responses in a TEA + 3,4-DAP treated preparation showing a positive 'spike' followed by slow deflexion which corresponds to Ca current. Upper trace is the control, lower trace, during ionophoretic TTX application. Diagrams show the position of recording and ionophoretic pipettes on motor endings.

To test this point, we applied ionophoretically Na or K channel blocking drugs to proximal or terminal parts of motor endings. Drug release was easily controlled by reducing or increasing the braking current (Katz & Miledi, 1968). The effect was always readily reversible. In the experiment illustrated in Fig. 5*A*, a TTX pipette was placed on the preterminal segment close to the recording electrode. Before drug application, the response consisted of two negative peaks preceded by a small positive deflexion which corresponds to passive local circuit current from nodes of Ranvier in the parent axon (trace 1). TTX application suppressed within a few seconds the first negative peak. The TTX-resistant negative deflexion (trace 2) is likely to correspond to the second component for two reasons: first, because there was a strict

concordance in peak time and, secondly, because it was suppressed by perfusing a TEA-containing solution (trace 3). This result indicates that the late component of the preterminal response does not correspond to Na current, and thereby rules out the first proposed explanation. We performed a complementary experiment by applying TEA instead of TTX to the preterminal segment. Fig. 5*B* shows that TEA failed to produce changes in the preterminal response. Potassium current responsible for generation of the late component must, then, occur elsewhere, probably at the terminal part of the endings. Local TEA application to one of the terminal branches abolished, indeed, the late component of the terminal response recorded by a nearby electrode but only depressed the corresponding component of the preterminal response recorded by a second electrode near the myelin end. The reason was that, while TEA blocked K channels under the pipette, it failed to affect K conductance in other terminal branches.

Invasion of the presynaptic terminals by the action potential

The results reported above have shown that active Na current is generated at the transition between the myelinated and non-myelinated parts of the axon and delayed K current at the terminal part of the endings. It is still unclear whether the action potential invades actively or passively the whole terminal arborization. As pointed out by Katz & Miledi (1965, p. 479) 'the configuration of the nerve spike near the extreme tip does not readily allow one to distinguish between active propagation and decremental spread'. In this context, local TTX application should be useful to reveal the presence of regenerative Na current at the terminal part of the endings.

In the experiment illustrated in Fig. 5*C*, a recording electrode was placed at the terminal part of the endings and a second one close to the myelinated end. Outward K current was suppressed by TEA (1 mM) and 3,4-DAP (50 μM) added to the bath. The TTX pipette was placed first at the proximal site to check that inward Na current could be reversibly suppressed in a few seconds by releasing the braking current (not illustrated). The bias was adjusted again and the pipette placed close to the distal electrode. The negative deflexion that follows the positive 'spike' (Fig. 5*C*) corresponds to inward Ca current which will be studied later in detail. Local TTX application failed to modify distal wave form configuration (Fig. 5*C*, lower trace). However, prolonged TTX application (one minute or more) decreased, probably by drug diffusion, the amplitude of distal and proximal responses. This result strongly suggests the absence of TTX-sensitive Na current at the terminal part of the endings. The positive 'spike' recorded at the terminal portion would, then, correspond to capacitive current spread from both the actively invaded preterminal portion and nodes of Ranvier in the parent axon.

The ability of local circuit currents generated in the parent axon to depolarize terminal branches is further shown by the experiment illustrated in Fig. 6. Both TTX and recording pipettes were placed at the transition between myelinated and non-myelinated portions. The amount of d-tubocurarine was reduced to allow the observation of end-plate current. The record in Fig. 6*A*, taken with the braking current turned on, displays both the nerve response, which consists of a relatively large outward passive current and of a two-component inward current, and the post-synaptic response. TTX application suppressed completely early inward current.

End-plate current decayed to less than 50 % but was not abolished. Two conditions are present in this mouse neuromuscular preparation, but not in frogs (Katz & Miledi, 1968), which allow efficient passive invasion of motor endings, namely, a short last internode (average 50 μm) and a short non-myelinated portion (20–30 μm). Nevertheless, the amout of transmitter released under such circumstances is unlikely to ensure sufficient neuromuscular transmission. Generation of an action potential at the preterminal segment must be necessary to obtain a high safety factor for transmission.

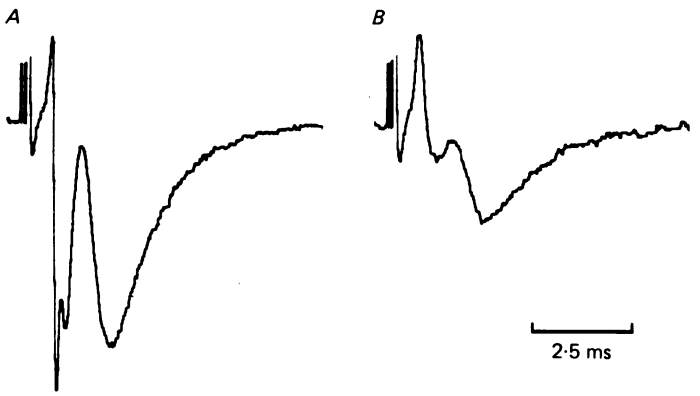

Fig. 6. The amount of d-tubocurarine has been reduced to show end-plate current. *A*, before and *B*, after ionophoretic TTX application to preterminal part of the endings which suppressed the first negative component of the preterminal response and reduced end-plate current amplitude. A small second negative component follows the passive positive 'spike'.

Calcium current

An essential step in the process of transmitter release is a potential-dependent Ca^{2+} influx into the presynaptic terminal (see Katz, 1969). The study of transmission in the giant synapse of the squid after pharmacological suppression of Na and K conductances revealed the presence of a self-regenerative Ca conductance (Katz & Miledi, 1967, 1969; Linás & Nicholson, 1975). Ca current has been further demonstrated and characterized in the voltage-clamped presynaptic element of the giant synapse of the squid (Llinás, Steinberg & Walton, 1981*a*, *b*).

Since, as shown above, the terminal portion of mouse motor endings lacks regenerative Na^+ conductance, we did not need to use TTX to avoid the complicating effect of Na current. We could, thus, stimulate the nerve in presence of K channel blockers to look for inward Ca current. As shown before, the addition of TEA, 4-AP, or 3,4-DAP to the standard Krebs solution failed to reveal the presence of inward current in recordings from the terminal branches. However, if the external Ca concentration ($[Ca]_o$) was increased to 10 or 50 mM we were able to demonstrate a conspicuous inward current. Fig. 7*A*1 is from an experiment performed in a solution containing 50 mM-Ca^{2+} and 10 mM-TEA added to standard saline solution. Nerve conduction time was increased probably because of the action of divalent cations on Na current (Hille, Woodhull & Shapiro, 1975). Inward current started immediately after the passive 'spike' and lasted approximately 2 ms. If instead of using a single

K channel blocker, we added a combination of TEA (1 mM) and 3,4-DAP (50-100 μM) to the bathing solution, inward current was visible with only 2 mM-Ca^{2+} present. Furthermore, inward current duration increased from 2 to 4 ms (Fig. 7*B*1). A probable explanation is that TEA alone was unable to suppress completely K conductance (see Katz & Miledi, 1971).

Since the bathing medium contained the normal Na (154 mM) concentration, the possibility exists that this cation could carry slow inward current. This has been ruled

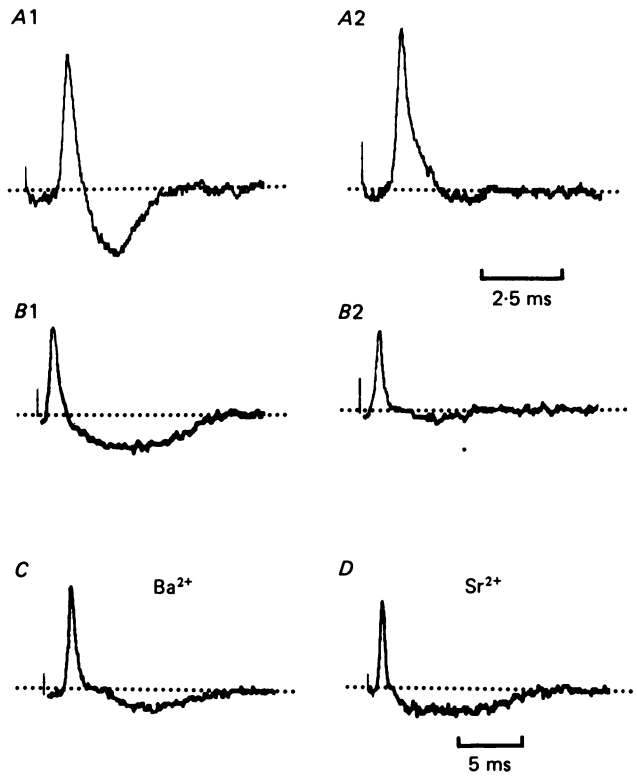

Fig. 7. Ca, Ba and Sr-dependent responses at the terminal part of motor endings. *A*1, in a medium containing 50 mM-Ca and 10 mM-TEA; *A*2, addition of 10 mM-Co; *B*1, 1 mM-TEA + 100 μM-3–4-DAP added to standard Krebs solution; *B*2, same with 10 mM-Co added; *C*, *D*, Ba or Sr substituted isosmolarly Ca in the bathing medium. Note prolonged response in Sr. Same time scale for *A*, *B* and *C*.

out by the previously described experiment illustrated in Fig. 5*C*, which shows that ionophoretic application of TTX close to a recording electrode positioned at the terminal part of the endings failed to affect neither the early positive 'spike' nor the late inward current.

Strong evidence for the involvement of Ca in the inward current we recorded from the terminal portion of the endings is furnished by the effect of Ca channel blockers which suppressed it in less than 3 min of perfusion. The effect of Co (10 mM) on inward current is shown in Fig. 7*A*2, *B*2). The residual negativity that follows the 'spike'

corresponds to the negative deflexion of diphasic capacitive current (Hodgkin & Huxley, 1952).

We tested next the ionic selectivity of the channel for inward current. Among divalent cations, only Ca, Ba and Sr are permeant through the Ca channel (Hagiwara, 1975) and support transmitter release (Dodge, Miledi & Rahamimoff, 1969; Katz & Miledi, 1969). Experiments performed in modified Krebs solutions in which Ca was isosmolarly replaced by Ba or Sr and delayed K conductance suppressed by

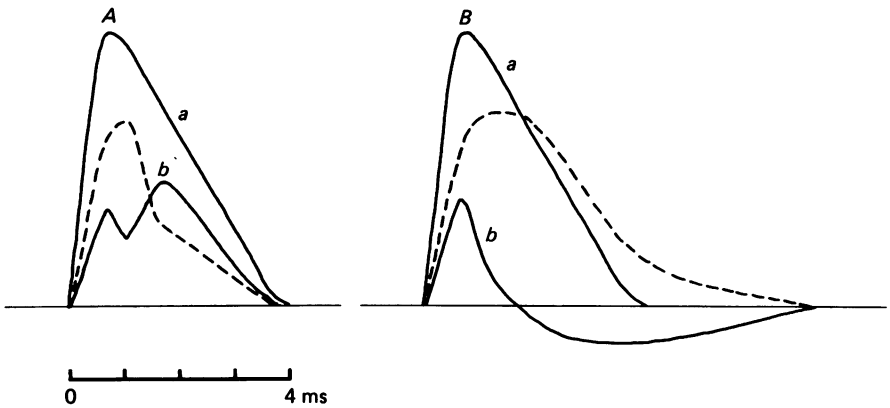

Fig. 8. Reconstruction of probable potential change at terminal part of the endings. *A*, standard Krebs solution; *B*, in presence of TEA+3,4-DAP. *a* is the action potential generated at the preterminal part drawn from data obtained by Chiu *et al.* (1979) in nodes of Ranvier at 14 °C, *b* is the terminal response recorded at 14 °C which is proportional to the current generated by the potential difference between preterminal and terminal parts. Terminal potential change (dashed line) was obtained from the difference between *a* and *b* multiplied by a proportionality factor; its amplitude corresponds to 70% electronic attenuation.

1 mM-TEA + 100 μM-3,4-DAP, showed, not unexpectedly, the presence of an inward current. Ba current was only slightly longer than that seen with 2 mM-Ca. In Sr Krebs, however, inward current was more than twice as long as that obtained with Ca (10 and 4 ms, respectively).

Although Ca and K 'currents' appear to occur simultaneously, a measurable difference in onset time exists between them. 'K current' started at the peak of the presynaptic potential change, which, presumably, corresponds to the dimple between first and second components of the terminal response, as will be shown below. Its latency was, on average, 350 μs measured from the foot of the positive 'spike'. The onset of the 'Ca current' was estimated by subtracting recordings obtained with and without Co added to the TEA+3,4-DAP-blocked preparations (see Fig. 7). Its average value was 700 μs, from the start of the positive deflexion, which is in close agreement with that found by Llinás *et al.* (1981*a*) in the giant synapse of the squid. It might seem surprising that 'Ca current' is activated later than 'K current', since the reverse occurs in membranes with Ca spike electrogenesis (see Hagiwara, 1975). A possible explanation is that the rate of potential change at the terminal part of mouse motor endings is slower than in cases of active depolarization, and, as shown

by Llinás *et al.* (1981*a*), Ca activation latency is very sensitive to parameters of presynaptic depolarization.

The preceding recordings were performed at the terminal part of the motor endings, i.e. that portion of the presynaptic terminal which occupies the synaptic gutters and releases transmitter. We looked then for the possible existence of inward Ca current at the proximal part of the endings. K conductance was blocked by TEA and 3,4-DAP but we failed to observe slow inward current following the fast Na current. This situation is similar to that reported for the squid giant synapse by Katz & Miledi (1969) where a high density of Ca channels exists only at the terminal part of the presynaptic axon. In favourable experiments in which 'Ca current' was large, we could observe a slow positive deflexion in the preterminal portion which looked like the inverted replica of the former. It can be regarded as the source of the local circuit promoted by the entry of Ca at the terminal part of the endings.

Potential change at the presynaptic terminal

In the absence of detectable Na current at the terminal portion of the endings, we ought to admit passive invasion of the latter. Since Ca influx and, therefore, transmitter release depend critically on potential changes, it is of considerable interest to know the amplitude and time course of presynaptic depolarization.

Let us assume that motor endings of mouse and frog have similar cable properties. Then, a space constant of 60 μm for transient potential changes, as estimated by Katz & Miledi (1968) for frog endings, would be twice as long as the maximal length of individual terminal branches in the mouse. The potential at the tip of terminal branches would, thus, be 50–60 % of its original amplitude. An approximate reconstruction of the shape of presynaptic depolarization at a point 20 μm away from the myelin end is shown in Fig. 8*A*. Action potential configuration (*a*) at the transition between myelinated and non-myelinated parts of the axon is based on that obtained in rabbit nodes of Ranvier by Chiu *et al.* (1979). Since the signal (*b*) recorded by a focal electrode placed on the terminal part is proportional to the potential difference between this site and the site that generates an active action potential, the time course of the potential change at the former would be given by the dashed line (see Eccles, 1964, p. 124). Note that the time course is affected not only by cable properties but also by a large K conductance which is present only at the terminal part. Fig. 8*B* shows a similar analysis of recordings obtained in TEA + 3,4-DAP. Terminal potential is increased in duration both by the suppression of K conductance and by the development of inward Ca current.

DISCUSSION

Our results indicate spatial differentiation of the presynaptic membrane regarding channel distribution: Ca and K channels being located at the terminal part and Na channels concentrated at the preterminal part of the endings. This statement is based on two lines of evidence: (i) wave form configuration and (ii) action of specific channel blockers on wave form components. Na channel activity is signalled at the preterminal part by a sharp negative deflexion which classically corresponds to inward current associated to the impulse. If voltage-sensitive K channels were also present at this

point one would expect a positive deflexion following the sharp negativity, but we observed, instead, a second negative component. Absence of a predominantly negative phase at the terminal part would indicate either closed-end effect or absence of active invasion. At the closed-end of a linear conductor membrane current becomes equal to longitudinal current and corresponds, thus, to the first derivative with respect to time of the intracellular potential change. An external electrode would see at the end a diphasic (positive-negative) wave instead of a predominantly negative triphasic one (Katz & Miledi, 1965). Closed-end effect cannot explain terminal responses of mouse endings for three reasons: first, we never recorded diphasic responses in standard Krebs solution, secondly, terminal responses were recorded not only near the tip, but also at 7–8 μm from the myelin end, that is, very close to the segment where we demonstrated active invasion, and thirdly, specific K and Ca, but not Na channel blockers affected the configuration of terminal responses. By contrast, the shape of the latter could be satisfactorily explained by assuming both an electrotonic distortion of the preterminal action potential and a large g_K increase restricted to the terminal part. The terminal response would be proportional to the current generated by the potential difference between terminal and preterminal parts (Fig. 8; see Eccles, 1964, p. 124).

Strong evidence for separate distribution of K and Ca channels on one part, and of Na channels on the other is furnished by results obtained by bath or ionophoretic application of specific channel blockers. We found a TTX-sensitive component restricted to the preterminal part of the endings whose time course (allowing for differences in temperature) closely agrees with that of the Na current reconstructed from voltage-clamp data by Chiu *et al.* (1979) in mammalian nodes of Ranvier. Ionophoretic TEA application to this part of the endings failed to reveal membrane current that could correspond to the K current. This observation points to a close similarity between the preterminal part of the endings and nodes of Ranvier in mammalian myelinated nerves (Chiu *et al.* 1979; Brismar, 1980). We failed to detect any TTX-sensitive component in membrane currents generated at the terminal part of the endings. Three possibilities can account for this absence. The first is that failure of active propagation occurs at the branching point where the safety margin is low, the second is the presence of TTX-resistant action potentials, as occurs in mammalian denervated muscle (Redfern & Thesleff, 1971) and the third, the absence or low density of Na channels. The first possibility seems unlikely since an action potential generated near the myelin end is expected to depolarize the terminal portion beyond the firing level by electronic spread. The second possibility is contradicted by focal depolarization experiments in the presence of TTX and TEA, in which we could obtain gradual post-synaptic responses related to presynaptic depolarizing current intensity (unpublished). This leaves only the third possibility, i.e. that Na channels are practically absent from the terminal part of the endings. The presence of a delayed potassium conductance, g_K at the terminal part is indicated by the action of TEA, 4-AP and 3,4-AP, since the only known property shared by these drugs is their ability to block K channels. The late component of the terminal response is, thus, homologous to the TEA or 4-AP-sensitive deflexion of the triphasic wave form from frog endings (Benoit & Mambrini, 1970) or from experimentally demyelinated internodes of rat nerves (Bostock *et al.* 1981). A restricted area between terminal and

preterminal portions shows triphasic (positive–negative–positive) wave forms similar to those obtained in cases of impulse propagation along a linear conductor when the action potential is terminated by a rapid increase in g_K. The suppression of the late positive deflexion of intermediate responses by 4-AP (Fig. 2*B*2) agrees, indeed, with this view. This observation indicates either overlapping Na and K channels or separated Na and K channel domains whose boundary could not be detected by our recording technique.

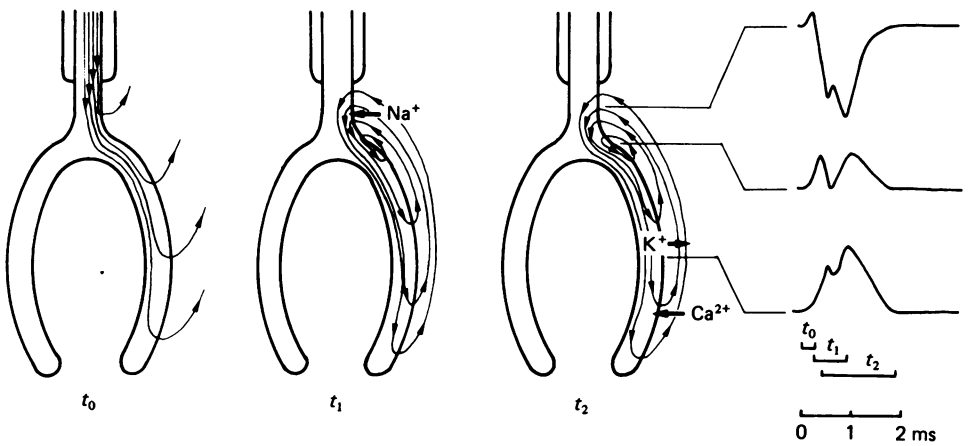

Fig. 9. Diagrammatic representation of current flow at motor endings in relation to recorded wave forms. At time t_0, local circuit current from upstream nodes of Ranvier leaves axon core at the endings. At time t_1, Na current enters the preterminal membrane and promotes a local circuit which depolarizes the terminal part. At time t_2, K and Ca currents flow in opposite directions across terminal membrane and promote local circuits which enter, or leave, the membrane at the preterminal part. Only K-dependent local circuit has been represented for clarity. Triphasic intermediate responses are recorded at that part of the endings which is close to the 'null' point of Na and K circuits.

Suppression of g_K at the terminal part by means of K channel blockers showed a slow inward current whose amplitude depended on $[Ca]_o$. Ca channel blockers suppressed it within a few minutes of perfusion but TTX failed to affect its amplitude or time course. We believe that this inward membrane current corresponds to Ca-dependent electrical activity investigated in squid presynaptic terminals by Katz & Miledi (1969), Llinás *et al.* (1981*a*) and Charlton, Smith & Zucker (1982), and in frog mononeurones by Barrett & Barrett (1976) and Alvarez-Leefmans & Miledi (1980). The apparent duration of 'Ca current' was strongly dependent on the concentration of K channel blocking agents, which suggests that in the terminal part of mouse motor endings, unlike in nodes of Ranvier, membrane repolarization depends highly on delayed g_K. One would, then, expect that in presence of adequate concentrations of TEA and DAP, motor nerve stimulation will induce plateau responses due to a maintained increase in g_{Ca} at the terminal part of the motor endings.

Ba^{2+} and Sr^{2+} could effectively replace Ca^{2+} as late inward current carriers. Both abnormal cations prolonged the current carried through the Ca channel, although the

action of Ba^{2+} was less marked. This effect is comparable to that reported previously for other neuronal systems (Katz & Miledi, 1969; Alvarez-Leefmans & Miledi, 1980 and Ashcroft & Stanfield, 1981). The mode of action of alkaline earths on the kinetics of Ca channel permeation is unknown. It is unlikely to be mediated by changes in surface potential because of the low concentrations used. A more likely explanation is either that inactivation depends on intracellular cation accumulation which may vary with the ionic species (Tillotson, 1979; Standen, 1981) or that channel properties are affected by the nature of permeant cations (Marty, 1980; Miledi & Parker, 1980; Ashcroft & Stanfield, 1981). The first explanation can be ruled out since it is unlikely that appreciable increase in $[Ca]_i$ could occur in our experimental conditions.

The sequence of electrical events at mouse motor endings can be described as follows (Fig. 9). Active depolarization at the last node of Ranvier invades electrotonically the presynaptic terminal and depolarizes it. An action potential is generated at the pre-terminal part if the depolarization reaches the threshold for excitation. Reversed membrane potential at this point promotes a local circuit which depolarizes further the terminal part of the endings by electrotonic spread. Outward K and inward Ca currents are initiated shortly there. Under normal conditions I_K overwhelms I_{Ca} and net outward ionic current repolarizes the membrane. A potential difference is, thus, established between terminal and proximal parts which behave as source and sink, respectively, of a local circuit. Passive current entering the proximal part during increased g_{Na} is not expected to affect appreciably the decay phase of the action potential.

It is worth noting important differences between mouse and frog motor endings. The latter being about ten times longer than the former, active impulse propagation is required to depolarize the whole length and release transmitter (Katz & Miledi, 1965, 1968; Braun & Schmidt, 1966). Interestingly, Na, K and Ca channels and acetylcholine release sites seem to be more or less uniformly distributed along frog motor endings while, in mouse, Na channels are concentrated exclusively at the preterminal part. This strategic Na channel emplacement leaves more room in terminal parts for other membrane components more directly involved in the release process.

REFERENCES

Alvarez-Leefmans, F. J. & Miledi, R. (1980). Voltage sensitive calcium entry in frog motoneurones. *J. Physiol.* **308**, 241–257.

Ashcroft, F. M. & Stanfield, P. R. (1981). Calcium dependence of the inactivation of calcium currents in skeletal muscle fibers of an Insect. *Science, N.Y.* **213**, 224–226.

Barrett, E. F. & Barrett, J. N. (1976). Separation of two voltage-sensitive potassium currents, and demonstration of a tetrodotoxin-resistant calcium current in frog motoneurones. *J. Physiol.* **255**, 737–774.

Benoit, P. R. & Mambrini, J. (1970). Modification of transmitter release by ions which prolong the presynaptic action potential. *J. Physiol.* **210**, 681–695.

Binah, O. & Palti, Y. (1981). Potassium channels in the nodal membrane of rat myelinated fibres. *Nature, Lond.* **290**, 598–600.

Bostock, H. & Sears, T. A. (1978). The internodal axon membrane: electrical excitability and continuous conduction in segmental demyelination. *J. Physiol.* **280**, 273–301.

Bostock, H., Sears, T. A. & Sherratt, R. M. (1981). The effects of 4-aminopyridine and tetraethyammonium ions on normal and demyelinated mammalian nerve fibres. *J. Physiol.* **313**, 301–315.

BRAUN, M. & SCHMIDT, R. F. (1966). Potential changes recorded from the frog motor nerve terminal during its activation. *Pflügers Arch.* **287**, 56–80.

BRISMAR, T. (1979). Potential clamp experiments on myelinated nerve fibres from alloxan diabetic rats. *Acta physiol. scand.* **105**, 384–386.

BRISMAR, T. (1980). Potential clamp analysis of membrane currents in rat myelinated nerve fibres. *J. Physiol.* **298**, 171–184.

BRISMAR, T. (1981). Specific permeability properties of demyelinated rat nerve fibres. *Acta physiol. scand.* **113**, 167–176.

CHARLTON, M. P., SMITH, S. J. & ZUCKER, R. S. (1982). Role of presynaptic calcium ions and channels in synaptic facilitation and depression at the squid giant synapse. *J. Physiol.* **323**, 173–193.

CHIU, S.Y. & RITCHIE, J. M. (1981). Evidence for the presence of potassium channels in the paranodal region of acutely demyelinated mammalian single nerve fibres. *J. Physiol.* **313**, 415–437.

CHIU, S. Y. & RITCHIE, J. M. (1982). Evidence for the presence of potassium channels in the internode of frog myelinated nerve fibres. *J. Physiol.* **322**, 485–501.

CHIU, S. Y. & RITCHIE, J. M., ROGART, R. B. & STAGG, D. (1979). A quantitative description of membrane currents in rabbit myelinated nerve. *J. Physiol.* **292**, 149–166.

COUTEAUX, R. (1958). Morphological and cytochemical observations on the post-synaptic membrane at motor end-plates and ganglionic synapses. *Expl Cell. Res.* suppl. **5**, 294–322.

DATYNER, N. B. & GAGE, P. W. (1980). Phasic secretion of acetylcholine at a mammalian neuromuscular junction. *J. Physiol.* **303**, 299–314.

DEL CASTILLO, J. & KATZ, B. (1956). Localization of active spots within the neuromuscular junction of the frog. *J. Physiol.* **132**, 630–649.

DODGE, F. A., MILEDI, R. & RAHAMIMOFF, R. (1969). Strontium and quantal release of the transmitter at the neuromuscular junction. *J. Physiol.* **200**, 267–283.

DUBOIS, J. M. (1981). Evidence for the existence of three types of potassium channels in the frog Ranvier node membrane. *J. Physiol.* **318**, 297–316.

DUDEL, J. (1965). The mechanism of presynaptic inhibition at the crayfish neuromuscular junction. *Pflügers Arch.* **284**, 66–80.

DUDEL, J. (1981). The effect of reduced calcium on quantal unit current and release at the crayfish neuromuscular junction. *Pflügers Arch.* **391**, 35–40.

ECCLES, J. C. (1964). *The Physiology of Synapses*, p. 124. New York: Academic Press.

FITZHUGH, R. (1960). Thresholds and plateaux in the Hodgkin-Huxley equations. *J. gen. Physiol.* **43**, 867–896.

FRANKENHAUSER, B. & HUXLEY, A. F. (1964). The action potential in the myelinated nerve fibre of *Xenopus laevis* as computed on the basis of voltage clamp data. *J. Physiol.* **171**, 302–315.

HAGIWARA, S. (1975). Ca-dependent action potential. In *Membranes: a series of advances*, vol. 3, ed. EISENMAN, G., pp. 359–381. New York: Dekker.

HILLE, B. (1967). The selective inhibition of delayed potassium currents in nerve by tetramethylammonium ion. *J. gen. Physiol.* **50**, 1287–1302.

HILLE, B., WOODHULL, A. M. & SHAPIRO, B. I. (1975). Negative surface charge near sodium channels of nerve: divalent ions, monovalent ions and pH. *Phil. Trans. R. Soc.* B **270**, 301–318.

HODGKIN, A. L. & HUXLEY, A. F. (1952). A quantitative description of membrane current and its application to conduction and excitation in nerve. *J. Physiol.* **117**, 500–544.

HUBBARD, J. I. & SCHMIDT, R. F. (1963). An electrophysiological investigation of mammalian motor nerve terminals. *J. Physiol.* **166**, 145–167.

KAARS, C. & FABER, D. S. (1981). Myelinated central vertebrate axon lacks voltage-sensitive potassium conductance. *Science, N.Y.* **212**, 1063–1065.

KATZ, B. (1969). The release of neural transmitter substances. Liverpool: Liverpool University Press.

KATZ, B. & MILEDI, R. (1965). Propagation of electric activity in motor nerve terminals. *Proc. R. Soc.* B **161**, 453–482.

KATZ, B. & MILEDI, R. (1967). A study of synaptic transmission in the absence of nerve impulses. *J. Physiol.* **192**, 407–436.

KATZ, B. & MILEDI, R. (1968). The effect of local blockage of motor nerve terminals. *J. Physiol.* **199**, 729–741.

KATZ, B. & MILEDI, R. (1969). Tetrodotoxin-resistant electric activity in presynaptic terminals. *J. Physiol.* **203**, 459–487.

KATZ, B. & MILEDI, R. (1971). The effect of prolonged depolarization on synaptic transfer in the stellate ganglion of the squid. *J. Physiol.* **216**, 503–512.

KIRSCH, G. E. & NARAHASHI, T. (1978). 3,4-diaminopyridine: a potent new potassium channel blocker. *Biophys. J.* **22**, 507–512.

KOCSIS, J. D. & WAXMAN, S. G. (1980). Absence of potassium conductance in central myelinated axons. *Nature, Lond.* **287**, 348–349.

LLINÁS, R. & NICHOLSON, C. (1975). Calcium role in depolarization-secretion coupling: an aequorin study in squid giant synapse. *Proc. natn. Acad. Sci. U.S.A.* **72**, 187–190.

LLINÁS, R., STEINBERG, I. Z. & WALTON, K. (1981*a*). Presynaptic calcium currents in squid giant synapse. *Biophys. J.* **33**, 289–322.

LLINÁS, R., STEINBERG, I. Z. & WALTON, K. (1981*b*). Relationship between presynaptic calcium current and postsynaptic potential in squid giant synapse. *Biophys. J.* **33**, 323–352.

LUNDH, H. (1978). Effects of 4-aminopyridine on neuromuscular transmission. *Brain Res.* **153**, 307–318.

MAMBRINI, J. & BENOIT, P. R. (1968). Modifications de la libération du médiateur à la jonction neuromusculaire sous l'action de l'ion uranyle. *C.r. hebd. Séanc. Acad. Sci., Paris* **266**, 1145–1148.

MARTY, A. (1980). Action of calcium ions on acetylcholine-sensitive channels in Aplysia neurones. *J. Physiol.* **76**, 523–527.

MCARDLE, J. J., ANGAUT-PETIT, D., MALLART, A., BOURNAUD, R., FAILLE, L. & BRIGANT, J. L. (1981). Advantages of the *triangularis sterni* muscle of the mouse for investigations of synaptic phenomena. *J. Neurosci. Meth.* **4**, 109–115.

MEVES, H. & PICHON, Y. (1977). The effect of internal and external 4-aminopyridine on the potassium currents in intracellularly perfused squid giant axons. *J. Physiol.* **268**, 511–532.

MILEDI, R. & PARKER, I. (1980). Effects of strontium ions on end-plate channel properties. *J. Physiol.* **306**, 567–577.

MOLGÓ, J., LUNDH, H. & THESLEFF, S. (1980). Potency of 3,4-diaminopyridine and 4-aminopyridine on mammalian neuromuscular transmission and the effect of pH changes. *Eur. J. Pharmac.* **61**, 25–34.

NEHER, E., SAKMANN, B. & STEINBACH, J. H. (1978). The extracellular patch clamp: a method for resolving currents through individual open chanels in biological membranes. *Pflügers Arch.* **375**, 219–228.

NONNER, W. & STÄMPFLI, R. (1969). A new voltage clamp method. In *Laboratory Techniques in Membrane Biophysics*, ed. PASSOW, H. & STÄMPFLI, R., pp. 171–175. Berlin: Springer-Verlag.

REDFERN, P. & THESLEFF, S. (1971). Action potential generation in denervated rat skeletal muscle. II. The action of tetrodotoxin. *Acta physiol. scand.* **82**, 70–78.

RITCHIE, J. M. & ROGART, R. B. (1977). Density of sodium channels in mammalian myelinated nerve fibers and nature of the axonal membrane under the myelin sheath. *Proc. natn. Acad. Sci. U.S.A.*, **74**, 211–215.

SCHWINDT, P. C. & CRILL, W. E. (1981). Differential effects of TEA and cations on outward ionic currents of cat motoneurons. *J. Neurophysiol.* **46**, 1–16.

SMITH, K. J. & SCHAUF, C. L. (1981). Size-dependent variation of nodal properties in myelinated nerve. *Nature, Lond.* **293**, 297–298.

STANDEN, N. B. (1981). Ca channel inactivation by intracellular Ca injection into Helix neurones. *Nature, Lond.* **293**, 158–159.

TAKEUCHI, A. & TAKEUCHI, N. (1962). Electrical changes in pre- and postsynaptic axons of the giant synapse of Loligo. *J. gen. Physiol.* **45**, 1181–1193.

TASAKI, I. (1959). Conduction of the nerve impulse. In *Handbook of Physiology*, vol. 1, ed. FIELD, J., pp. 75–121. Washington: American Physiological Society.

TILLOTSON, D. (1979). Inactivation of Ca conductance dependent on entry of Ca ions in molluscan neurons. *Proc. natn. Acad. Sci. U.S.A.* **76**, 1497–1500.

ULBRICHT, W. & WAGNER, H. H. (1976). Block of potassium channels of the nodal membrane by 4-aminopyridine and its partial removal on depolarization. *Pflügers Arch.* **367**, 77–87.

WAXMAN, S. G. & FOSTER, R. E. (1980). Ionic channel distribution and heterogeneity of the axon membrane in myelinate fibers. *Brain Res. Rev.* **2**, 205–234.

ZUCKER, R. S. (1974). Crayfish neuromuscular facilitation activated by constant presynaptic action potentials and depolarizing pulses. *J. Physiol.* **241**, 69–89.