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VOLTAGE SENSITIVE CALCIUM ENTRY IN FROG MOTONEURONES

BY F. J. ALVAREZ-LEEFMANS* AND R. MILEDI

From the Department of Biophysics, University College London, Gower Street, London WC1E 6BT

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SUMMARY

1. The electrical properties of motoneurone membrane were investigated in the isolated and hemisected spinal cord of frogs, using intracellular recording techniques.

2. TTX (1×10^{-6} g/ml.) blocked action potentials produced either by intracellular depolarizing current pulses or ventral root stimuli. Voltage–current relations from these cells showed a diminishing slope for depolarizing current pulses of increasing intensity.

3. If TEA (5–10 mM) was added to the media containing TTX, intracellular depolarizing pulses elicited prolonged regenerative depolarizations characterized by a peak of variable amplitude and a repolarizing phase preceded by a prolonged plateau of variable duration.

4. During the plateau of the response, the membrane conductance was increased above its resting value.

5. The response was shortened during repetitive stimulation and could be curtailed by applying a hyperpolarizing pulse during the plateau.

6. The response depended on the presence of external Ca^{2+} and increased in size and duration with increasing Ca^{2+} concentration. Sr^{2+} substituted effectively for Ca^{2+}. Sr^{2+}-dependent responses were considerably longer than the Ca^{2+}-dependent ones. Ca^{2+} or Sr^{2+} dependent responses persisted in Na^{+}-free media containing isotonic TEA, and were abolished by addition of Co^{2+}.

7. Ca^{2+} or Sr^{2+}-dependent regenerative responses were followed by a hyperpolarization which could last several seconds. The current responsible for this after-hyperpolarization was TTX and TEA resistant.

8. It is concluded that the TTX-resistant regenerative response is probably generated in the soma-dendritic membrane, and is due to influx of Ca^{2+} or Sr^{2+} through voltage sensitive channels different to those through which Na^{+} permeates during generation of 'normal' action potentials. In addition it is shown that the hyperpolarization following 'Ca spikes', and which might be due to an increase in K^{+} conductance can also be triggered by Sr^{2+}.

* Present address and reprint requests: Departamento de Neurociencias, Centro de Investigación del IPN, Apartado postal 14–740, México 14, D.F.

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INTRODUCTION

During the last two decades research on vertebrate motoneurones has yielded some qualitative information on the ionic mechanisms underlying generation of action potentials in these cells. For instance, the motoneurone soma-dendritic spike is blocked by tetrodotoxin (TTX), which suggests that the inward current is carried by Na^+ (Blankenship, 1968; Barrett & Barrett, 1976). There is also evidence for a K^+ conductance being responsible for the repolarizing phase of the spike (Eccles, 1957; Washizu, 1959; Barrett & Barrett, 1976). In addition, the soma-dendritic spike of vertebrate motoneurones is followed by a prolonged after-hyperpolarization lasting 60–250 msec (Brock, Coombs & Eccles, 1952; Araki, Otani & Furukawa, 1953), which plays a major role in controlling the firing behaviour of these cells (Kernell, 1965; Kernell & Sjöholm, 1973; Baldissera & Gustafsson, 1974). In cat and frog motoneurones, the slow after-hyperpolarization is generated by an increase in the membrane conductance to K^+ (Coombs, Eccles & Fatt, 1955; Barrett & Barrett, 1976). More recent observations suggest that the increase in K^+ conductance underlying the slow after-hyperpolarization is triggered by an increase in the concentration of ionized calcium at the inner surface of the cell membrane (Krnjević & Lisiewicz, 1972; Meech, 1972, 1978), due to an influx of Ca during the action potential (Barrett & Barrett, 1976; Krnjević, Puil & Werman, 1978; Alvarez-Leefmans, De Santis & Miledi, 1979).

Ca^{2+} might enter the cell through Na^+ channels (Baker, Hodgkin & Ridgway, 1971; Meves & Vogel, 1973), or through different channels resembling those which occur at presynaptic nerve terminals (Katz & Miledi, 1967, 1969*a*, *b*) and axons (Baker *et al.* 1971). Preliminary observations have been briefly reported, which suggest the existence of voltage sensitive Ca^{2+} channels in motoneurones (Barrett & Barrett, 1976). The present paper provides clear evidence that regenerative Ca^{2+} influx can be promoted by depolarization of the surface membrane of motoneurones in which Na^+ and K^+ channels have been blocked, and describes studies on the nature of the channels through which these Ca^{2+} may enter these vertebrate nerve cells. A preliminary account of some of these results has been the subject of a brief communication to the Physiological Society (Alvarez-Leefmans & Miledi, 1979).

METHODS

Experiments were performed on the isolated and hemisected spinal cord of the frog (*Rana temporaria*), dissected and mounted in a cooled chamber (approx. 3·5 ml.) as previously described (Katz & Miledi, 1963; Alvarez-Leefmans, De Santis & Miledi, 1979). The bath temperature was usually 4 °C, varying in different experiments between 3 and 7·5 °C.

The location of the motor nucleus was determined while the cord was in 'normal Ringer'. Motoneurones from segments 8–10 (Katz & Miledi, 1963) were impaled with electrodes filled with 3 M-KCl. With the electrode inside a motoneurone, the bathing fluid was changed to one containing tetrodotoxin (TTX) and tetraethylammonium (TEA). The preparation was continuously superfused with oxygenated solutions. However, it was frequently necessary to stop the flow to reduce the risk of cell damage while the electrode was inside a motoneurone. On a few occasions it was possible to follow the same cell throughout changes in the bathing solution, but in most cases the electrode was dislodged. Sometimes it was possible to impale again the same cell or adjacent ones with a slight movement of the electrode.

The composition of the normal Ringer solution was (mM) NaCl 114, KCl 2, $CaCl_2$ 2, HEPES

(N-2-hydroxyethypiperazine-N'-2-ethane sulphonic acid) 10, and D-glucose 10. The final pH was adjusted to 7·2 after bubbling with 95% O_2 + 5% CO_2 for 30 min. Variations in the composition of the normal Ringer solution are indicated where appropriate, and included elevation of Ca^{2+} up to 10 mM, substitution of Ca^{2+} by 2–10 mM-Sr^{2+}, addition of $CoCl_2$ (8 mM), addition of 5–10 mM-TEA bromide (Eastman) and TTX 10^{-6} g/ml. (Sankyo). This dose of TTX was 100–1000 times larger than the one needed to block axonic and soma-dendritic Na^+ dependent action potentials. In Na-free Ringer, NaCl was replaced by TEA chloride.

Signals were led through standard intracellular recording electronics to a dual beam oscilloscope and a DC pen recorder. Intracellular injected current was applied through a conventional Wheatstone-bridge circuit. Injected currents were measured by monitoring the voltage drop across a series 5×10^8 Ω resistor. Input conductance in the resting state (G_r) and during active regenerative responses (G_t) was determined by injecting small hyperpolarizing current pulses (20–50 msec) into the motoneurone at various times after the end of a depolarizing current pulse (10–20 msec). The resulting voltage changes were measured just before the end of each applied pulse. The change in input conductance was expressed as the ratio G_t/G_r, and plotted against tim (t). The end of the depolarizing current pulse was taken as $t = 0$ (e.g. Fig. 3).

In some experiments depolarizing currents were passed through the cut end of dorsal or ventral roots, while recording intracellularly from motoneurones. In these cases, the close fitting suction electrode was connected to a current source. The current flowing between this source and the grounded bathing solution was monitored through a 10 kΩ resistor.

RESULTS

The production of TTX-resistant regenerative responses in motoneurones

In agreement with previous observations (Colomo & Erulkar, 1968; Barrett & Barrett, 1976), TTX (1×10^{-6} g/ml.) abolished the motoneuronal soma-dendritic spike evoked either by ventral root stimulation or by intracellularly applied depolarizing current pulses within 1–3 min after its admission to the bath. The voltage–current relation from motoneurones bathed in Ringer containing 2–10 mM-Ca^{2+} and TTX, showed a diminishing slope for depolarizing current pulses (50–100 msec) of increasing intensity. This behaviour is probably due to the voltage dependent increase in K^+ conductance (Hodgkin & Huxley, 1952; Araki & Terzuolo, 1962), which is not eliminated by TTX (Narahashi, Moore & Scott, 1964; cf. also Katz & Miledi, 1967). An increase in K^+ conductance during membrane depolarization could prevent a small inward Ca^{2+} current from becoming regenerative, we therefore used TEA to block the voltage sensitive K^+ channels and eliminate the delayed rectification (cf. Armstrong & Binstock, 1965; Hille, 1967; Katz & Miledi, 1967, 1969*a*).

A few minutes after TEA bromide (5–10 mM) was added to the bathing fluid, already containing TTX and 10 mM-Ca^{2+}, the responses to depolarizing current steps were very different. Not only did a current pulse produce a maintained depolarization but above a certain strength, the pulse evoked a regenerative electric potential change, frequently outlasting for hundreds of milliseconds the duration of the imposed current step (Figs. 1, 2 and 4). These responses were characterized by an inflexion on the rising phase, a peak, and a slowly declining plateau of variable duration, followed by a more rapid repolarization. In general, the shape of these responses, as well as some of their other properties (see later) were remarkably similar to those described in crustacean muscle (Fatt & Katz, 1953; Fatt & Ginsborg, 1958) and presynaptic terminals of the squid (Katz & Miledi, 1969*a*, 1971).

As in the presynaptic terminals of the squid (Katz & Miledi, 1969*a*), the voltage–current relation obtained from motoneurones in this medium (TTX–TEA–10 mM-Ca^{2+}) showed an 'upward swing'. That is to say, an increasing slope over a certain range of depolarization (Fig. 1*G*, filled circles). This jump from subthreshold to supra-threshold levels of depolarization can be clearly seen in the series illustrated in Fig. 1 (*C*, *D*). Note that once the threshold was reached (*D*), further increases in the strength of the depolarizing pulse decreased the latency of onset of the regenerative response (Fig. 1*D–F*). The 'all or none' nature of the TTX–TEA resistant response

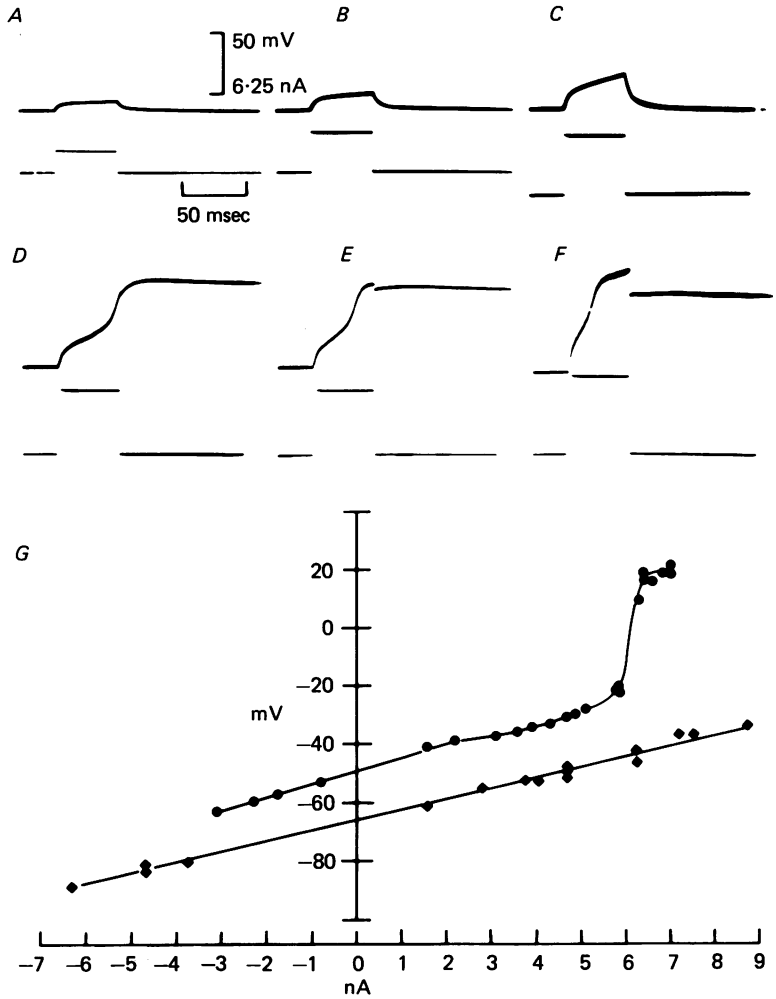

Fig. 1. *A–F*, motoneurone response to depolarizing pulses of constant duration (50 msec) and increasing intensity. Ca concentration 10 mM. All records in this and following Figures, were obtained by intracellular recording from motoneurones of spinal cords superfused with TEA (5 mM) and TTX (10^{-6} g/ml.). Temperature was 5 °C, and resting potential −50 mV. In each block, top trace shows intracellular potential and lower trace current pulse. *G*, voltage–current relation (final potential, pulse duration 50 msec). Circles, plot of series partly illustrated in *A–C*. Squares, 10 min after the Ca concentration was reduced to 2 mM, and Co (8 mM) was added to the bathing solution (temp. 8 °C).

was also evident when depolarizing pulses of constant strength but increasing duration were applied. This rheobasic current threshold is illustrated in Fig. 2.

Regenerative responses similar to the ones described, persisted for several hours when the preparation was bathed in a solution in which all the Na^+ was replaced by TEA^+, and $[Ca^{2+}]$ was kept at 10 mM. This suggests that if these responses are due to an inward movement of positive charge across the surface membrane, in this ionic environment, only TEA^+ or Ca^{2+} could carry the current. Since there is no evidence suggesting that TEA^+ is an effective charge carrier in nerve membranes, the inward current is presumably produced by Ca^{2+} influx.

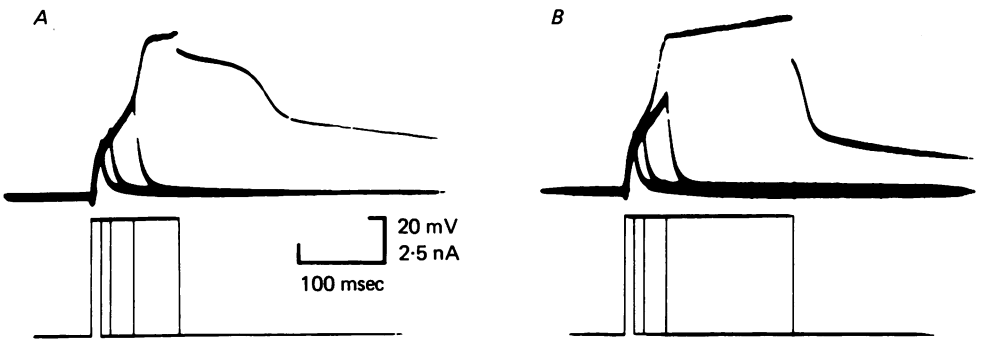

Fig. 2. *A* and *B*, effect of depolarizing injected current pulses of constant strength and varying duration. Note that the response had a rheobasic current threshold, and once triggered, it crossed the 'zero potential' line. Current pulses (bottom traces) were retouched for clarity. Temp. 5 °C (same motoneurone as in Fig. 1).

Regenerative responses were abolished if $[Ca^{2+}]_0$ was reduced to 2 mM and Co^{2+} (8 mM) was added to the media containing TTX and TEA (5 mM). An example of a typical current–voltage relation obtained in the presence of Co^{2+} is shown in Fig. 1*G* (squares). Unfortunately, the voltage displacement produced by depolarizing current steps stronger than 10 nA could not be accurately determined because of inevitable bridge inbalance. However, in this, and many other experiments, current steps up to 25 nA, and 50–100 msec duration, failed to produce any sort of regenerative activity in this Co^{2+}-containing medium.

Conductance changes underlying the TTX-resistant regenerative response

The results presented in the previous section suggest that the TTX-resistant regenerative response is due to an inward Ca^{2+} current. An alternative explanation is that the regenerative response results from a sudden fall in K^+ conductance, over a certain potential range (i.e. inward-going rectification of the outward current channels (Nelson & Frank, 1967)). In order to distinguish between these two possibilities, the input conductance was assessed with small hyperpolarizing repetitive current pulses applied before, during and after a regenerative response evoked by a short (10 msec) depolarizing pulse. The result of one such experiment is shown in Fig. 3. In this case, the regenerative response showed a peak which crossed the 'zero potential line', after which the membrane potential fell to a new value from which it started returning towards the resting level at a relatively slow rate, forming a 'plateau' (Fig. 3*A–C*).

From the amplitude of the modulating potentials it is evident that the input conductance was greatly increased during the peak of the response (Fig. 3*C* inset), and during the early part of the plateau (Fig. 3*A*, *B*). This was followed by a gradual recovery throughout the later part of the plateau, until the eventual and relatively abrupt repolarization. The time course of the input conductance change during the regenerative response is illustrated in the plot shown in Fig. 3*C*. Note that at the end of the plateau, the input conductance had recovered almost to its resting value. These findings suggest that the regenerative response is due to an increase in membrane conductance, probably reflecting an increase in Ca^{2+} permeability.

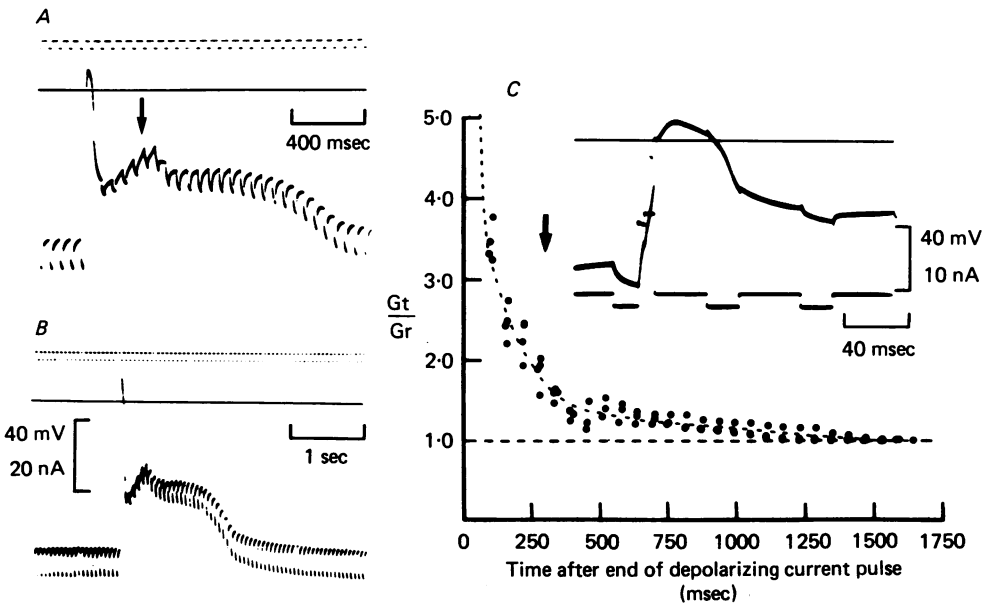

Fig. 3. Input conductance during regenerative response produced by a depolarizing pulse lasting 10 msec. *A*, *B* and inset *C* show examples of the regenerative response with hyperpolarizing pulse modulation. All records came from the same cell, and are shown on different time bases. The graph shows the time course of the change in input conductance; measured from records obtained from the same cell illustrated in *A*, *B* and inset *C* (temp. 6 °C). Arrow in the graph *C*, indicates the time during the response which is marked by the arrow in *A*. The 'unmodulated' response of the same cell can be seen in Fig. 4*F*. Ringer contained 10 mM-Ca^{2+}, TTX and TEA.

Shape, amplitude and time course of the TTX-resistant regenerative response

TTX-resistant regenerative responses were extremely variable in shape, amplitude and time course, not only from motoneurone to motoneurone, but also in different trials in the same cell. In spite of such variability, it was possible to measure some common features. These are (i) the active membrane potential V_a (the potential attained just at the end of the depolarizing current pulse); (ii) the total duration *TD*, measured from the end of the depolarizing pulse to the point in which the falling phase crossed the resting potential V_r; and (iii) the amplitude of the regenerative response ($V_a - V_r$).

TD and particularly V_a were strongly dependent on the external [Ca^{2+}]. Although

the quantitative relationship between external $[Ca^{2+}]$, V_a and TD, was not determined in the present experiments, it was nevertheless found that when $[Ca^{2+}]_0$ was 2 mM, the amplitude of the regenerative responses ranged from 15 to 30 mV, V_a ranged from -25 to -20 mV (i.e. there was no overshoot), and TD ranged from 250 msec to 1 sec.

With 10 mM-$[Ca^{2+}]_0$, depolarizing current pulses (20 msec) of threshold or suprathreshold strength evoked much larger and longer regenerative responses. V_a ranged from $+2$ to $+18$ mV (mean = 10·6 mV), TD ranged between 80 msec and 3·8 sec (mean = 733 msec), amplitude ranged from 32 to 78 mV (mean = 54·4 mV) (n = 24 observations made in eight cells). In another series of experiments in which the depolarizing pulses had a duration of 50 and 100 msec, the following values were obtained: mean V_a was $+10·5$ mV (range $+3·1$ to $+18·8$ mV), mean TD 985 msec (range 440 msec to 2 sec), and mean amplitude 49 mV (range 33 to 64·8 mV) (n = 9 observations made in three cells).

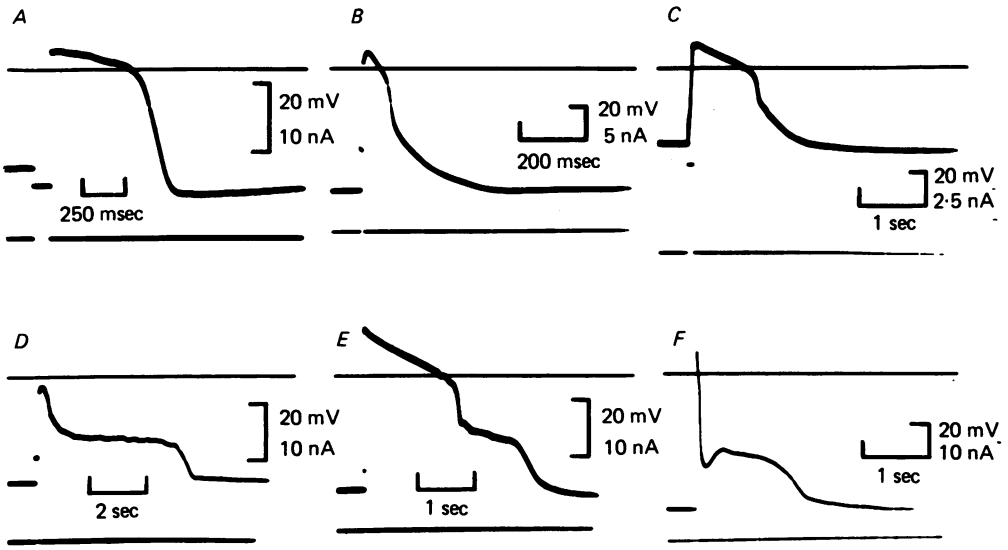

Fig. 4. Examples of regenerative responses showing diversity of shape. All traces were obtained from different cells, with the exception of *B* and *F*, which came from the same cell shown in Fig. 3. Top trace 'zero potential line'; middle trace, intracellular potential; bottom trace, applied current pulses. Temperature 4–6 °C. Ringer contained TTX, TEA and 10 mM-Ca^{2+}. First peak of the responses is indicated by black arrows, and subsequent steps by white arrows.

The shape of the regenerative responses varied from cell to cell (Fig. 4). Sometimes, the response appeared as a fairly rectangular action potential whose plateau terminated abruptly when the potential had declined to a certain level (e.g. Fig. 4*A*). The repolarizing phase of these responses was either fast and without inflexions (Fig. 4*A*) or, more frequently, showed an initial fast component followed by a relatively slow component (Fig. 4*B*, *C*). Most commonly the responses fell to the resting level in two or three steps of different amplitudes and durations (Fig. 4*D–F*).

In most cases the response declined in steps in the first trial (e.g. Fig. 9*B*), but subsequent responses became shorter, and the steps disappeared or were replaced

by inflexions in the repolarizing phase (e.g. Fig. 9*B–D*). Sometimes responses became stepped only after several pulses had been applied to the same cell (e.g. compare trace *B* and *F* in Fig. 4). This fractionation of the response into different steps resembles that seen in the squid giant synapse, where the different steps were attributed to differences in the plateau duration of responses produced in different terminal branches of the axon (Katz & Miledi, 1969*a*). In the present case, the stepped response suggests by analogy with squid nerve terminals, that the regenerative activity extends over different dendrites (cf. Llinás & Hess, 1976), or that it occurs in various discrete patches of the somatic membrane which have a relatively high density of 'Ca channels'.

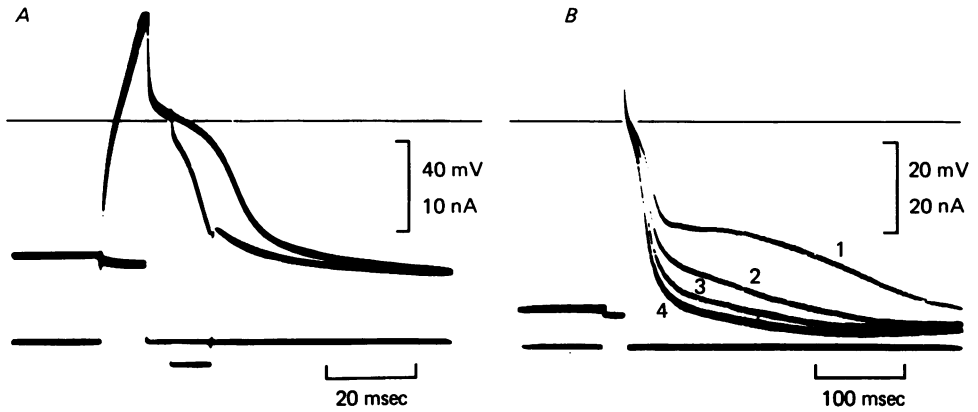

Fig. 5. *A*, effect of a hyperpolarizing current pulse in cutting short the regenerative response. *B*, effect of pulse repetition at 3 sec intervals. Numbers indicate the sequence in which the responses were recorded. *A* and *B* come from different motoneurones superfused with TTX, TEA and 10 mM-Ca^{2+}. Temp. 6 °C.

Some factors affecting the duration of the plateau

The duration of the plateau was very sensitive to repetitive activation. *TD* decreased as the interval between responses was reduced. Fig. 5*B* shows the progressive shortening of the response during a series of pulses repeated every 3 sec. Fig. 9*B*, *C* illustrates the effect even when pulses are delivered at intervals of several minutes. This phenomenon is analogous to that observed in Ca regenerative responses found in squid nerve terminals (Katz & Miledi, 1969*a*). Its detailed mechanism is still unknown, but it could be a consequence of an increase in intracellular free Ca caused by previous regenerative responses.

As with other action potentials of similar shape (Weidmann, 1951; Fatt & Katz, 1953; Katz & Miledi, 1969*a*), the response could be curtailed by applying a hyperpolarizing pulse during the plateau as shown in Fig. 5*A*.

TTX–TEA-resistant long lasting hyperpolarization

The regenerative response was usually followed by a slow hyperpolarization lasting from 500 msec up to 10 sec (mean = 3·9 sec, n = 10 observations, t = 5–6 °C, $[Ca^{2+}]_0$ = 10 mM). The amplitude and duration of the long lasting hyperpolarization varied not only from cell to cell, but also from one trial to the next in the same cell,

and did not seem to be simply related to the amplitude or duration of the regenerative response. This is seen for instance in Fig. 6*A* where an abortive spike was accompanied by an after-hyperpolarization larger than the one following a full regenerative response.

The variability of the responses precluded measurement of the equilibrium potential of the after-hyperpolarization. However, the largest after-hyperpolarizations (20 mV) were found in cells with low resting potential (-30 to -35 mV), and the relation of after-hyperpolarization amplitude to initial resting potential in different cells suggested an equilibrium potential of about -70 mV.

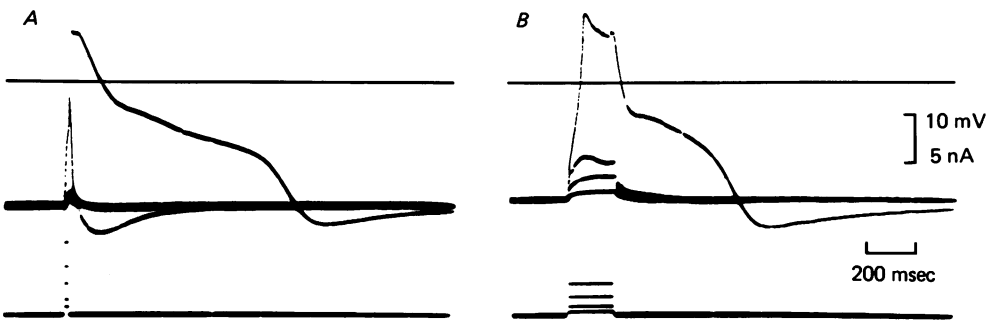

Fig. 6. Effect of depolarizing injected current pulses of varying strength and fixed short (*A*) and long (*B*) durations. Note the slow after-hyperpolarization in *A* and *B*. In *A*, one of the depolarizing pulses produced an abortive spike which was followed by an after-hyperpolarization. All the records came from the same motoneurone in the presence of TTX, TEA and 10 mM-Ca^{2+}. Temp. 4 °C.

TTX-resistant regenerative responses evoked by dorsal or ventral root polarization

TTX-resistant regenerative responses could also be generated in motoneurones by applying depolarizing current pulses (50 msec) to the cut ends of either dorsal or ventral roots. Examples of these action potentials are shown in Fig. 7. The series shown on the left (*A–F*) was obtained in 2 mM-Ca^{2+}; that on the right (*H–K*) in 10 mM-Ca^{2+}. The dorsal root stimulus strength was gradually increased from *A* to *D*. The first sign of activity evoked by either dorsal or ventral root polarizing pulses was a local response (e.g. *B* and *H*) from which the regenerative response arose (e.g. thick arrows in *C* and *I*). In *H* to *K*, the ventral root stimulus strength was fixed at threshold value. Note the fluctuations in latency of the regenerative response, as well as the inflexion in the repolarizing phase (thin arrows), similar to those described for responses generated by intracellularly applied current steps. The responses evoked by root stimulation were also followed by long lasting hyperpolarizations.

It is important to emphasize that responses evoked by root polarization occurred in the absence of 'Na^+ spikes' in either dorsal or ventral roots. Responses evoked by ventral root polarization are probably produced by electrotonic spread of the depolarizing pulse from the zone of root entry to the motoneurone soma, while those produced by dorsal root polarization are likely to arise either from electrotonic spread via electrical synapses (Alvarez-Leefmans, De Santis & Miledi, 1978, 1979; Shapovalov, Shiriaev & Velumian, 1978), or by regenerative Ca^{2+} influx in dorsal

root presynaptic terminals, which in turn would depolarize the motoneurones by transmitter action producing a post-synaptic regenerative response.

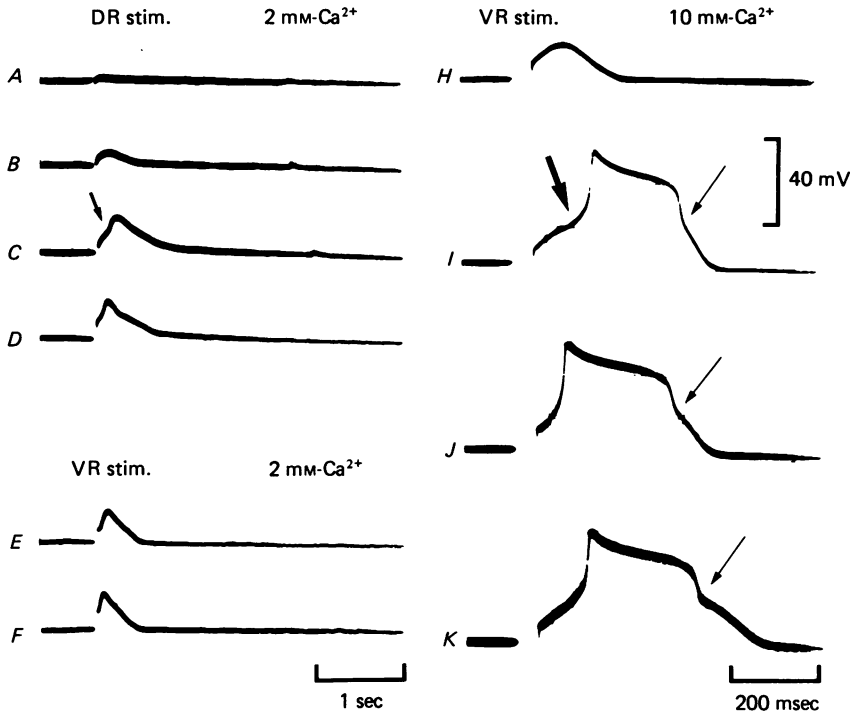

Fig. 7. Effect of polarizing current steps applied to ventral and dorsal roots on intracellularly recorded potential from motoneurones in the presence of TTX, TEA and low (2 mM) and high (10 mM) Ca concentration. *A–D*, dorsal root (DR) stimulation, and *E–F*, ventral root (VR) stimulation in the presence of 2 mM-Ca^{2+}. Depolarizing pulses (50 msec) of increasing strength from *A* to *D*, and from *E* to *F*. Resting potential in *A–F* was −46·5 mV. *H–K*, ventral root stimulation with depolarizing current pulses of fixed threshold strength (50 msec) in the prsence of 10 mM-Ca^{2+}. Resting potential was −58 mV. Records *A–D* came from a different cell than those shown in *H–K*.

Production of regenerative responses in solutions in which Ca^{2+} was replaced by Sr^{2+}

It is known that in other excitable cells Sr^{2+} can pass through voltage sensitive Ca^{2+} channels (Fatt & Ginsborg, 1958; Katz & Miledi, 1969*a*; Hagiwara, 1975). In the present experiments, depolarizing current pulses in motoneurones bathed in Ca^{2+}-free medium containing 10 mM-Sr^{2+}, TTX and TEA, evoked regenerative responses which were much longer than those seen with 10 mM-Ca^{2+} (Figs. 8 and 9).

The 'Sr^{2+} responses' usually became progressively smaller and shorter with each stimulus, until their eventual abolition, which happened even without appreciable changes in the resting potential. This state of refractoriness could not be reversed by increasing the duration or the intensity of the depolarizing current pulses. The decline of the 'Sr^{2+} responses' occurred more rapidly and with fewer stimuli than in the case of 'Ca^{2+} responses'.

Fig. 9*B–D* shows three successive responses recorded from the same cell. Fig. 9*B* shows the step-wise repolarization like that seen in Ca media. A second stimulus applied 11 min later produced the much shorter response shown in *C*. Finally in *D*, the response was shortened further, and the steps in the falling phase almost disappeared leaving only an inflexion.

Sr^{2+}-dependent regenerative responses evoked by depolarizing current steps (pulse duration of 50–100 msec; 4–6 °C; sixteen observations, twelve motoneurones), attained active membrane potentials ranging between −6·4 and +22 mV (mean = +5·5 mV), and mean amplitude of 61·5 mV (range 37·6 to 84 mV). *TD* ranged from 2·2 up to 400 sec, with a mean of 59·5 sec.

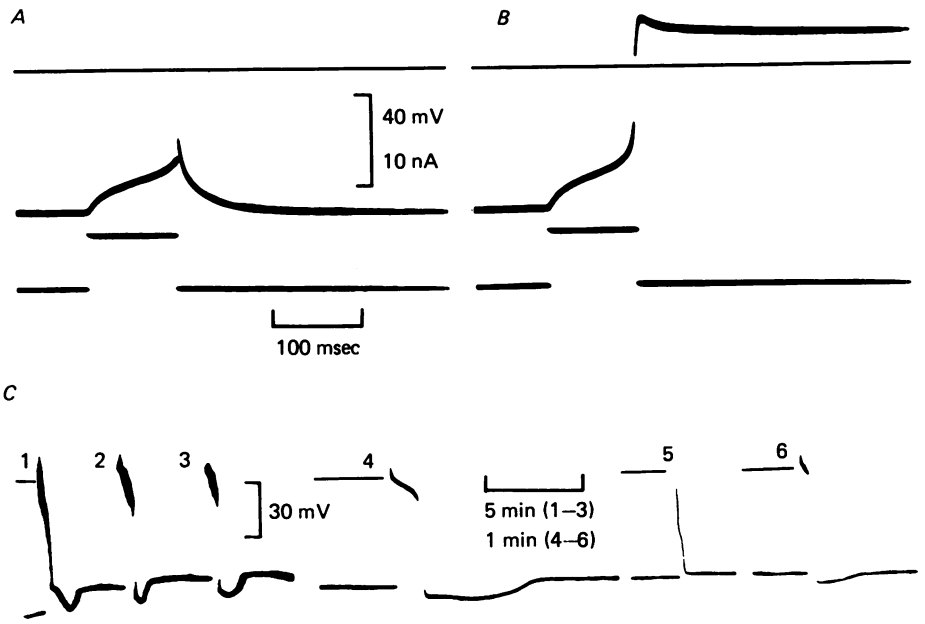

Fig. 8. Regenerative rsponses obtained in motoneurones superfused with 'Ca^{2+}-free' Ringer containing 10 mM-Sr^{2}, TTX and TEA. *A*, subthreshold and *B*, threshold for regenerative response. *C* 1–6, series of responses from pen recorder showing slow after-hyperpolarization. Note changes in time base indicated in calibration bars. The approximate time interval between responses 3 and 4 was 2·5 min, between 4 and 5 was 3·5 min, and between 5 and 6 was 2·5 min. Temp. 4 °C.

The responses in Sr^{2+}-containing media were followed by a slow hyperpolarization (Fig. 8*C*), which lasted much longer than the one following the 'Ca^{2+} responses'. The mean duration of the after-hyperpolarization in Sr medium was 23 sec (range 1·4 to 70 sec, t = 4 to 7·5 °C, n = 14 observations, five motoneurones), and their mean amplitude was 5·5 mV (range 1·8 to 12 mV).

DISCUSSION

The present results show that amphibian motoneurones treated with TTX and TEA to block voltage sensitive Na^+ and K^+ channels, can produce prolonged Ca^{2+}-dependent 'action potentials' (Barrett & Barrett, 1976). Similar, but even longer

responses occur when Sr^{2+} is used to replace Ca^{2+}. The regenerative responses are followed by a prolonged hyperpolarization which may be attributable to a Ca^{2+} (or Sr^{2+}) activated K^+ conductance. Without TEA, no regenerative activity can be elicited, and the usual delayed rectification is observed (Hodgkin & Huxley, 1952; Araki & Terzuolo, 1962).

The amplitude of the TTX resistant regenerative response increases with the concentration of external Ca^{2+} or Sr^{2+}. With 10 mM-Ca^{2+} or Sr^{2+}, the membrane potential becomes positive during the peak of the response. In addition, the input conductance is greatly increased during the peak and the plateau of the response. These findings indicate that the response involves an increase in membrane permeability to one or more ions, having a positive equilibrium potential, and whose

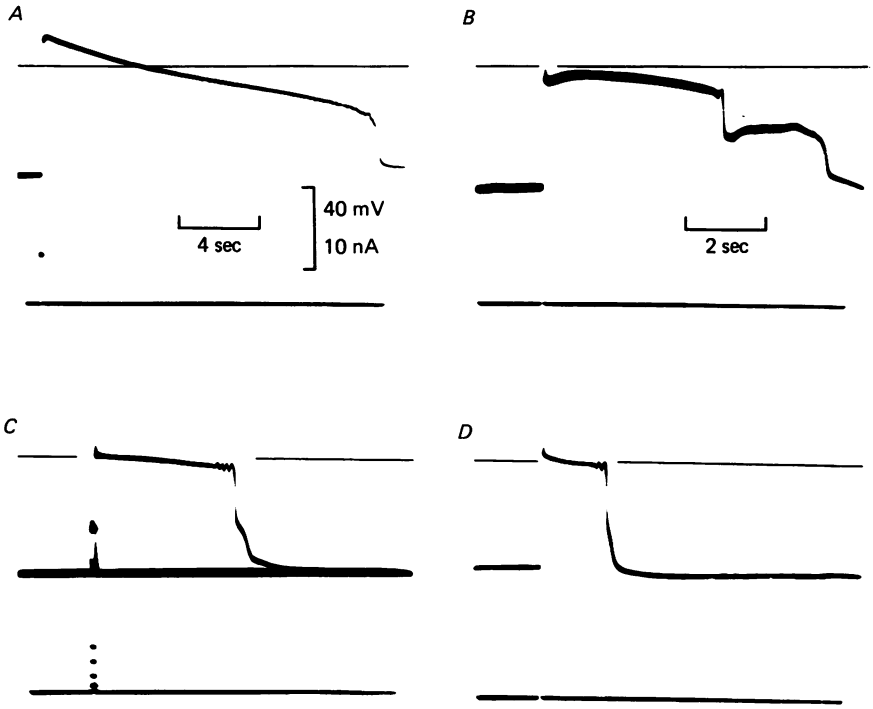

Fig. 9. Examples of regenerative responses evoked by depolarizing injected current pulses in motoneurones bathed in Ca^{2+}-free Ringer containing 10 mM-Sr^{2+}, TTX and TEA. Temperature in *A* was 3 °C, and in *B–D*, 6 °C. *B–D* were responses evoked in the same cell. Time interval between *B* and *C* was 11 min, and between *C* and *D* was 8 min. Time calibration in *B* applies to *C* and *D*.

influx does not occur through voltage sensitive Na^+ or K^+ channels. Similar responses are elicited in solutions in which all the Na^+ is replaced by TEA^+, suggesting that the inward current is carried mainly, if not exclusively by Ca^{2+} (or Sr^{2+}). The TTX and TEA resistant responses can be blocked by Co^{2+}, which further supports the suggestion that the regenerative current most likely flows through voltage sensitive Ca channels.

The present results resemble those obtained under similar conditions in TTX poisoned presynaptic terminals of the squid (Katz & Miledi, 1969*a*), where Ca^{2+}-

dependent regenerative responses can be elicited only in the presence of TEA. The most likely explanation is that TEA allows the Ca^{2+} current to become regenerative by suppressing a K^+ outward current that normally cancels the effect of the Ca^{2+} influx (Katz & Miledi, 1969*a*; see also Ross & Stuart, 1978; McAfee & Yarowsky, 1979).

TEA is not always needed to show Ca^{2+} dependent regenerative responses in neurones (e.g. Koketsu & Nishi, 1969; Standen, 1975; North & Nishi, 1976; Schwartzkroin & Slawsky, 1977; Dichter & Fischbach, 1977). This difference between neurones in which TEA is required to produce regenerative Ca^{2+} influx and those in which it is not, is probably due to variations in the relative magnitude and time course of the Ca^{2+} and K^+ currents. It is also possible that TEA might modify the permeability of other channels normally present in the membrane, making them 'abnormally permeable' to Ca^{2+} (Kleinhaus & Prichard, 1975), or TEA^+ itself might carry inward current. These alternative explanations seem unlikely since the size of the regenerative response varied with external $[Ca^{2+}]$ but not $[TEA^+]$, and the response was abolished by Co^{2+}, even in the presence of high concentrations of TEA. Moreover, TEA at the concentrations used here (5–10 mM) totally abolishes delayed K^+ currents in axons of amphibian motoneurones without affecting Na^+ or leakage conductances (Hille, 1967), and in the presence of TTX and isotonic TEA all ionic currents are abolished showing that TEA does not carry inward current (Hille, 1971).

Assuming that the motoneurone membrane becomes selectively permeable to Ca^{2+} during the peak of the regenerative response, the overshoot potential (V_a) should approach the equilibrium potential for Ca^{2+} (E_{Ca}), calculated from the Nernst equation:

$$E_{Ca} = \frac{RT}{2Z} \ln \frac{[Ca]_o}{[Ca]_i}.$$

E_{Ca} cannot be calculated without knowing the intracellular free $[Ca^{2+}]$. However, in almost all cells for which an estimate exists, the intracellular ionized Ca seems to be less than 1 μM (Katz & Miledi, 1969*a*; Baker *et al.* 1971; Meech & Standen, 1975; Di Polo *et al.* 1976), and the only available data for frog motoneurones indicate a level well below 10 μM (Bührle, Richter & Sonnhof, 1978). Taking a value of $[Ca^{2+}]_i = 1$ μM, the predicted E_{Ca} at 5 °C when $[Ca^{2+}]_o = 10$ mM, is around 110 mV inside positive. However the present results indicate that the mean amplitude of V_a was +10 mV and never exceeded +18 mV, which is well below the predicted E_{Ca}. This situation is similar to that found in other membranes displaying calcium electrogenesis (Reuter, 1973) where the difference is due to an outward K^+ current (I_K) overlapping with the Ca^{2+} inward current (I_{Ca}) resulting in a reduction of the overshoot and plateau potentials. In the present case, at least four possibilities exist, namely (i) the Ca^{2+} channels are not perfectly selective for Ca^{2+} against K^+ (e.g. Reuter & Scholz, 1977), (ii) TEA does not totally block delayed rectification, (iii) the Ca^{2+} permeability rise is not sufficient to 'overwhelm' the effect of the resting K^+ permeability, and (iv) there are TEA insensitive K^+ channels which are activated during the 'spike' (Thompson, 1977; Connor, 1979; Moolenaar & Spector, 1979). We have no evidence for or against the first possibility. The second possibility seems unlikely because in some experiments we used isotonic TEA and it is known that 5 mM-TEA is enough to abolish

measurable delayed rectification in motor axons (Hille, 1967). However, the values obtained for V_a could adequately be explained by the third possibility considered here.

Assuming a simplified model in which the resting K conductance (G_K) remains in parallel with the activated Ca conductance (G_{Ca}), and if during the peak of the response the net current flow is zero, i.e. $I_{Ca}+I_K = 0$, then V_a will be approximated by the following equation

$$V_a = \frac{E_K G_K + E_{Ca} G_{Ca}}{G_K + G_{Ca}}.$$

Taking $E_K = -70$ mV, and $E_{Ca} = +110$ mV (10 mM-Ca^{2+}), the results obtained would be explained if during the active state $G_{Ca}/G_K \approx 0{\cdot}8$.

There are some reasons to suspect that in addition to the effect of the resting K^+ permeability, the fourth possibility considered here, namely the existence of TEA insensitive K^+ channels which are activated during the 'spike' might also account for the results discussed, particularly for the reduction in amplitude of the plateau. This kind of K^+ channel has been found on invertebrate neurones (Thompson, 1977; Connor, 1979) and seems to be activated by an increase in the intracellular [Ca^{2+}] resulting from calcium influx (Meech, 1977; Lew & Ferreira, 1978). Furthermore, motoneurone 'Ca spikes' are followed by a slow hyperpolarization lasting for several seconds, similar to those found in other nerve cells (Meech, 1977). This after-hyperpolarization is due to an increase in K^+ conductance activated by Ca^{2+} (Meech, 1972, 1977; Krnjević & Lisiewicz, 1972; Gorman & Hermann, 1979). Furthermore, it has been shown that such an increase in K^+ permeability can also be triggered by Sr^{2+} (Alvarez-Leefmans *et al.* 1979; Gorman & Hermann, 1979; but see Moolenaar & Spector, 1979). Therefore it is tempting to think that the hyperpolarization following Ca or Sr dependent 'spikes' in the present experiments is due to an increase in K^+ conductance activated by these cations.

The shape and duration of the TTX-resistant regenerative depolarization suggest that as in other nerve cells (Katz & Miledi, 1971; Baker *et al.* 1973; Llinás, Steinberg & Walton, 1976; Eckert & Lux, 1976; Kostyuk & Krishtal, 1977; Connor, 1979), motoneurone Ca^{2+} inward current inactivates very slowly, and/or incompletely. Repolarization could result from a slowly developing K^+ outward current triggered by Ca^{2+}, or slow inactivation of the Ca^{2+} channels, or a mixture of both mechanisms. Recently it has been suggested that inactivation of Ca^{2+} channels can result from the increased intracellular Ca^{2+} levels resulting from Ca^{2+} entry (Brehm & Eckert, 1978; Tillotson, 1979; see also Kostyuk & Krishtal, 1977). Sr^{2+} appears much less effective than Ca^{2+} in producing inactivation (Brehm & Eckert, 1978), and in triggering the increase in K^+ permeability (Gorman & Hermann, 1979), and this could explain the much longer responses seen in motoneurones bathed in Sr^{2+} medium.

In conclusion, our results suggest that motoneurones have in addition to usual Na^+ and K^+ channels, voltage sensitive Ca^{2+} channels with properties similar to those of Ca^{2+} channels in presynaptic terminals (Katz & Miledi, 1967, 1969*a*, 1971). These channels may be activated during normal action potentials, allowing an influx of Ca^{2+}. However, with a normal ionic environment, nearly all the inward current is carried by Na^+, and this explains why the shape of the soma-dendritic spike is not modified by changing the concentration of external Ca^{2+} or substituting it by other

divalent cations within the concentration range used in the present experiments (Alvarez-Leefmans *et al.* 1979). Although small, the influx of Ca^{2+} during normal action potentials is enough to trigger an increase in K^+ permeability responsible for the slow hyperpolarization which follows normal spikes, and which is so crucial in controlling the firing behaviour of these nerve cells.

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