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POTASSIUM AND CALCIUM CONDUCTANCE IN SLOW MUSCLE FIBRES OF THE TOAD

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SUMMARY

1. Slow muscle fibres in isotonic potassium sulphate saline could be easily repolarized to −90 mV. From this membrane potential a regenerative response could be elicited with short depolarizing pulses.

2. This response is blocked by TEA, suggesting that potassium is the main ion involved.

3. In the presence of TEA, a transient depolarization is recorded when the steady hyperpolarization is withdrawn. This anode break response is dependent upon the external calcium and is blocked by cobalt, suggesting that it is due to a calcium conductance.

4. The membrane conductance change was continuously recorded with short pulses at the end of the hyperpolarization. The membrane conductance decayed with at least two components with an average $t_{\frac{1}{2}}$ of 1·2 and 6·8 sec. TEA blocked the slow component, and the fast one was dependent upon calcium and was blocked by cobalt.

INTRODUCTION

When excitable cells are bathed in high potassium solution, regenerative responses due to an increase in potassium conductance can be elicited if the cell membrane is artificially hyperpolarized by injecting intracellularly negative current (Mueller, 1958; Moore 1959; Grundfest, 1961; Luttgau, 1960; Nakajima & Kusano, 1966). To observe the same phenomenon in twitch muscle fibres, it is necessary to eliminate the shunt of the inward rectifying channel (anomalous rectification), in order to allow a more effective cell hyperpolarization. This can be done for example with formaldehyde (Hutter, 1969). Slow muscle fibres can be particularly suitable for these experiments since the inward rectifying channel is absent and

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the fibres can be easily hyperpolarized in high potassium saline (Stefani & Steinbach, 1969). The aim of the experiments described in the present paper was to investigate further the electrical properties of slow muscle fibres in isotonic potassium sulphate solution and to analyse the time course of the potassium conductance change. During these experiments we confirmed, as in other excitable cells, the presence of a potassium regenerative response, and furthermore we found a second type of response that was obtained at the end of the hyperpolarization when tetraethylammonium ions (TEA) were present. This response was found to be dependent on the external calcium, suggesting that it was mainly due to an increase of membrane calcium conductance. This can be an interesting finding since it has recently been suggested that calcium entry could have a role in the excitation-contraction coupling mechanism in skeletal muscle fibres (Suarez-Kurtz, Reuben, Brandt & Grundfest, 1972; Ford & Podolsky, 1972; Chiarandini & Stefani, 1973; Stefani & Chiarandini, 1973). Furthermore, calcium currents associated with calcium entry have recently been described in the presynaptic terminal of the squid giant axon, in squid giant axons, in barnacle muscle and in heart muscle (Katz & Miledi, 1969; Hagiwara, Hayashi & Takahashi, 1969; Baker, Hodgkin & Ridgway, 1971; Baker, Meves & Ridgway, 1973*a*, *b*; Meves & Vogel, 1973; Keynes, Rojas, Taylor & Vergara, 1973; for recent reviews see Baker, 1972; Reuter, 1973).

METHODS

Experiments were made on piriformis muscle of the toad (*Bufo arenarum*). Slow muscle fibres were easily found in the cutaneous face of the muscle along the external border. The muscle was mounted on a polyethylene rod; one tendon of the muscle was fixed to the rod, and by turning the rod, the muscle was rolled and simultaneously stretched to about 150% of its *in situ* length (Stefani & Schmidt, 1972). With this mounting procedure mechanical artifacts due to muscle contraction were considerably diminished.

Conventional techniques for intracellular recording and stimulation were used. Recording micropipettes were filled with 3 M potassium chloride and were selected for low tip potential. They were connected to the input grid of a differential cathode follower with an input capacity of about 1 pF. The potential was differentially recorded between the micropipette and a silver–silver chloride wire in the bath. Current passing micropipettes were filled with 2 M potassium citrate and were connected to a constant current source. The resistance between the inside and the outside of the fibre (effective resistance, R_{eff}) was measured in the middle of the fibre length. For this measurement, the recording and the current micropipettes were inserted in the same fibre at a distance of 50–100 μm. The injected current was measured with a second silver–silver chloride wire placed in the bath, connected to earth via a high input impedance operational amplifier in the ammeter configuration. With 1 MΩ resistor in the feed-back, a current of 1 nA gave an output of 1 mV. The signals were displayed on a dual beam oscilloscope and photographed or directly registered with a curvilinear pen-recorder.

Solutions. The composition of the normal saline was 115 mM-NaCl, 2·5 mM-KCl and 1·8 mM-$CaCl_2$. The potassium sulphate saline contained 95 mM-K_2SO_4, 9 mM-$CaSO_4$ and 350 mM sucrose, added to reduce the contraction (Howarth, 1958). TEA was added to the saline from a 25% stock solution of TEA hydroxide (B.D.H.), previously neutralized with sulphuric acid. Stock solution of TEA had a significant sodium contamination, which produced a final concentration of about 3 mM sodium in the saline with 30 mM $(TEA)_2SO_4$.

Calcium free solutions were prepared by omitting calcium and adding 1 mM-EGTA (ethylene glycol-bis-(β-aminoethyl ether) *N,N'*-tetracetic acid) neutralized to pH 7·0 with sodium hydroxide. To prevent depolarization caused by removal of calcium (Luttgau, 1963) 4 mM magnesium sulphate was added to the calcium-free solution. Cobalt was added to the experimental solution as solid cobalt sulphate.

All solutions were prepared before each experiment and were buffered to pH 7·3 with 2 mM- Tris (hydroxymethyl)-aminomethane sulphate. The experiments were performed at room temperature (20–26° C). Results are calculated as mean ± S.E.

RESULTS

Regenerative response in slow fibres

Frog twitch muscle fibres in isotonic potassium have anomalous rectification: a decrease in membrane resistance with hyperpolarizing pulses (inward current) (Katz, 1949). Contrary to twitch fibres, slow muscle fibres have an ohmic membrane resistance in high potassium. This property was used as a reliable criterion to electrically recognize slow muscle fibres (Stefani & Steinbach, 1969). We have confirmed this observation in the slow muscle fibres of the toad. In all cases we found that fibres having membrane properties of slow fibres in normal saline ($R_{eff} > 2 \times 10^6\ \Omega$ and membrane time constant > 200 msec) had an ohmic resistance in the potassium sulphate saline. The absence of anomalous rectification was used in these experiments to electrically recognize slow muscle fibres.

The resting potential (E_{rp}) of slow fibres in the normal saline was $-88{\cdot}3 \pm 2{\cdot}4$ mV (mean ± S.E., seven fibres) and the average R_{eff} was $4{\cdot}77 \pm 1{\cdot}2 \times 10^6\ \Omega$ (seventeen fibres). As expected the addition of the potassium sulphate saline depolarized the cells and reduced the R_{eff}. The E_{rp} was $+2{\cdot}8 \pm 0{\cdot}31$ mV (eight fibres). When 350 mM sucrose was added to the isotonic potassium sulphate, the muscle fibres hyperpolarized to an E_{rp} of $-11{\cdot}1 \pm 0{\cdot}86$ mV (fifteen fibres) and they had an average R_{eff} of $1{\cdot}24 \pm 0{\cdot}16 \times 10^6\ \Omega$ (fifteen fibres). Under these conditions the fibres could be easily polarized to about -90 mV by injecting generally less than -100 nA. In the experiment of Fig. 1 the E_{rp} was driven by injecting current intracellularly to a membrane potential of *ca.* -100 mV. It can be clearly seen that when short pulses of outward current were injected a regenerative response was triggered. This response has a threshold of about -50 mV (Fig. 1*A* and *B*), and develops more rapidly with stronger stimuli (Fig. 1*A*). It lasted several seconds, and reached a plateau value of about -10 to

-20 mV. Then the response slowly declined to a certain critical level of about -40 mV from where it abruptly fell (Fig. 1*B*, *C*). When using solutions without sucrose this response was associated with a strong contraction, which generally produced membrane damage at the point of micropipette insertion. These responses were similar to those observed in

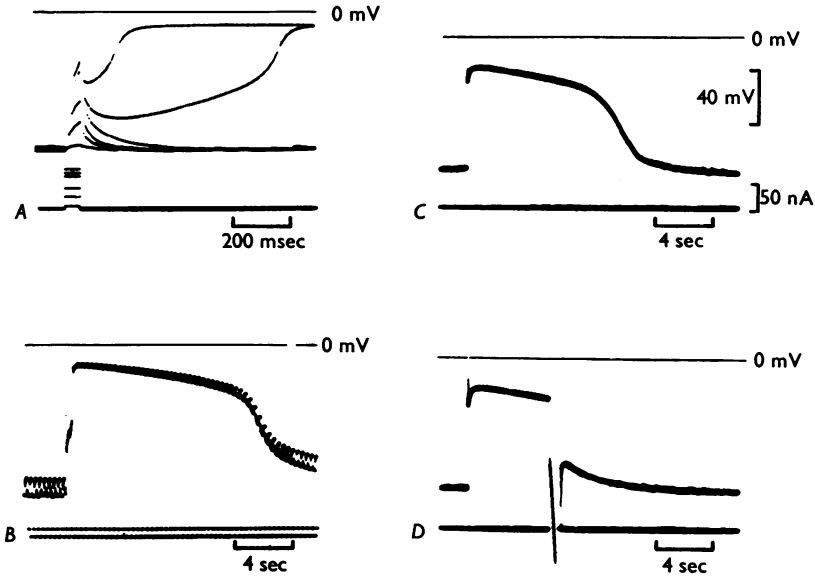

Fig. 1. Regenerative response in potassium sulphate media. Two different muscle fibres *A*, *B* and *C*, *D*. The muscle fibres were hyperpolarized to a membrane potential *ca.* -100 mV by DC current of 90 nA. Upper trace: zero reference line for the voltage. Middle trace: voltage record. Lower trace: current record. In *A* short outward current pulses of different intensity were injected and regenerative responses elicited (superimposed traces). In *B* small hyperpolarizing pulses were continuously injected and a regenerative response was triggered showing a great increase of membrane conductance at the beginning of the response and its decrease associated with the declining phase of the plateau. In *C*, regenerative response which could be cut off by strong hyperpolarizing pulse as shown in *D*.

twitch muscle fibres pre-treated with formaldehyde to block anomalous rectification (Hutter, 1969), in Fig. 1*B*, small pulses of current were passed continuously through the cell membrane to monitor membrane conductance. It can be clearly seen that there is a large increase of conductance at the beginning of the response and that the declining phase of the plateau is associated with a progressive reduction of conductance. The response can be cut off by a strong hyperpolarizing pulse as shown in Fig. 1*D*.

The duration of this response depends upon the level and duration of the previous conditioning hyperpolarization. Fig. 2 shows that the response is larger and more prolonged as the fibre is hyperpolarized to a more negative membrane potential during a longer time. On the other hand, the membrane potential at the peak of the response reached similar values with different conditioning hyperpolarizations ($-14{\cdot}8 \pm 1{\cdot}4$ mV, eight fibres).

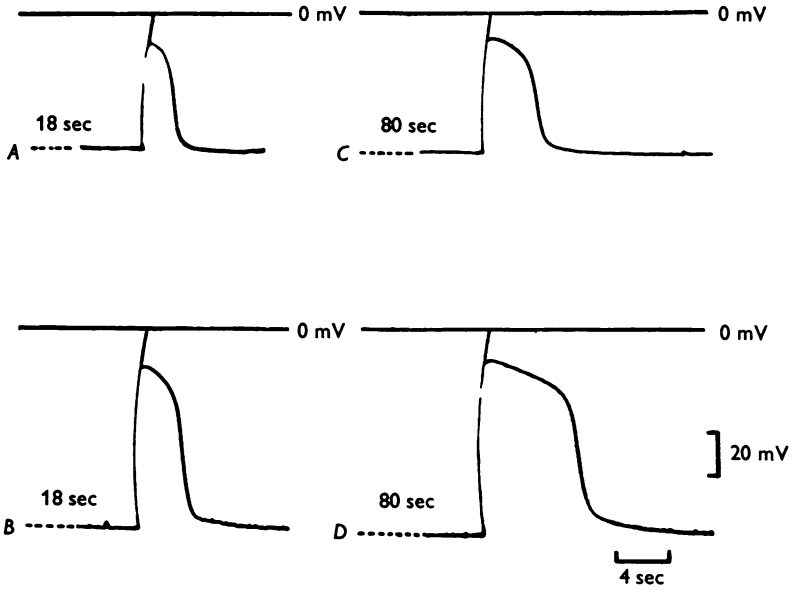

Fig. 2. Regenerative responses from the same muscle fibre in potassium sulphate media with different conditioning steady hyperpolarizations. *A* and *B* were hyperpolarized during 18 sec to -60 and -90 mV respectively. *C* and *D* were hyperpolarized during 80 sec to -60 and -90 mV respectively.

Effect of TEA on the regenerative response

The fact that this response is obtained in a saline in which potassium is the major permeant ion suggests that it is mainly due to an increase in potassium conductance. This was further confirmed by using TEA which selectively blocks potassium conductance (Hille, 1967; Armstrong, 1969; Stanfield, 1970). Muscles were incubated for 1–3 hr in the potassium sulphate solution with 30 mM-$(TEA)_2SO_4$. The E_{rp} and the effective resistance were not modified by TEA. The values were $-11{\cdot}2 \pm 0{\cdot}94$ mV (eighteen fibres) and $1{\cdot}6 \pm 0{\cdot}17 \times 10^6\ \Omega$ (eighteen fibres), respectively. However, TEA blocked the regenerative response. Fig. 3*A* shows the control response; with TEA the response was completely blocked and only with

larger pulses was a partial response obtained (Fig. 3*B*). The blocking action of TEA indicates that potassium is the main ion participating in this regenerative response.

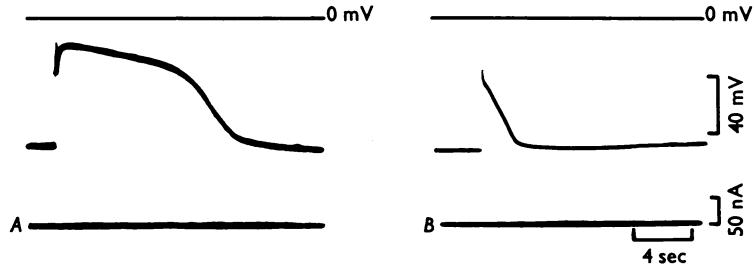

Fig. 3. Effect of TEA on the regenerative response. In *A* control response. In *B* after adding 30 mM-$(TEA)_2SO_4$.

Anode break response in potassium sulphate with TEA

In the potassium sulphate solution the membrane potential always returned to the original value of E_{rp} (about -11 mV) after the hyperpolarizing current was terminated. However when TEA was added to the saline we found a slow and transient depolarization at the end of the hyperpolarization. Fig. 4*A* shows the regenerative potassium response described above in the potassium sulphate saline. The fibre was steadily hyperpolarized for 60 sec and when a short depolarizing stimulus was

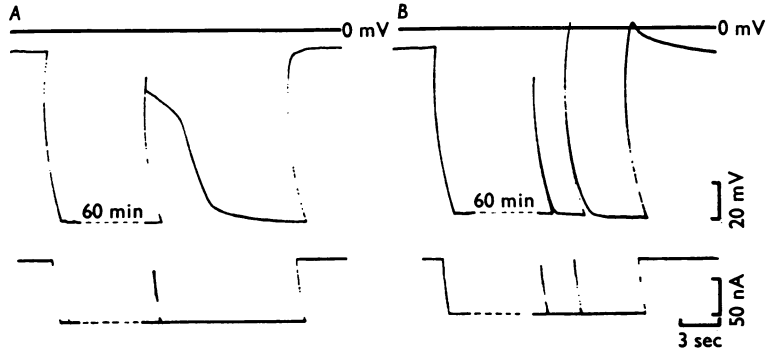

Fig. 4. Effect of TEA on the anode break. In *A* the fibre incubated in potassium sulphate was hyperpolarized to -92 mV during 60 sec. Then a regenerative response was elicited. When the steady hyperpolarization was withdrawn the potential returned to its initial value. In *B* TEA was added, the same fibre was hyperpolarized to -90 mV during 60 sec. Strong depolarizing pulses only elicited a very small response. The cut of the hyperpolarizing current elicited a slow depolarization of 12 mV amplitude (anode break response).

applied the potassium response was elicited. When the hyperpolarizing current was withdrawn, the membrane potential returned to its initial value. In *B*, with 30 mM-$(TEA)_2SO_4$, the potassium response was blocked but a response that reversed the membrane potential appeared at the end of the hyperpolarizing pulse. We shall call this depolarization anode break response.

The anode break response increased with longer conditioning hyperpolarizations. Fig. 5 shows three anode break responses in a fibre that was

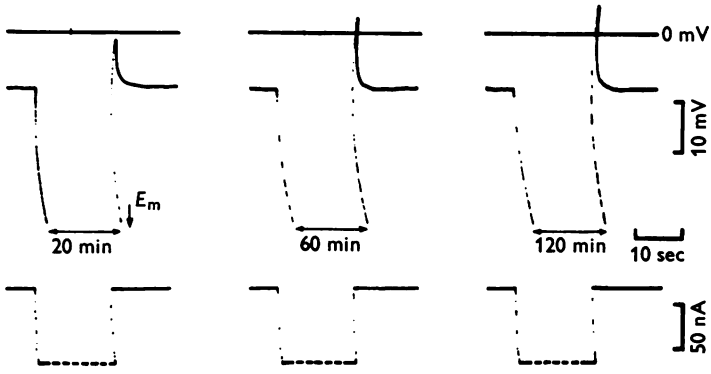

Fig. 5. Anode break responses produced in the same fibre at the end of conditioning pulses of −90 mV amplitude and of different durations.

held at −90 mV during different periods. In eighteen fibres the average amplitude of the anode break response with 60 sec hyperpolarizations of −90 to −100 mV was 12·8 ± 0·71 mV, reaching an average membrane potential of +1·6 mV. When using saline without sucrose this response was associated with a strong contraction.

Membrane conductance during the anode break response

To study the time course of the membrane conductance during the anode break, the fibres were steadily hyperpolarized between −90 to −100 mV during 60 sec, and the membrane resistance was monitored by injecting small pulses of current through the membrane at a frequency of 2–4/sec. The insert of Fig. 6 shows one of these experiments. In the control saline there is a reduction of the pulse amplitude at the end of the hyperpolarization (insert Fig. 6*A*), indicating an increase of the membrane conductance probably due to potassium ions. After adding TEA (insert Fig. 6*b*), the anode break response appears and a less marked reduction of the pulse amplitude was observed.

The graph of Fig. 6 shows in the same fibre the time course of the

conductance change after the end of the hyperpolarizing pulse in the control potassium sulphate (open circles) and with 30 mM-$(TEA)_2SO_4$ (filled circles). The change of membrane conductance is expressed as the difference between the membrane conductance ($1/R_{eff}$) measured at different times after the anode break (G_t) and the resting membrane conductance

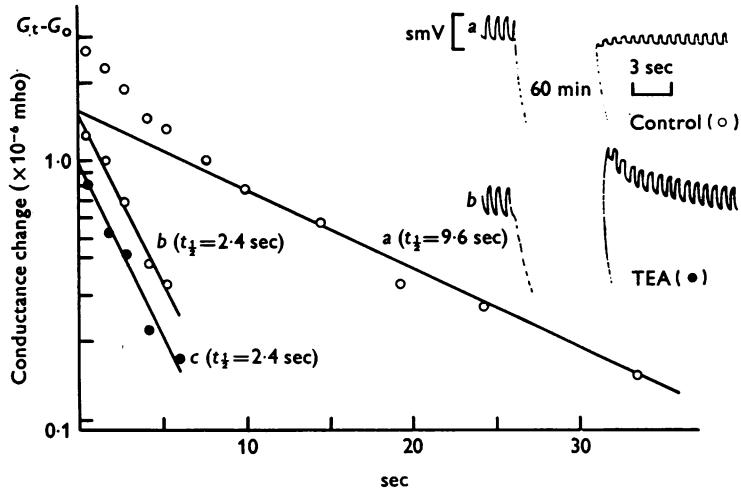

Fig. 6. Graph showing the time course of the membrane conductance change after a conditioning hyperpolarization of −95 mV during 60 sec, in the same fibre before (open circles) and after (filled circles) adding TEA to the bath. The experimental points were obtained from the experiment shown in the insert. G_t is the membrane conductance at different times after the break and G_o is the resting membrane conductance.

measured before applying the conditioning hyperpolarization (G_o). The experimental curve (open circles) obtained in control saline was resolved into the sum of two negative exponential functions with half-times ($t_{\frac{1}{2}}$) of 9·6 sec (curve *a*) and 2·4 sec (curve *b*). The addition of TEA blocked the slow component and the decay of the membrane conductance followed a single negative exponential with a $t_{\frac{1}{2}}$ of 2·4 sec (curve *c*). Similar results were found in nine other fibres, with an average $t_{\frac{1}{2}}$ of $6{\cdot}8 \pm 0{\cdot}6$ sec for the slow component and $1{\cdot}2 \pm 0{\cdot}2$ sec for the fast one. In all cases TEA abolished the slow component while the amplitude of the fast one was slightly reduced changing the time course with a $t_{\frac{1}{2}}$ of $1{\cdot}5 \pm 0{\cdot}1$ sec.

Calcium and cobalt action on the anode break response

The muscle fibres in potassium sulphate solution have an E_{rp} approximately equal to the potassium equilibrium potential (E_K). The fact that the anode break response drives the potential to more positive values than

the E_K suggests that an ion conductance with a positive equilibrium potential is activated.

To analyse a possible role of calcium-ions in the development of the response, slow muscle fibres were incubated during more than 1 hr in potassium sulphate calcium-free solution with TEA and 4 mM magnesium added. The removal of calcium did not affect the E_{rp} ($-11{\cdot}00 \pm 1{\cdot}27$ mV, ten fibres) but reduced the effective resistance ($1{\cdot}09 \pm 0{\cdot}12 \times 10^6\ \Omega$, ten fibres) ($P < 0{\cdot}01$). In this saline the anode break response could not be elicited, and furthermore, no change in membrane conductance was detected at the end of the hyperpolarizing pulse.

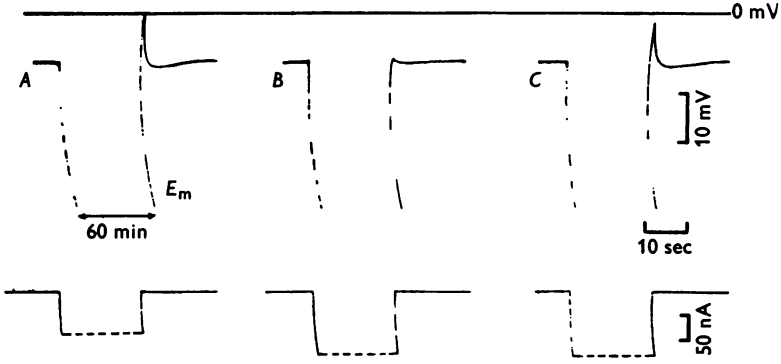

Fig. 7. Action of calcium-free solution on the anode break response. The membrane potential during the conditioning pulses was -95 mV. In *A* an anode break response in potassium sulphate TEA solution. In *B* the fibre was incubated during 20 min in potassium sulphate TEA calcium-free solutions. In *C* 10 min after returning to control solution. *A*, *B*, and *C* are records from the same fibre. In *B* and *C* it was necessary to inject a stronger current to drive the potential to the same level of hyperpolarization as in *A*.

Fig. 7*A* shows the control anode break response; in the calcium-free solution the response disappeared (*B*), and recovered in the control saline (*C*). These results suggest that a calcium current is involved during the anode break response. To confirm this hypothesis we tried the effect of cobalt, a specific blocking agent of calcium current (Hagiwara & Takahashi, 1967; Kohlhardt, Bauer, Krausse & Fleckenstein, 1973; Baker *et al.* 1973*a*, *b*). Fig. 8 shows a control anode break response (*A*), which was blocked by 30 mM cobalt sulphate (*B*). The response did not completely recover after washing for 15 min (*C*). The addition of cobalt also abolished the increase of membrane conductance normally observed at the end of the conditioning hyperpolarizing pulse.

Tetrodotoxin and sodium action on the anode response

Baker *et al.* (1973*a*, *b*) observed in squid axons a tetrodotoxin sensitive calcium influx, and postulated that this influx would occur through the sodium channel. With the aim of studying this possibility, the effect of tetrodotoxin and sodium on the anode break response was analysed.

In eight fibres from three different muscles, the anode break response was unmodified by tetrodotoxin by 10^{-7} mg/ml. The addition of 30 mM sodium sulphate also did not affect the anode break response. These results indicate that a sodium channel is not involved in the generation of the anode break response.

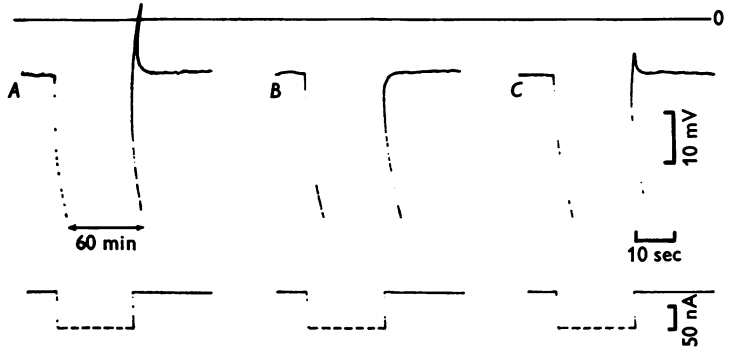

Fig. 8. Action of cobalt on the anode break response. The membrane potential of the conditioning pulses was -92 mV. In *A* an anode break response in potassium sulphate TEA solution. In *B* 30 mM cobalt sulphate was added to the bath. In *C* 20 min after returning to control solution. *A*, *B*, and *C* are records from the same fibre.

DISCUSSION

Similar to the frog, the piriformis muscle of the toad has two types of muscle fibres, twitch and slow fibres. According to Stefani & Steinbach (1969), in high potassium solution slow fibres were electrically identified by the absence of anomalous rectification. In isotonic potassium sulphate, slow fibres had the expected E_{rp} of *ca.* $+3$ mV for a potassium electrode, assuming an internal potassium concentration $[K]_i$ of 140 mM. When the saline was made hypertonic by the addition of 350 mM sucrose, the cells hyperpolarized to about -11 mV. This hyperpolarization can be explained by an increase of $[K]_i$ due to the loss of water from the cell because of the increase in external osmolarity. In the hypertonic saline the calculated $[K]_i$ would be 260 mM.

Slow fibres were able to produce regenerative responses to short depolarizing pulses if they were continuously hyperpolarized to *ca.* -100 mV by passing current intracellularly. This response is similar to those observed in various excitable membranes under conditions in which an inward potassium gradient existed. They were explained as voltage dependent increase of potassium conductance which allows an inward potassium current to flow down the electrochemical gradient (Luttgau, 1963; Nakajima & Kusano, 1966; Hutter, 1969). Similarly, an increase of the potassium conductance can explain this response of slow fibres. In effect, by passing brief and small pulses of current at a frequency of 2–4 sec, the membrane conductance could be continuously monitored, and it was clearly seen that an increase of the membrane conductance occurred during the response (Fig. 1*B*). Furthermore this response was abolished by TEA, a selective blocker of potassium conductance (Hille, 1967; Armstrong, 1969). The conductance change was also studied at the end of the steady hyperpolarization. In a similar way, small and brief pulses of current were delivered intracellularly. At the end of the hyperpolarization the conductance increased about fourfold, and then decayed with at least a fast and slow component with average $t_{\frac{1}{2}}$ of 1·2 and 6·8 sec respectively. The addition of TEA blocked only the slow component. TEA also produced the anode break, i.e. a transient depolarization when the steady hyperpolarizing current is withdrawn. The anode break response was abolished in calcium-free saline or with cobalt, suggesting that a calcium conductance may be activated and that calcium may enter following its electrochemical gradient. Furthermore, the fact that TEA abolished only the slow component of the conductance change, and that the withdrawal of calcium or the addition of cobalt blocked the fast one, suggests that this fast component may be due to calcium. The participation of calcium in the anode break response was further supported by the lack of effect of tetrodotoxin or sodium. These results are essentially similar to the findings of Katz & Miledi (1969) in the presynaptic terminal of the giant synapse of the squid. They found a calcium dependent action potential by blocking the sodium current with tetrodotoxin and diminishing the potassium rectification with TEA.

Baker *et al.* (1971, 1973*a*, *b*) observed that calcium entry associated with depolarization in squid giant axons can be divided into an early component which can be blocked by tetrodotoxin, and a late component which is unaffected by tetrodotoxin, and is abolished by cobalt, manganese and D-600. The calcium conductance in slow muscle fibres might be similar to the second component of the squid axon, since similarly it is unaffected by tetrodotoxin and is blocked by cobalt.

An attempt can be made to calculate the activated G_{Ca} and the calcium

current (I_{Ca}) from an equivalent circuit in which G_{Ca} and E_{Ca} are in parallel with G_K and E_K. In this circuit

$$G_{Ca} = G_K \frac{E - E_K}{E_{Ca} - E} \tag{1}$$

and the resultant calcium current is

$$I_{Ca} = G_{Ca}(E - E_{Ca}). \tag{2}$$

In these equations E is the membrane potential at the peak of the anode break response (+1·6 mV) and E_K is equal to the resting potential. The calcium equilibrium potential can be calculated from $E_{Ca} = RT/Fz \ln [Ca]_o/[Ca]_i$. In the saline with 9 mM calcium sulphate only approximately 0·5 mM calcium would be ionized assuming a dissociation constant of $5{\cdot}3 \times 10^{-3}$ mole/l. (Brink, 1954). The internal calcium concentration could be considered to be between 10^{-8} and 10^{-7} M, since a $[Ca]_i$ of 0·3–1·2 $\times 10^{-7}$ M induces threshold tension (Ebashi & Endo, 1968; Hellam & Podolsky, 1969). Therefore the resultant E_{Ca} would be between +107 and +136 mV. To calculate G_K, the potassium conductance during the anode break, it is assumed that G_K does not increase during the anode break. This assumption is valid since TEA blocked the slow component and did not affect the fast component which was dependent upon calcium and was blocked by cobalt. The G_K would be equal to the specific membrane conductance G_m since in the potassium sulphate saline practically all the current through the membrane is carried by potassium. The values of G_m or G_K can be calculated from the infinite cable model which can be used in high potassium saline since there is a reduction of the membrane resistance of slow fibres and the space constant becomes much smaller than the fibre length (Stefani & Steinbach, 1969). With the current (I_o) and voltage (V_o) electrodes in the middle of the muscle fibre length, G_m (mho/cm^2) can be calculated from

$$G_K = G_m = \left(\frac{I_o}{V_o}\right)^2 \frac{1}{\pi^2 d^3 G_i}, \tag{3}$$

I_o/V_o is the effective membrane conductance ($1/R_{eff}$, d, the fibre diameter and G_i the internal conductance. Assuming G_i $5{\cdot}9 \times 10^{-3}$ mho/cm (Hodgkin & Nakajima, 1972) and a diameter of 80 μ, with the average value of V_o/I_o ($1{\cdot}6 \times 10^6$ Ω), the resultant G_m is $1{\cdot}3 \times 10^{-5}$ mho/cm^2. From the eqns. (1) and (2) the calculated G_{Ca} and I_{Ca} are $1{\cdot}3 \times 10^{-6}$ mho/cm^3 and $0{\cdot}18 \times 10^{-6}$ A/cm^2. These values are much smaller than those obtained for barnacle muscle fibres (Keynes *et al.* 1973) for the presynaptic terminal of the giant synapse of the squid (Katz & Miledi, 1969), and for the giant axon of the squid (Meves & Vogel, 1973).

An interesting question remains whether this calcium current might

have some role in excitation–contraction coupling in slow muscle fibres, since it has been recently suggested that an inward calcium current could have a direct role in mechanical activation in frog twitch muscle fibres (Chiarandini & Stefani, 1973; Stefani & Chiarandini, 1973).

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