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Role of ion conductance changes and of the sodium-pump in adrenaline-induced hyperpolarization of rat diaphragm muscle fibres

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1 The ionic mechanism of membrane hyperpolarization induced by adrenaline in rat diaphragm muscle fibres was studied.

2 Removal of the extracellular K^+ ($[K^+]_o$) from Krebs-Ringer solution initially increased the resting membrane potential and then caused an increase in the intracellular Na^+ activity ($[Na^+]_i$) and a decrease in the intracellular K^+ activity ($[K^+]_i$). All the changes were maintained for more than 3 h.

3 Application of ouabain (0.1 mM) or lowering the temperature rapidly reduced the resting potential by about 10 mV in the K^+-free solution. It then produced further progressive decreases in resting potential and in $[K^+]_i$ and a progressive increase in $[Na^+]_i$. These observations indicate that an electrogenic Na-pump operates in the K^+-free solution.

4 Removal of most of the Cl^- in the K^+-free solution did not affect the resting potential or the magnitude of the initial decrease produced by ouabain, despite an increased input resistance; this result implies a passive distribution of Cl^-.

5 Adrenaline (30–60 μM) either added to the bathing solution or applied to the membrane by ionophoresis produced a hyperpolarization (3–10 mV: adrenaline hyperpolarization), the amplitude of which was decreased with a rise in $[K^+]_o$ and increased with a reduction in $[K^+]_o$, but unaffected by the removal of Cl^-.

6 Adrenaline produced an increase in input resistance, the relative magnitude (17–18%) of which was constant whether external K^+ or Cl^- was removed. In contrast, a conditioning membrane hyperpolarization hardly affected the resistance.

7 Ouabain (0.1 mM) or low temperature (8–10°C) abolished both the hyperpolarization and the increased input resistance induced by adrenaline.

8 The $[K^+]_i$, $[Na^+]_i$ and the peak of the action potential remained unchanged after a 20 min exposure to adrenaline (30 μM).

9 The hyperpolarization induced by the replacement of all Na^+ with Tris (Tris-hyperpolarization) in the K^+-free solution was depressed by 39% during the early period (4–31 min) of exposure to adrenaline (30 μM), while it was enhanced by 26% during the later period (80–130 min). The initial depression suggested a decrease in the ratio of the membrane permeability for Na^+ (P_{Na}) to that for K^+ (P_K).

10 These results suggest that the adrenaline hyperpolarization is generated largely by a decrease in P_{Na}/P_K, which is associated with the activity of the Na-pump.

Introduction

Adrenaline produces a hyperpolarization of mammalian skeletal muscle fibres (Bowman & Raper, 1967; Somlyo & Somlyo, 1969; Kuba, 1970; Clausen & Flatman, 1977) which may be one mode of autonomic regulation of skeletal muscle activity (cf. Bowman, 1982). Adrenaline-induced hyperpolarization (adrenaline hyperpolarization) in the rat soleus muscle fibres has been suggested to result from an increase in the electrogenic activity of the Na-pump (Tashiro, 1973; Clausen & Flatman, 1977).

In the rat diaphragm muscle, however, adrenaline hyperpolarization is augmented by the removal of K^+

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from the Krebs solution, and is accompanied by an increased membrane resistance (Kuba, 1970; Kuba *et al.*, 1978). These results do not support the idea that stimulation of the electrogenic Na-pump is responsible for the hyperpolarization, but rather suggest a mechanism involving a change in the passive ionic permeability of the membrane. For this reason, we have re-investigated the ionic mechanism underlying the adrenaline hyperpolarization in the rat diaphragm muscle. The present experiments suggest that the adrenaline hyperpolarization is induced largely by a decrease in the ratio of the membrane permeability for Na^+ (P_{Na}) to that for K^+ (P_K), which depends on the operation of the Na-pump. Some of the results have been published in an abstract form (Nohmi & Kuba, 1980).

Methods

Both sides of the hemidiaphragm with the phrenic nerve attached were dissected from Albino rats of either sex under ether anaesthesia. They were cut radially along fibres into a strip 1.5–2.0 cm in width with the nerve (1.5 cm) attached. One of the muscle strips was mounted in a lucite chamber (5.0 ml in volume) and perfused with a modified Krebs solution equilibrated with 95% O_2 and 5% CO_2 at 32.0–34.0°C unless otherwise specified, while the other strip was kept in oxygenated Krebs solution at 20–24°C and used for a subsequent experiment. An interval of 2–5 min was required for a 90% change of the perfusing solution in the recording chamber. The composition of the modified Krebs solution used was (mM): Na^+ 137, K^+ 5.9, Mg^{2+} 1.2, Ca^{2+} 2.5, Cl^- 134, $H_2PO_4^-$ 1.2, HCO_3^- 15.5 and glucose 11.4. The tonicity of the K^+-free or high K^+ solution was adjusted by increasing or decreasing the Na^+ concentration. The K^+-free, Cl^--deficient (7.5 mM) solution was prepared by replacing all the NaCl with an equimolar amount of Na-isethionate. A K^+-free, Tris solution was made by the replacement of all the Na^+ with 137 mM Tris (Tris-hydroxymethylaminomethane) and the pH of the solution was adjusted to 7.0 with 0.1 N HCl.

A conventional intracellular recording technique was employed, as described previously (Kuba, 1970). Recording electrodes were filled with 3 M KCl (resistance 20–50 MΩ). The input resistance of the muscle fibre was measured by penetration with two independent electrodes, one for recording and the other for passing a current. The current electrode was filled with 1 M K-citrate (resistance 20–30 MΩ). In all the experiments, intracellular electrodes were inserted through the membrane at least 2–3 mm away from the endplate region. This was confirmed by the absence of miniature endplate potentials in the recording.

The ionophoretic application of adrenaline to a muscle fibre was made by a micropipette filled with 0.5 M adrenaline bitartrate (pH 5.0 adjusted with 0.1 N HCl: the tip resistance was 200 MΩ) and by passing a long-lasting current (100–400 nA) for several minutes. To obtain the maximum response, the adrenaline-filled electrode was positioned 30–100 μm above the muscle fibre (in which a recording electrode was impaled) and 50–100 μm in the upstream of the flow of solution. An electrode filled with 0.5 M Na-bitartrate (pH 5.0) was also used to assess that the effect of ionophoresis of adrenaline on the muscle fibre did not result from the ionophoretic current itself (resistance 30–100 MΩ).

Ion-sensitive electrodes were made as described by Fujimoto & Kubota (1976). Micropipettes were pulled from Pyrex capillary tubing (Narishige GD-1.2) on a conventional horizontal puller so as to have a tip resistance of 20 MΩ when filled with 3 M KCl. After siliconization with 0.15–0.5% silicon oil (KF-96, Shin-etsu Chemical Ind. Japan) in trichlorethylene, the tip of the pipette was filled up to 200–500 μm in length with a K^+-exchanger (WPI) or a Na^+-exchanger which consisted of a 10% (w/v) neutral carrier (1,1,1-Tris-(1'-(2'-oxa-4'-oxo-5'-aza-5'-methyl) dodecanyl) propan: FLUKA 71732) in *o*-nitrophenyl-*n*-octyl-ether (FLUKA 73732) with 0.5% (w/w) of sodium tetraphenyl borate (FLUKA 72020: cf. Steiner *et al.*, 1979). The reference solution placed above the ion exchanger was 0.1 M NaCl for Na^+-sensitive electrodes and 0.5 M KCl for K^+-sensitive electrodes. The slope of the relationship between the K^+ activity (0.01–0.5 M) and the voltage across the K^+-exchanger in the electrode (measured with a high-input impedance amplifier (AD 331J)) was 54.7 ± 1.4 mV ($n = 11$) per 10 fold change in K^+ activity. The selectivity constant of the K^+-sensitive electrode to Na^+ (cf. Fujimoto & Kubota, 1976) was around 0.014. The sensitivity of Na^+-sensitive electrodes was 58.5 ± 3.2 mV ($n = 26$) per 10 fold change in Na^+ activity (0.01–0.3 M). The selectivity to K^+ similarly calculated was 0.0097. Calibration of the electrode was usually carried out before and after the penetration of muscle fibres. Intracellular activity of Na^+ or K^+ was measured from a voltage difference between an ion-sensitive electrode and an intracellular reference voltage electrode filled with 3 M KCl. The sensitivity of either type of ion-sensitive electrode remained essentially unchanged after penetration of muscle fibres.

Effects of various treatments of the muscle fibres were observed in three ways: (1) in some experiments while recording resting potentials or in all the experiments when measuring the input resistance of the fibre, the effects were observed in a single fibre and their net changes were presented. (2) Resting membrane potentials were also recorded from many fibres in a considerable number of muscles before and during various treatments of the muscle and the mean values

of all the pooled data in each condition were compared. (3) Intracellular Na^+ or K^+ activities were measured from individual fibres in single muscles before and during treatments, averaged for two conditions in each muscle, and the means of the averaged Na^+ or K^+ activities of each muscle were compared. All the data are expressed mean ± s.e.mean. Numbers of fibres and muscles are expressed as n_f and n_m, respectively. Statistical significance was examined by use of Student's t test and a P value less than 0.001 was considered significant.

Adrenaline bitartrate, strophanthin-G (ouabain), Tris-hydroxyaminomethane and Na-isethionate were obtained from Sigma, Merck, Wako Pure Chemical (Japan) and Tokyo Kasei Ind. (Japan), respectively.

Results

The contribution of the electrogenic Na-pump to the resting membrane potential in a K^+-free solution

It was previously shown (Kuba *et al.*, 1978) that adrenaline hyperpolarization in the rat diaphragm muscle fibre was greatest in a K^+-free solution, a condition known to block the activity of the Na-pump. One possible mechanism for this effect is enhancement of the electrogenic effect of the Na-pump by adrenaline (Somlyo & Somlyo, 1969; Tashiro, 1973; Clausen & Flatman, 1977). It was therefore necessary to test whether the Na-pump is active and electrogenic in a K^+-free solution.

The evidence for the activity of the Na-pump and its electrogenicity in K^+-free solution When K^+ was removed from the Krebs solution, the membrane was hyperpolarized from the normal value of -74.8 ± 0.2 mV mean ± s.e.mean, ($n_f = 443$) to -91.7 ± 0.7 mV ($n_f = 668$) within 20 min, and the hyperpolarization was maintained for more than 3 h. Under this condition, the intracellular K^+ activity ($[K^+]_i$) decreased gradually from the normal value of 72.2 ± 3.7 mM ($n_m = 11$) to a steady value of 60.3 ± 2.0 mM ($n_m = 17$) in 1–1.5 h, and the intra-cellular Na^+ activity ($[Na^+]_i$) increased with a similar time course from 18.2 ± 3.0 mM ($n_m = 10$) to 25.5 ± 2.4 mM ($n_m = 14$). The $[K^+]_i$ and $[Na^+]_i$ in a K^+-free solution were maintained for more than 3 h.

The addition of ouabain (0.1 mM) to a K^+-free solution rather abruptly reduced the resting potential by about 10 mV (Figure 1a; see Table 1), and then decreased the $[K^+]_i$ progressively (Figure 1b) and likewise increased the $[Na^+]_i$ (Figure 1c). Furthermore, lowering temperature (from 34°C to 8–10°C) significantly decreased the resting potential in a K^+-free solution (Table 1). These effects of ouabain and low temperature, as well as the maintenance of a high resting potential in a K^+-free solution, indicate that the Na-pump was still operating and electrogenic in a K^+-free solution in the rat diaphragm muscle fibre even though the Na-pump activity was low, as evidenced by the decreased $[K^+]_i$ and increased $[Na^+]_i$.

The potential induced by the electrogenic Na-pump is independent of Cl^- It is well known that the electrogenic (normally-hyperpolarizing) effect of the Na-pump is augmented by the replacement of Cl^- with non-permeable anions. This arises as a result of the reduction in the short-circuiting effect of Cl^- (Rang & Ritchie, 1968). Alternatively, however, if Cl^- is pas-

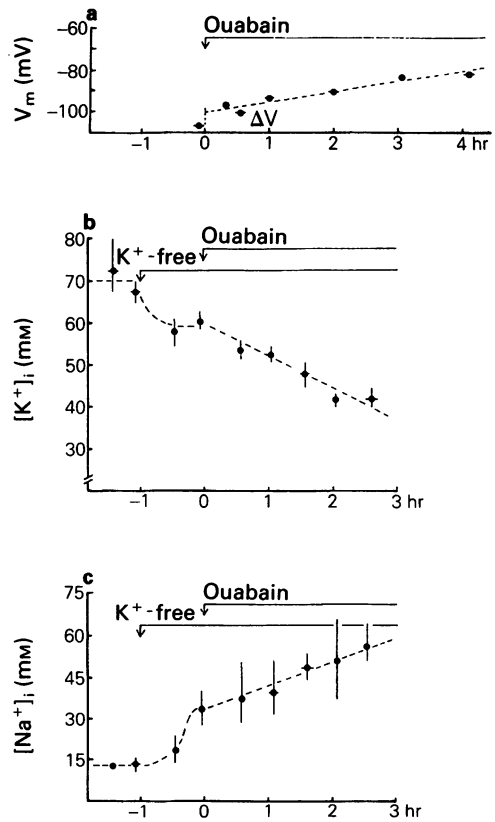

Figure 1 Effects of ouabain (0.1 mM) on (a) resting potential, (V_m), (b) $[K^+]_i$ and (c) $[Na^+]_i$ in a K^+-free solution. In (a) ΔV: the membrane depolarization induced by the 'instantaneous' effect of ouabain which was obtained by a linear extrapolation of the data to the beginning of the time of application using a least-square fitting. It presumably indicates the magnitude of the membrane potential induced by the electrogenic effect of the Na-pump.

Table 1 Effects of adrenaline on resting membrane potentials (mV) in various ionic solutions

	Temp. (°C)	*Control*		*Adrenaline*		*Δ Hp*
Krebs (K^+ 5.9 mM)	34	− 74.8 ± 0.2	(443)	− 78.7 ± 0.2	(417)	− 3.9*
K^+-free	34	− 91.7 ± 0.7	(668)	− 98.3 ± 0.6	(763)	− 6.6*
K^+-free	20	− 96.4 ± 0.5	(108)	− 100.2 ± 0.4	(120)	− 3.8*
K^+-free	10	− 81.2 ± 1.4	(60)	− 82.1 ± 0.4	(102)	− 0.9
K^+-free Cl^--deficient (7.5 mM)	34	− 92.6 ± 0.2	(337)	− 98.4 ± 0.3	(183)	− 5.8*
K^+-free, ouabain (0.1 mM)	34	− 82.1 ± 0.5	(161)	− 82.1 ± 0.4	(294)	− 0.1

Data were obtained by random penetrations of muscle fibres and the effect of adrenaline (30–60 μM) was observed between 5 and 30 min after perfusion with the solution containing adrenaline. ΔHp is net change in membrane potential by adrenaline. *Values significantly different from zero. The numbers in parentheses represent the number of impalements.

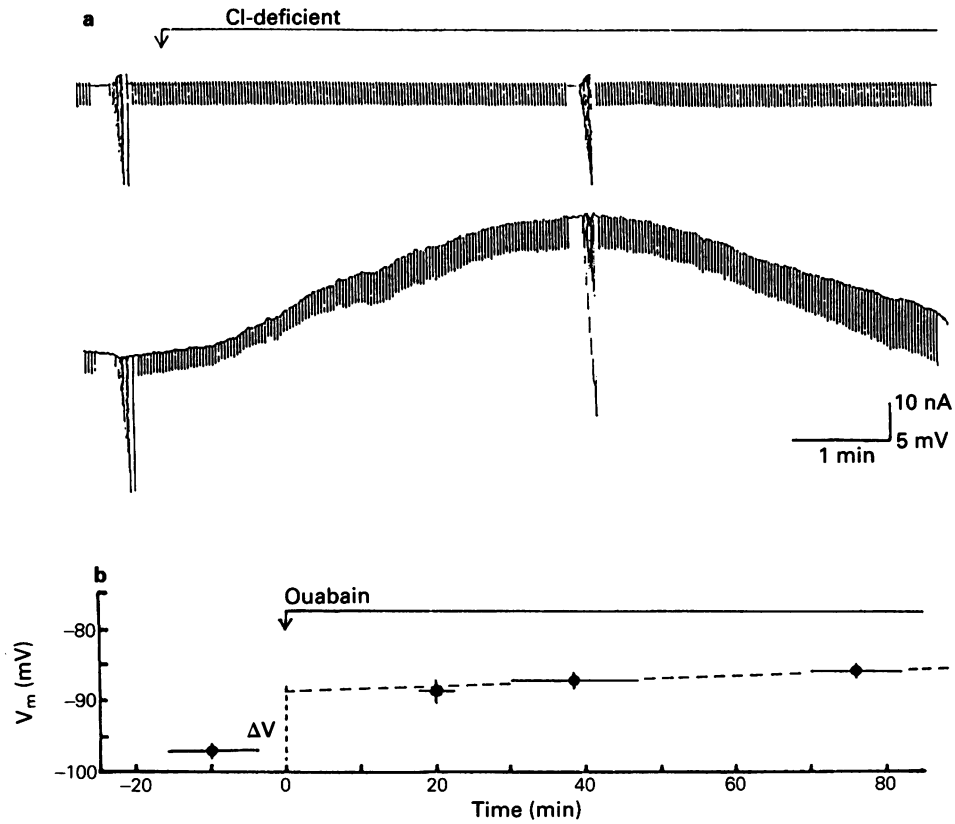

Figure 2 (a) Effects of replacement of 126.5 mM Cl^- with isethionate ions on the resting potential and input resistance of a single fibre. Hyperpolarizing current pulses (10 nA and 50 ms) were constantly applied, except for the period during which current pulses of different intensities were applied, to obtain current-voltage relationships such as those shown in Figure 5. (b) Effects of ouabain (0.1 mM) on the resting potentials (V_m) sampled by random penetrations in a K^+-free, Cl^--deficient solution. The broken line was drawn by the least-square fitting method. For explanation of ΔV see Figure 1 legend.

sively distributed in the rat diaphragm muscle fibre (Palade & Barch, 1977; Dulhunty, 1978), then, as long as the membrane is in a steady state, no net-Cl^- current would flow across the cell membrane. Accordingly, the removal of Cl^- from the perfusing solution would not affect the potential generated by the Na-pump after equilibration (cf. Sjodin, 1984). The results shown in Figure 2 indicate that this is the case.

When 95% of Cl^- in the K^+-free solution was slowly replaced with isethionate ions, there was a small membrane depolarization unaccompanied by action potentials (albeit some fibres showed spontaneous action potentials and twitches due to a large depolarization of the membrane: see Hodgkin & Horowicz, 1959), which subsequently returned to the previous value (Figure 2a; Table 1). Under this condition, the input resistance of the fibre increased to 219% of the control at the low extracellular Cl^- concentration (Figure 2a). In addition, ouabain (0.1 mM) decreased the resting potential in the K^+-free, Cl^--deficient solution by 8.7 ± 1.6 mV ($n_m = 6$) (Figure 2b). These results suggest that the Na-pump is active in the K^+-free, Cl^--deficient solution, and that its electrogenic effect is independent of Cl^- conductance. Thus, as in amphibian muscle fibres (cf. Sjodin, 1984), Cl^- appears to be passively distributed even in the K^+-free solution.

The mechanism of adrenaline hyperpolarization

Adrenaline hyperpolarization and its dependence on $[K^+]_o$. When adrenaline was ionophoresed through a micropipette in normal Krebs solution (100–400 nA d.c.), the fibre membrane was hyperpolarized (Figure 3Aa), while the ionophoresis of Na^+ had no effect (Figure 3Ab). The hyperpolarization reached a peak (4.9 ± 0.3 mV, $n_f = 26$) about 2 min after the start of ionophoresis, and subsided slowly after the ionophoresis had stopped. Superfusion of adrenaline (30–60 μM) to the muscle hyperpolarized the fibre membrane by 4.7 ± 0.7 mV ($n_f = 15$) in Krebs solution, as seen Figure 3Ba. The adrenaline-induced hyperpolarization (adrenaline hyperpolarization) was also observed when sampling resting potentials from many different fibres (Table 1). These findings agree with those from previous experiments by Kuba (1970), but

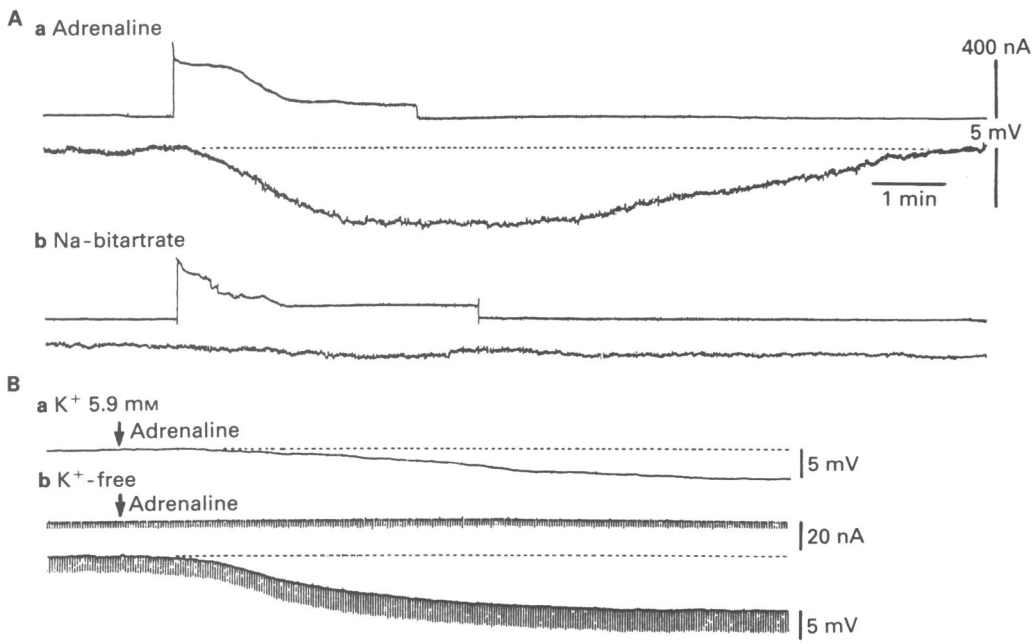

Figure 3 Adrenaline hyperpolarizations induced by two different methods in different ionic solutions. (A) Ionophoretic applications of adrenaline (0.5 M; a) and Na^+ (Na-bitartrate, 0.5 M; b) in Krebs solution. Upper and lower traces represent current for ionophoresis and membrane potential, respectively. (B) Membrane hyperpolarizations and changes in input resistance induced by perfusion of a solution containing adrenaline (30 μM) in normal Krebs (a: the latter effect is not shown), and K^+-free (b) solution. Upper traces in (b) represent pulses (50 ms in duration) passed to the membrane, while the trace in (a) and lower trace in (b) show membrane potential.

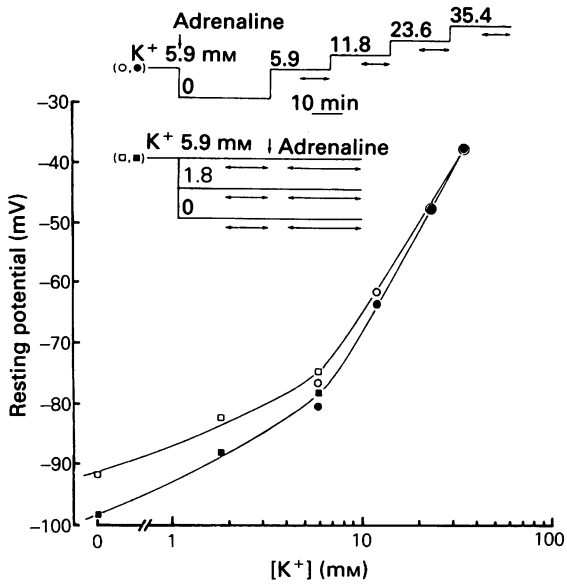

Figure 4 Effects of varying the extracellular K^+ concentration on resting potentials in the absence (O, □) and presence (●, ■) of adrenaline (30–60 μM). Two different procedures were employed for changes in extracellular K^+ concentration as shown by time-charts in inset. Sampling of resting membrane potentials from different fibres were made during periods indicated by horizontal arrows in inset. Each value represents the mean of data pooled from 97–763 fibres in 6–30 muscles. The s.e.mean of each point is within the size of the symbol.

not with that of Bray *et al.* (1976), presumably because the latter study was done at room temperature (see below).

When adrenaline (30 μM) was superfused to the muscle in the K^+-free solution, it produced a membrane hyperpolarization (5.8 ± 1.0 mV, $n_f = 9$) greater than that in normal Krebs solution (Figure 3Bb). A similar result was obtained when recording resting potentials from many different fibres (Table 1). Figure 4 summarizes the effects of adrenaline (30–60 μM) on resting membrane potentials at different concentrations of extracellular K^+ ($[K^+]_o$). The adrenaline hyperpolarization was enhanced by a decrease in $[K^+]_o$ and reached a maximum value (4–10 mV) in the K^+-free solution, whereas it was decreased by increasing $[K^+]_o$. Therefore, the ionic mechanism underlying adrenaline hyperpolarization was hereafter analysed in the K^+-free solution.

The reduction of the membrane conductance during adrenaline hyperpolarization The adrenaline hyperpolarization in the K^+-free solution was accompanied by an increase in the effective resistance of the fibre, which was 117 ± 2% ($n_f = 12$) of the control (Figure 5a; see also Figure 3Bb) and similar in magnitude to that observed in normal Krebs solution (117%; Kuba, 1970). The increased input resistance was not the result of the membrane hyperpolarization caused by adrenaline, since an equal conditioning hyperpolarization raised the effective resistance by only 2 ± 0.7% ($n_f = 25$; Figure 5b). Assuming that adrenaline does not affect the longitudinal myoplasmic resistance and the fibre diameter, the adrenaline-induced alteration

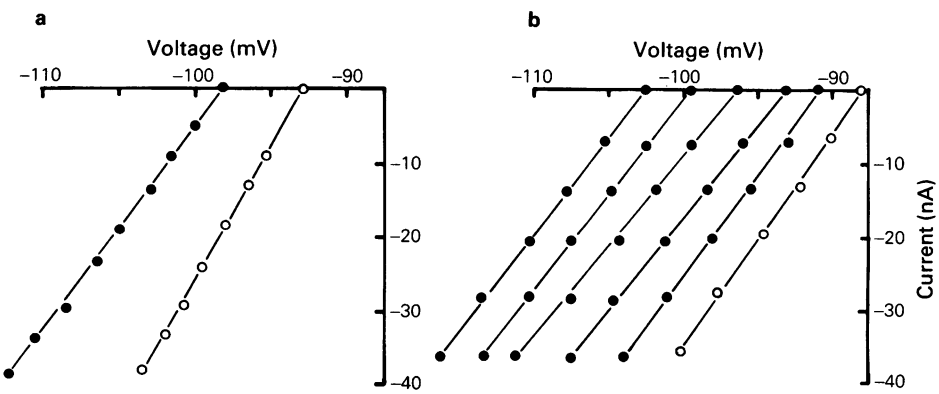

Figure 5 Effects of adrenaline (30 μM) and conditioning hyperpolarization on current-voltage relationships. (a) Effects of adrenaline in a K^+-free solution. (b) Effects of conditioning hyperpolarizations in a K^+-free solution. Membrane potential was displaced to different levels by passing constant hyperpolarizing currents. The conditioning currents are not shown in the current axis. (O) Indicate the current-voltage relationships without adrenaline or conditioning hyperpolarizations, while (●) represent those in the presence of adrenaline (a) or during conditioning hyperpolarizations (b).

in the effective resistance (R_e and $R_{e'}$ in the absence and presence of adrenaline, respectively) was converted to a fractional decrease (g_m(Ad)) in the membrane conductance (g_m) by the equation (cf. Hodgkin & Rushton, 1946),

$$g_m(Ad) = (1 - (R_e/R_{e'})^2) \times 100.$$

This was 27% of the control.

Na-pump inhibitors abolish both adrenaline hyperpolarization and associated conductance decrease When ouabain (0.1 mM) was added to the perfusing solution, or the temperature was lowered (8–10°C), adrenaline no longer produced hyperpolarization (Figure 6a,b; Table 1) or the associated increase in resistance (Figure 6a,b). At a moderately low temperature (20°C), adrenaline hyperpolarization was reduced to a 58% (3.8 mV) of that at 34°C, although the resting potential was not altered (Table 1). These results strongly suggest that the adrenaline hyperpolarization, as well as the increased membrane resistance in the rat innervated diaphragm muscle fibre, depend on the activity of the Na-pump.

Adrenaline does not alter $[Na^+]_i$ and $[K^+]_i$ If adrenaline enhances Na-pump activity, the intracellular Na^+ and K^+ activity would be expected to change. In contrast to the previous findings obtained in Krebs solution in other mammalian muscles (Clausen & Flatman, 1977), adrenaline (30 μM) superfused for 20 min did not affect $[Na^+]_i$ or $[K^+]_i$ of the rat diaphragm muscle fibres in K^+-free solution. These were 22.2 ± 3.8 mM (n_m = 5, 9–13 fibres for each muscle) and 62.3 ± 2.7 mM (n_m = 5, 7–14 fibres for each), respectively, before application of adrenaline and 21.3 ± 2.7 mM (n_m = 5, 7–13 fibres for each muscle) and 60.4 ± 2.9 mM (n_m = 5, 7–18 fibres for each), respectively, after application. These results conform to the findings that adrenaline (30 μM) affected neither the peak amplitude of the action potential (control; 37.9 ± 0.9 mV, n_f = 44: adrenaline applied for 7–40 min; 36.7 ± 0.7 mV, n_f = 51), which is an indication of the Na^+ gradient across the membrane (Hodgkin & Katz, 1949), nor the resting potential at a high $[K^+]_o$ which should be almost totally generated by the K^+-diffusion potential (Figure 4).

Lack of involvement of Cl^- in the mechanism of adrenaline hyperpolarization The removal of most of the Cl^- from the K^+-free solution did not affect the adrenaline hyperpolarization (Figure 6c, Table 1); the accompanying increase in the effective resistance was 18 ± 5%, (n_f = 4), and the fractional decrease in the g_m was 28%. Constant decreases in g_m (Ad) at the normal and low extracellular concentrations of Cl^-, despite a markedly increased input resistance under the latter condition, suggest that adrenaline decreased g_K and g_{Cl} to a similar extent.

A possible change in P_{Na}/P_K during adrenaline hyperpolarization If P_{Na} contributes to the generation of resting potential in the K^+-free solution, a rapid replacement of total Na^+ with non-permeable ions, Tris ion, for a short period (less than 10 min) should

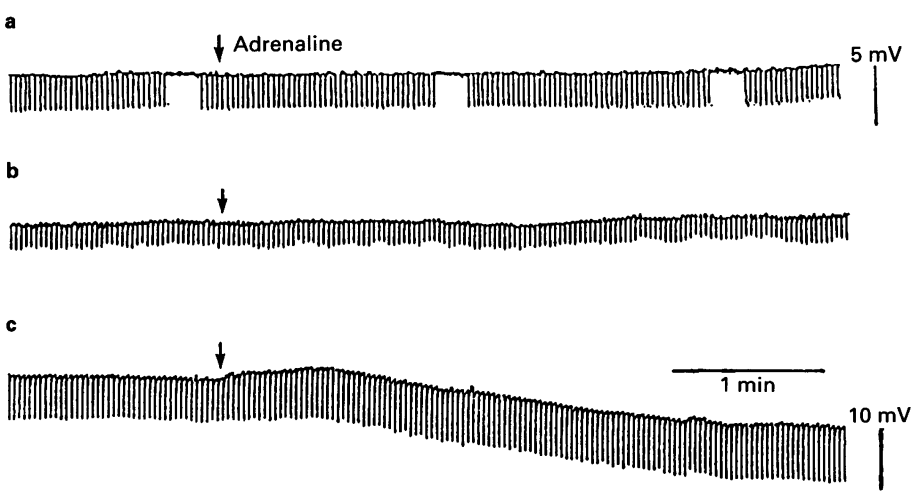

Figure 6 Effects of adrenaline (30 μM) on the resting potential and input resistance at low temperature in a K^+-free solution (8°C; a), in a K^+-free solution containing ouabain (0.1 mM; b) and in a K^+-free, Cl^--deficient (7.5 mM) solution (c). Current pulses (12 nA (a), 15 nA (b) and 10 nA (c) and 50 ms in duration), which are not shown, were applied constantly except for some periods in (a).

hyperpolarize the membrane for a decreased (or reversed) Na^+-concentration gradient. This was in fact observed. When all the Na^+ was replaced with Tris ion in the K^+-free solution, the muscle membrane hyperpolarized (inset records of Figure 7). Accordingly, since both the $[Na^+]_i$ and $[K^+]_i$ would not change during the initial phase of this hyperpolarization (Tris-hyperpolarization), a change in its amplitude may represent a change in the P_{Na}/P_K of the membrane. The amplitude of Tris-hyperpolarization in the K^+-free solution (8.4 ± 0.5 mV, $n_f = 46$) remained constant for more than 2 h (Figure 7A). Thus, the Tris-hyperpolarization may be used to examine a change in P_{Na}/P_K, provided that $[K^+]_i$ and $[Na^+]_i$ are constant.

When adrenaline (30 μM) was superfused onto the muscle, the amplitude of Tris-hyperpolarization was decreased from the control value of 8.4 mV to 5.1 ± 0.4 mV ($n_f = 10$; Figure 7Ba) during the early part (4–31 min) of exposure, but in the latter part (80–130 min) it was progressively enhanced (10.6 ± 0.9 mV, $n_f = 15$; Figure 7Bb). These results which were obtained from the pooled data of 34 muscles were confirmed in other types of experiments, where the effects were examined in the same muscle. The reduction in the amplitude of Tris-hyperpolarization by adrenaline would indicate a decrease in the P_{Na}/P_K, since $[Na^+]_i$ and $[K^+]_i$ remained unchanged during this period (see Discussion). On the other hand, an increased Tris-hyperpolarization during a later period of treatment with adrenaline would imply an increase in the equilibrium potential for K^+ (E_K; presumably because the possible enhancement of Na-pump under this condition would decrease the K^+ concentration close to the extracellular surface of the membrane), or an increased P_{Na}/P_K. Almost constant resting potentials throughout this period would rule out the latter possibility since an increased P_{Na}/P_K would have depolarized the membrane (cf. the previous section). On the other hand, an increase in E_K, arising from a decrease in $[K^+]_o$, would not affect the resting potential significantly, since at a low $[K^+]_o$ the resting potential is less sensitive to a change in $[K^+]_o$ (see Figure 4), but it would augment the hyperpolarization produced by a reduced $[Na^+]_o$.

Discussion

Operation of Na-pump and its electrogenicity in a K^+-free solution

The present study has shown that a high resting membrane potential is maintained in a K^+-free solution for more than three hours. Under this condition, $[K^+]_i$ gradually decreased during the first 60 min and $[Na^+]_i$ similarly increased; subsequently $[K^+]_i$ and $[Na^+]_i$ were maintained at a constant level. Furthermore, applying ouabain or lowering the temperature

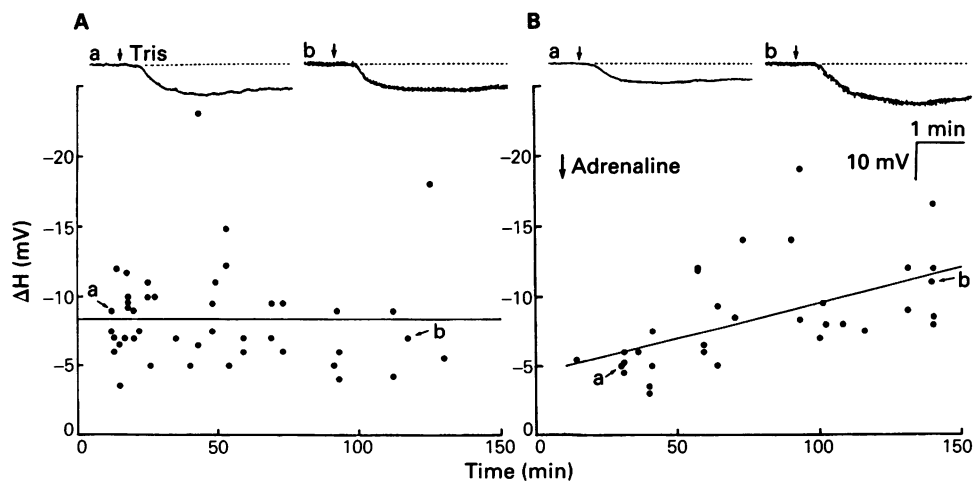

Figure 7 Tris-hyperpolarizations in the absence (A) and presence (B) of adrenaline (30 μM). The inset records (a and b) correspond to the points marked by the same symbol in the graphs. Graphs indicate the relationships between the peak amplitude of Tris-hyperpolarization (ΔH) and the time after perfusion with the K^+-free solution with (B) and without (A) adrenaline. Data in (A) and (B) were obtained from 3 and 5 preparations, respectively, in which Tris-hyperpolarizations were repeatedly induced by a transient replacement (less than 10 min) of all the Na^+ in the K^+-free solution with Tris ions. Correlation coefficients for (A) and (B) are 0.03 and 0.63, respectively, while lines are $y = -0.003x - 8.53$ and $y = -0.05x - 4.98$, respectively.

immediately decreased the resting potential in the K^+-free solution, and produced a further reduction of resting potential and a progressive decrease and increase in $[K^+]_i$ and $[Na^+]_i$, respectively. These results suggest that the Na-pump operates in a K^+-free solution and that it is electrogenic in nature, although its activity is suppressed to a considerable extent, as evidenced by a reduced $[K^+]_i$ and an increased $[Na^+]_i$ and a relatively small drop in membrane potential (10 mV) after the application of ouabain compared with that in normal Krebs solution (20 mV: Bray *et al.*, 1976). This is rather an unexpected finding, since the Na-pump in most cells is markedly depressed or blocked by the removal of K^+ from the bathing fluid (Kerkut & Thomas, 1965; Tomita & Yamamoto, 1971; Koketsu, 1971; Thomas, 1972). Rat soleus muscle fibres (Akaike, 1975) and a certain part of frog muscle fibres (Akiyama & Grundfest, 1971) are exceptions. The Na-pump of the rat diaphragm muscle in a K^+-free solution appears to be activated by K^+ that leaks out of the fibres and accumulates at the outer surface of the membrane. Such an accumulation of K^+ would occur mainly in the transverse tubular systems, since, in diaphragm muscle detubulated by glycerol treatment, the removal of K^+ from the perfusing solution resulted in a progressive decrease in the resting potential after an initial transient hyperpolarization, whereas the resting potential of such fibres in normal Krebs solution was maintained at the normal value (Nohmi, Kuba, Chiba & Murata, unpublished observations).

Den Hertog & Mooij (1976) showed membrane depolarization to occur in the rat diaphragm muscle fibre on removal of K^+ from a Cl^--free solution, and that this depolarizing effect of K^+-removal did not occur in the presence of the normal concentration of Cl^-. This is inconsistent with the present findings as well as with those by Palade & Barch (1977) and Dulhunty (1978), all of which indicate a passive distribution of Cl^- in the rat diaphragm muscle fibre. A notable difference between the experimental conditions of Den Hertog & Mooij (1976) and ours is that their experiments were done at room temperature (20–22°C). Although this might explain the differences in the observations, the exact reasons are unknown.

The ionic mechanism of adrenaline hyperpolarizations

There are at least two possible mechanisms for the adrenaline hyperpolarization in the rat diaphragm muscle fibre; (1) a reduced P_{Na}/P_K of the fibre membrane and (2) an increase in the electrogenic effect of the Na-pump by an increased membrane resistance and/or enhanced activity of the pump.

The blockade of adrenaline hyperpolarization by the procedures that inhibit the Na-pump, e.g. application of ouabain and lowering temperature, would suggest the second mechanism; that is augmentation of the electrogenic effect of the Na-pump by adrenaline, and this is consistent with the earlier conclusions reached using other muscle fibres (Tashiro, 1973; Bressler *et al.*, 1975; Koketsu & Ohta, 1976; Clausen & Flatman, 1977; Kaibara *et al.*, 1982; see also Phillis & Wu, 1981). Evidence obtained in the present study, however, favours the idea that the first mechanism is predominant for the adrenaline hyperpolarization in the rat diaphragm muscle fibres.

Firstly, there was no change in $[Na^+]_i$ and $[K^+]_i$ during adrenaline hyperpolarization. This differs from the findings of Clausen & Flatman (1977), who observed a significant decrease in $[Na^+]_i$ and increase in $[K^+]_i$, suggesting the activation of the Na-pump by adrenaline.

Secondly, the lower the $[K^+]_o$, the greater was the adrenaline hyperpolarization; adrenaline hyperpolarization was largest in the K^+-free solution. This does not agree with the observation on the soleus muscle by Tashiro (1973), who observed that the hyperpolarizing effect of adrenaline disappeared in a K^+-free solution, although under such a condition the fibre showed a resting potential of -96 mV which is similar to that seen in the present study. Thus, if adrenaline were to hyperpolarize the membrane of the rat diaphragm muscle fibre by enhancing the Na-pump activity, its effect would have been depressed or blocked in a K^+-free solution, as found in the soleus muscle.

Thirdly, Tris-hyperpolarization induced by the replacement of Na^+ with Tris ion in the perfusing solution was decreased during the initial phase of the adrenaline action. Since the amplitude of Tris-hyperpolarization would represent a relative measure of P_{Na}/P_K (because a component of membrane potential induced by electrochemical potential in a Na^+-free solution would mainly be due to that of K^+, and $[K^+]_i$ and $[Na^+]_i$ would be unchanged during this period), its reduction during adrenaline hyperpolarization suggests a decrease in P_{Na}/P_K by adrenaline. Figure 8 is a schematic representation of the effect of the rapid removal of extracellular Na^+ on membrane potential in a K^+-free solution. The difference between the resting membrane potentials recorded during Tris-hyperpolarizations in the absence (-101.1 mV) and presence (-103.4 mV) of adrenaline indicates the magnitude of adrenaline hyperpolarization, which would be induced in a Na^+-free (or low Na^+), K^+-free solution with constant $[Na^+]_i$ (for the measurements were done immediately after the removal or reduction of $[Na^+]_o$). This adrenaline hyperpolarization (2.3 mV) at zero or lowered $[Na^+]_o$ is much smaller than that (6.6 mV) at the normal $[Na^+]_o$. If adrenaline hyperpolarization were induced solely by the enhancement of the electrogenic effect of the Na-pump, it would have been unchanged or potentiated (since the activity

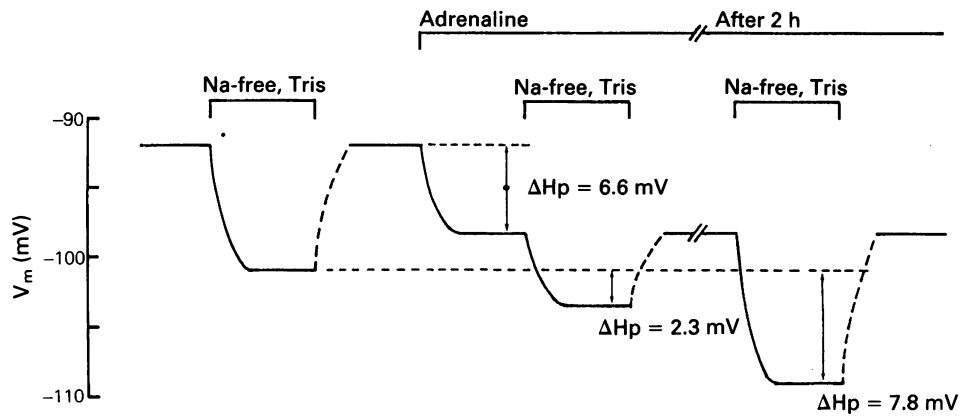

Figure 8 Schematic representation of the effect of the rapid removal (or lowering) of the extracellular Na^+ on the magnitude of the hyperpolarizations (ΔHp) induced by adrenaline in a K^+-free solution. ΔHp was decreased in the Na^+-free, Tris solution during the initial phase of the adrenaline action, but increased in the later period.

of the Na-pump would be enhanced when the thermodynamic load to the pump is reduced by decreasing or reversing the gradient of Na^+ across the membrane: Weer 1984; Gadsby *et al.*, 1985). This expectation is not consistent with the results obtained during the initial phase of the action of adrenaline, but may be relevant to the effects observed after prolonged treatment with adrenaline (see below).

Consequently, consideration of the results obtained in the present study favours the idea that the adrenaline-induced hyperpolarization of the rat diaphragm muscle fibre, seen during the initial phase of adrenaline application, is induced to a large extent by a reduction in P_{Na}/P_K of the membrane. This mechanism resembles that of adrenaline hyperpolarization in the spinal motoneurones (Marshall & Engberg, 1979). An increase in the electrogenic effects of the pump, however, cannot be completely ruled out; it could contribute, to some extent, to the generation of adrenaline hyperpolarization, since the increase in Tris-hyperpolarization, after prolonged treatment with adrenaline, can be interpreted as an enhancement of the Na-pump by adrenaline (see Figure 8). Furthermore, the activation of the Na-pump by adrenaline is enhanced when the muscle is denervated, as evidenced by the large ouabain-sensitive hyperpolarization induced by adrenaline in the denervated rat diaphragm (Bray *et al.*, 1976).

One problem remains to be discussed; why adrenaline hyperpolarization was abolished by the treatment with ouabain or by lowering the temperature? The decreases in the membrane permeabilities to Na^+, K^+ and Cl^- (preferentially for Na^+) by adrenaline were also abolished by procedures that inhibit the Na-pump. Thus, the reduction in the passive ionic permeability of the membrane induced by adrenaline must be associated with (or depend on) the Na-pump activity. It is not known how the operation of the Na-pump is involved in the effect of adrenaline on the Na^+, K^+ and Cl^- channels, although this is an interesting problem.

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References

AKAIKE, N. (1975). Contribution of an electrogenic sodium pump to membrane potential in mammalian skeletal muscle fibres. *J. Physiol.*, **245**, 499–520.

AKIYAMA, T. & GRUNDFEST, H. (1971). The hyperpolarization of frog skeletal muscle fibres induced by removing potassium from the bathing medium. *J. Physiol.*, **217**, 33–60.

BOWMAN, W.C. (1982). Effects of adrenaline activators and inhibitors on the skeletal muscle. In *Handbook of Experimental Pharmacology, Adrenergic activators and inhibitors*, Vol 54, part II, ed. Szekers, L. pp. 47–128, Berlin: Springer-Verlag.

BOWMAN, W.C. & RAPER, C. (1967). Adrenotropic receptors in skeletal muscle. *A. New York Acad. Sci.*, **139**, 741–753.

BRAY, J.J., HAWKEN, M.J., HUBBARD, J.I., POCKET, S. & WILSON, L. (1976). The membrane potential of rat

diaphragm muscle fibres and the effect of denervation. *J. Physiol.*, **255**, 651–667.

BRESSLER, B.H., PHILLIS, J.W. & KOZACHUK, W. (1975). Noradrenaline stimulation of a membrane pump in frog skeletal muscle. *Eur. J. Pharmac.*, **33**, 201–204.

CLAUSEN, T. & FLATMAN, J.A. (1977). The effect of catecholamines on Na-K transport and membrane potential in rat soleus muscle. *J. Physiol.*, **270**, 383–414.

DEN HERTOG, A. & MOOIJ, J.J.A. (1976). Potassium induced potential changes in rat diaphragm muscle. *Pflügers Arch.* **362**, 69–79.

DULHUNTY, A.F. (1978). The dependence of membrane potential on extracellular chloride concentration in mammalian skeletal muscle fibres. *J. Physiol.*, **276**, 67–82.

FUJIMOTO, M. & KUBOTA, T. (1976). Physicochemical properties of a liquid ion exchanger microelectrode and its application to biological fluids. *Jap. J. Physiol.*, **26**, 631–650.

GADSBY, D.C., KIMURA, J. & NOMA, A. (1985). Voltage dependence of Na/K pump current in isolated heart cells. *Nature*, **315**, 63–65.

HODGKIN, A.L. & HOROWICZ, P. (1959). The influence of potassium and chloride ions on the membrane potential of single muscle fibres. *J. Physiol.*, **148**, 127–160.

HODGKIN, A.L. & KATZ, B. (1949). The effect of sodium ions on the electrical activity of the giant axon of the squid. *J. Physiol.*, **108**, 37–77.

HODGKIN, A.L. & RUSHTON, A.H. (1946). The electrical constants of a crustacean nerve fibre. *Proc. R. Soc. B.* **133**, 444–479.

KAIBARA, K., KOKETSU, K., AKASU, T. & MIYAGAWA, M. (1982). A kinetic analysis of the facilitatory action of adrenaline. *Pflügers Arch.*, **392**, 304–306.

KERKUT, G.A. & THOMAS, R.C. (1965). An electrogenic sodium pump in snail nerve cells. *Comp. Biochem. Physiol.*, **14**, 167–183.

KOKETSU, K. (1971). The electrogenic sodium pump. *Adv. Biophys.*, **2**, 77–112.

KOKETSU, K. & OHTA, Y. (1976). Acceleration of the electrogenic Na pump by adrenaline in frog skeletal muscle fibres. *Life Sci.*, **19**, 1009–1014.

KUBA, K. (1970). Effects of catecholamines on the neuromuscular junction in the rat diaphragm. *J. Physiol.*, **211**, 551–570.

KUBA, K., KUBA, M. & KOKETSU, K. (1978). Adrenaline hyperpolarization in rat diaphragm muscle fibres. *J. Physiol. Soc. Jap.*, **40**, 377–380.

MARSHALL, K.C. & ENGBERG, I. (1979). Reversal potential for noradrenaline-induced hyperpolarization of spinal motoneurons. *Science*, **205**, 422–424.

NOHMI, M. & KUBA, K. (1980). The mechanism of adrenaline hyperpolarization in the rat diaphragm. *J. Physiol. Soc. Jap.*, **42**, 54.

PALADE, P.T. & BARCH, R.L. (1977). Characteristics of the chloride conductance in muscle fibres of the rat diaphragm. *J. gen. Physiol.*, **69**, 325–342.

PHILLIS, J.W. & WU, P.H. (1981). Catecholamines and the sodium pump in excitable cells. *Prog. Neurobiol.*, **17**, 141–181.

RANG, H.P. & RITCHIE, J.M. (1968). On the electrogenic sodium pump in mammalian non-myelinated nerve fibres and its activation by various external cations. *J. Physiol.*, **196**, 183–221.

SJODIN, R.A. (1984). Contributions of electrogenic pumps to resting membrane potentials: the theory of electrogenic potentials. In *Electrogenic Transport: Fundamental Principles and Physiological Implications*, ed. Blaustein, M.P. & Lieberman, M. pp. 105–127, New York: Raven Press.

SOMLYO, A.P. & SOMLYO, A.V. (1969). Pharmacology of exitation-contraction coupling in vascular smooth muscle and in avian slow muscle. *Fedn. Proc.*, **28**, 1634–1642.

STEINER, R.A., OEHME, M., AMMANN, D. & SIMON, W. (1979). Neutral carrier sodium ion-selective microelectrode for intracellular studies. *Analyt. Chem.*, **51**, 351–353.

TASHIRO, N. (1973). Effects of isoprenaline on contractures of directly stimulated fast and slow skeletal muscles of guinea-pig. *Br. J. Pharmac.*, **48**, 121–131.

THOMAS, R.C. (1972). Electrogenic sodium pump in nerve and muscle cells. *Physiol. Rev.*, **52**, 563–594.

TOMITA, T. & YAMAMOTO, T. (1971). Effects of removing the external potassium on the smooth muscle of guinea-pig taenia coli. *J. Physiol.*, **212**, 851–868.

WEER, P.W. (1984). Electrogenic pumps: theoretical and practical considerations. In *Electrogenic Transport: Fundamental Principles and Physiological Implications*, ed. Blaustein, M.P. & Lieberman, M. New York: Raven Press.

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