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IONIC BASIS OF MEMBRANE POTENTIAL AND OF ACETYLCHOLINE-INDUCED CURRENTS IN THE CELL BODY OF THE COCKROACH FAST COXAL DEPRESSOR MOTOR NEURONE

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Summary

The ionic basis of the resting potential and of the response to acetylcholine (ACh) has been investigated in the cell body membrane of the fast coxal depressor motor neurone in the metathoracic ganglion of the cockroach *Periplaneta americana*. By means of ion-sensitive microelectrodes, intracellular concentrations of three ion species were estimated (mmol l^{-1}): $[K^+]_i$, 144±3; $[Na^+]_i$, 9±1; $[Cl^-]_i$, 7±1. The resting potential of continuously superfused cells was −75.6±1.9 mV at 22°C. A change in resting potential of 42.0±2.5 mV accompanied a decade change in $[K^+]_o$. Experiments with (10^{-4} mol l^{-1}) ouabain, Na^+ injection, low temperature (10°C) and non-superfused cells indicated the presence of an electrogenic sodium pump.

Under current-clamp, the cell body membrane was depolarized by sequentially applied, ionophoretic pulses (500 ms duration) of ACh. Under voltage-clamp, such doses of ACh resulted in an inward current which was abolished in low-Na^+ saline. Ion-sensitive electrodes revealed an increase in $[Na^+]_i$ but no change in $[Cl^-]_i$ in response to externally applied ACh. The ACh-induced current–voltage relationship was shifted in a negative direction by low-K^+ saline. The ACh-induced inward current was usually followed by a delayed outward current which reversed at E_K. Low-K^+ saline had the same effect on this outward component as depolarizing the membrane. This suggests that the outward current component is carried by K^+.

The ACh-induced inward current and the delayed outward current were potentiated either when $[Ca^{2+}]_i$ was lowered by injecting the calcium chelator BAPTA or by exposure of the cell to low-Ca^{2+} saline. High-Ca^{2+} saline reduced the inward component of the response and produced a negative shift in the ACh-induced current–voltage relationship. The amplitude of the delayed outward

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current was enhanced initially and then depressed when the cell was exposed to high-Ca^{2+} saline.

The ACh-induced current in this neuronal cell body appears to be carried by Na^+, K^+ and, to a lesser extent, Ca^{2+}. It is suggested that the delayed outward component is due to a combination of (i) Ca^{2+} entering the cell and activating a K^+ conductance and (ii) Na^+ entering the cell and stimulating the Na^+/K^+ pump. Ca^{2+} appears to regulate the permeability of the ACh-receptor-operated ion channel. Reducing the level of intracellular or extracellular Ca^{2+} increases the ACh-induced current. Possible mechanisms are discussed.

Introduction

A neuronal nicotinic acetylcholine (ACh) receptor, for which α-bungarotoxin is a specific, high-affinity blocker, has been characterized in insects using radiolabelled ligand binding to CNS extracts (Sattelle, 1980), electrophysiological studies on identifiable neurones (Harrow *et al.* 1982; Sattelle, 1985) and molecular biology approaches (Marshall *et al.* 1988). The binding site for α-bungarotoxin is present in the CNS of many insect species, including: the cockroach *Periplaneta americana* (Gepner *et al.* 1978; Lummis and Sattelle, 1985); the fruit fly *Drosophila melanogaster* (Schmidt-Nielsen *et al.* 1977) and the locust *Locusta migratoria* (Breer, 1981). For *Periplaneta*, there is evidence that this binding site is a constituent of functional postsynaptic cholinergic receptors at synapses in the sixth abdominal ganglion between cercal mechanosensory neurones and giant interneurones 2 and 3 (GI2, GI3) (Sattelle *et al.* 1983), and at synapses in the metathoracic ganglion between trochanteral hair-plate mechanosensory neurones and the slow coxal depressor motor neurone (D_s) (Carr and Fourtner, 1980). Cell body (extrasynaptic) membranes of GI2 (Harrow and Sattelle, 1983) and the fast coxal depressor motor neurone (D_f) (David and Sattelle, 1984) also contain neuronal nicotinic ACh receptors sensitive to α-bungarotoxin, and the peripheral location of these identifiable neurones permits the application of voltage-clamp techniques.

Little is known of the ion channel gated by the nicotinic receptor in insects. A role for Na^+ in mediating the depolarizing response to ACh of unidentified cells in the dorsal midline of the sixth abdominal ganglion of the cockroach *Periplaneta americana* has been proposed, based on the substantial reduction of ACh-induced depolarizations in Na^+-free saline (Kerkut *et al.* 1969). Preliminary experiments on the voltage-clamped cell body of motor neurone D_f of the cockroach metathoracic ganglion showed that the ACh-induced inward current was reversibly blocked in Na^+-free saline (Harrow *et al.* 1982). However, reversal potentials (E_{ACh}) for ACh-induced responses of cockroach central neurones, determined by extrapolation, depart from those expected for a pure Na^+ current. Examples include: -35 to -40 mV for the cell bodies of motor neurone D_f (David and Pitman, 1982) and for unidentified cell bodies in the dorsal midline of the sixth abdominal (A6) ganglion (Pitman and Kerkut, 1970); approximately -35 mV for synaptic membranes of unidentified giant interneurones also in the A6 ganglion

(Callec, 1974); and approximately −40 mV for the cell body of embryonic giant interneurone 2 (Blagburn *et al.* 1985). However, embryonic dorsal unpaired median (DUM) neurones of the grasshopper (*Schistocerca nitens*) show reversal potentials in excess of +20 mV (Goodman and Spitzer, 1980).

In the present study, the ionic basis of the resting potential and of ACh-induced current in the cell body of the fast coxal depressor motor neurone (D_f) in the cockroach *Periplaneta americana* has been investigated, using voltage-clamp techniques and ion-sensitive microelectrodes.

Materials and methods

Adult male cockroaches (*Periplaneta americana*) reared at 27°C were used throughout this investigation. The cell body of the cockroach fast coxal depressor motor neurone (D_f) was visually located in isolated, desheathed metathoracic ganglia. The composition of normal saline was (in mmol l^{-1}): NaCl, 214.0; KCl, 3.1; $CaCl_2$, 9.0; sucrose, 50.0; Tes, 10.0 (pH 7.2, adjusted with 1.0 mol l^{-1} NaOH, or 1.0 mol l^{-1} KOH in the case of sodium-free saline). For salines in which the concentrations of either KCl or $CaCl_2$ were varied, iso-osmolarity was maintained by adjusting the concentration of NaCl. To make low-Na^+ or zero-Na^+ saline, NaCl was substituted by either sucrose or Tris–HCl. The desheathed ganglion was mounted under normal saline in a Perspex experimental chamber (volume 0.5 ml) and the cell body was impaled by two microelectrodes filled with 2.0 mol l^{-1} potassium acetate. In voltage-clamp studies the clamp current was recorded directly from a voltage-clamp amplifier (model 3500, Dagan). Electrodes of resistance 10–15 MΩ were used for current injection, and the gain of the clamp was set between 4×10^3 and 6×10^3. This provided voltage control to within ±0.2 mV determined by a second, independent voltage electrode, even during the application of large concentrations (10^{-3} mol l^{-1}) of ACh. Following electrode impalement, cells were bathed in saline, either circulated by a jet delivering a 95 % O_2, 5 % CO_2 mixture positioned at an oblique angle above the surface, which resulted in a relatively slow movement of saline past the preparation (termed stationary, oxygenated saline in the text), or continuously perfused at a rate of 2.0 ml min^{-1}. The temperature of the saline was monitored by means of a thermocouple placed in the bath. Cells were left for 60 min before experimentation to allow equilibration to these conditions. ACh was applied ionophoretically to the surface of the cell body from micropipettes filled with a 1.0 mol l^{-1} solution of the chloride salt. A retaining current of 45 nA was used to prevent leakage of the transmitter from the ionophoretic pipette.

Ion-sensitive microelectrodes prepared from acid-cleaned, thin-walled glass (GC 150T Clark Electromedical, UK) coated with tri-*n*-butyl-chlorosilane (Phase Separations Ltd, UK) were normally filled with 1.0 mol l^{-1} KCl. The tip was placed in a droplet of the appropriate ion-sensitive sensor. A syringe was used to draw the sensor into the electrode tip (see Rink, 1981). For K^+-sensitive electrodes, the sensor was either the 'classical' liquid ion exchanger (Corning

477317), or the valinomycin-based, neutral ion carrier (Laming and Djamgoz, 1983; Oehme and Simon, 1976). For Na^+ detection, electrodes contained the Na^+-sensitive neutral ion carrier ETH227 (Steiner *et al.* 1979), and in this case the remainder of the electrode was filled with 1.0 mol l^{-1} NaCl. The Corning liquid ion exchanger 477315 was employed in Cl^--sensitive electrodes. Calibration solutions for Na^+-sensitive and K^+-sensitive electrodes were normal saline, with $CaCl_2$ excluded and 1.0 mmol l^{-1} EGTA added, in which the activities of Na^+ and K^+ were varied (at constant ionic strength). The electrodes had the following slopes for a 10-fold change in the concentration range of the relevant ion species: Corning 477317, slope=50–54 mV ([K^+], range 100–300 mmol l^{-1}); valinomycin-based neutral ion carrier, slope=50–53 mV ([K^+], range 100–200 mmol l^{-1}); Na^+, slope=33–43 mV ([Na^+], range 2–10 mmol l^{-1}); Cl^-, slope=43–50 mV ([Cl^-], range 1–10 mmol l^{-1}). The potential recordings from each preparation were converted to ionic activities by direct reference to electrode calibration graphs. These ionic activities have been converted to concentrations in the text by assuming an activity coefficient of 0.71 for Na^+, K^+ and Cl^-

For intracellular injection of the calcium chelator 1,2-bis-(2-aminophenoxy)ethane *N*,*N*,*N*′,*N*′-tetra-acetic acid (BAPTA), microelectrodes were filled with a 0.1 mol l^{-1} solution of the tetrapotassium salt. Current pulses (1 Hz, 500 ms) sufficient to hyperpolarize the cell by 20–40 mV were passed through the electrode. The injection was continued until calcium-dependent action potentials were induced in response to depolarizing pulses.

Results

Ion activities and resting potential in an insect motor neurone cell body

Following penetration of the cell body by an ion-sensitive electrode (recorded with reference to a previously inserted acetate-filled microelectrode), the recording was allowed to stabilize. This required 1–5 min for K^+-sensitive and Cl^--sensitive electrodes and 5–20 min for Na^+-sensitive electrodes. Ion activities and calculated equilibrium potentials are summarized in Table 1. Potassium ion activity (a_K^i) was not significantly modified when a reference electrode filled with 2.0 mol l^{-1} sodium acetate was used. The value for a_K^i was much higher using the liquid ion exchanger (477317) compared to values obtained with the valinomycin-based neutral ion carrier. This probably reflects the greater sensitivity of the liquid ion exchanger to interferent ions such as quaternary ammonium (Oehme and Simon, 1976).

For 52 cells maintained in stationary, oxygenated saline, a mean resting potential of -63.0 ± 0.94 mV (mean±S.E.M.) was obtained. Following superfusion in normal saline at 2.0 ml min^{-1} for 60 min, a much higher mean resting potential (-75.6 ± 1.9 mV) was observed (see Fig. 1A). The Na^+/K^+-ATPase inhibitor ouabain (10^{-4} mol l^{-1}) depolarized cells maintained in perfused saline by 10–15 mV (range of values, $N=3$) (see, for example, Fig. 1B). This effect was accompanied by a three- to sixfold reduction in the membrane resistance of cells

Table 1. Intracellular ion activities determined for the fast coxal depressor motor neurone (D_f)

Ion	Activity ($mmol\,l^{-1}$)			Concentration ($mmol\,l^{-1}$)	Equilibrium potential (mV)	Sensor
Potasium	(a^i_K)	179±10	(N=10)	251±14	−111	Corning liquid ion exchanger 477317
Potassium	(a^i_K)	102±2	(N=6)	144±3	−97	Neutral ion carrier (valinomycin-based cocktail)
Sodium	(a^i_{Na})	6±1	(N=6)	9±1	+80	Simon sodium-sensitive resin ETH227
Chloride	(a^i_{Cl})	5±1	(N=6)	7±1	−88	Corning liquid ion exchanger 4772315

Values are mean±S.E.M

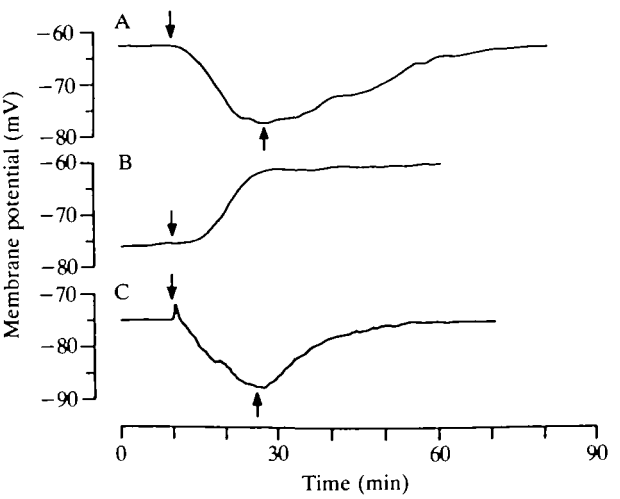

Fig. 1. Evidence for an electrogenic component to the resting potential of the cell body of motor neurone D_f. (A) Effects of saline flow on the resting potential. The initial resting potential was −60 mV in stationary, oxygenated saline. Arrows denote the onset (downward) and termination (upward) of perfusion. The perfusion rate was $2.0\,ml\,min^{-1}$. (B) Effects of ouabain ($10^{-4}\,mol\,l^{-1}$) on the resting potential. Arrow indicates the addition of ouabain, which was present thereafter in the record shown. (C) Effects of sodium injection on the resting potential. The cell was penetrated by a coarse microelectrode containing $1.0\,mol\,l^{-1}$ sodium acetate (first arrow). This electrode was removed after the membrane potential had reached a steady state (second arrow).

voltage-clamped at the original resting potential. Conversely, the membrane resistance of cells maintained in stationary saline was increased by up to threefold following perfusion at a rate of 2.0 ml min^{-1} (see Fig. 6). Ouabain (10^{-4} mol l^{-1}) was without effect on cells maintained in stationary saline. When cells continuously bathed in normal saline at 16°C were cooled to 10°C the membrane depolarized by approximately 10 mV and recovered as the temperature was returned to the initial value. Two cells were injected with Na^+ by diffusion from a relatively coarse microelectrode containing 1.0 mol l^{-1} sodium acetate. In both cases a 10–15 mV hyperpolarization of the cell membrane was noted (Fig. 1C). The simplest interpretation of these findings is that an electrogenic sodium pump contributes to the resting potential of neurones maintained under the experimental conditions described. Since this cell appears to be normally permeable to Na^+ (see below), the hyperpolarization resulting from the injection of Na^+ could also be attributable to a reduction in the transmembrane sodium gradient.

Raising the external potassium concentration depolarized the cell. Following exposures to $[K^+]_o$ in the range 0.31–100 mmol l^{-1}, the resting potential returned to normal when the cell was again bathed in normal saline containing 3.1 mmol l^{-1} K^+. The relationship between the resting potential and $\log[K^+]_o$ is shown for a single cell (Fig. 2). For the same cell, a_K^i was monitored and did not vary significantly for $[K^+]_o$ values up to 100 mmol l^{-1}. The resting potential, if determined exclusively by K^+, would be expected to follow the Nernst equation, yielding a 58 mV change for a decade change in $[K^+]_o$. The observed change (between 10 and 100 mmol l^{-1} K^+) was 42.0±2.5 mV (mean±S.E.M., N=6). The resting potential amplitude was less negative than the apparent equilibrium potential for K^+ (E_K=−97 mV, see Table 1) and exposure to Na^+-free saline

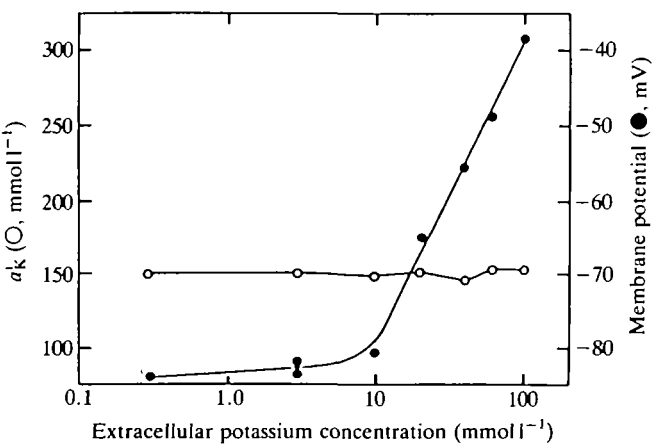

Fig. 2. Potassium-dependence of the resting potential. The dependence on $[K^+]_o$ of the resting potential is shown (●). Intracellular potassium a_K^i (mmol l^{-1}, ○) was monitored at several values of $[K^+]_o$ using a valinomycin-based neutral ion carrier as sensor. Data shown are for a single cell. From experiments on six cells, the mean membrane potential change for an increase in $[K^+]_o$ from 10.0 to 100.0 mmol l^{-1} was −42.0±2.5 mV.

containing an equimolar substitution of Tris ions resulted in an 8–15 mV hyperpolarization accompanied by a 4–6 mmol l^{-1} fall in $[Na^+]_i$. These results are consistent with a resting Na^+ permeability of the cell body membrane.

The resting potential was also dependent on $[Ca^{2+}]_o$. In saline containing 27 mmol l^{-1} calcium (three times the normal level), the membrane was hyperpolarized by 8–12 mV, and the input resistance was reduced by a factor of 5–6, presumably due to the opening of calcium-activated potassium channels (I_C). In Ca^{2+}-free salines either containing no divalent cations or with L-lysine substituted, the membrane depolarized and a drop in input resistance was observed. These effects were probably the result of an increase in sodium permeability (Frankenhaeuser and Hodgkin, 1957; Tomita and Watanabe, 1973).

Response to acetylcholine (ACh) and its dependence on $[Na^+]_o$

Acetylcholine was applied by ionophoresis to the cell body of motor neurone D_f under current clamp. When applying successive doses of ACh, a 2 min interval between each application was allowed to prevent desensitization. ACh produced a depolarizing response which increased both with increasing dose and on hyperpolarizing the membrane. Steady-state recordings at membrane potentials more positive than −50 mV could not be obtained owing to the large increase in K^+ conductance which resulted from the opening of voltage-dependent, Ca^{2+}-mediated K^+ channels (Thomas, 1984). Since the ACh-induced response is dependent on $[Ca^{2+}]_i$ (see below), analysis was confined to membrane potentials more negative than −50 mV. With the cell body voltage-clamped close to the resting potential, ionophoretically applied ACh resulted in an inward current which increased in amplitude with membrane hyperpolarization (see Figs 4, 5). This current appeared to be carried largely by Na^+ for three main reasons. First, during application of ACh, a Na^+-sensitive microelectrode located inside a voltage-clamped cell recorded an increase in a^i_{Na}. In the example shown in Fig. 3B, the peak increase in a^i_{Na} was 6.2 mmol l^{-1}. The peak amplitude of the inward current just preceding the peak increase in $[Na^+]_i$ was 20 nA (Fig. 3A). The observed ACh-induced increase in $[Na^+]_i$ was necessarily highly localized, since it related only to the vicinity (unknown) of the electrode tip within the cell. Second, when NaCl in the saline was replaced by either Tris–chloride or sucrose, both the ACh-induced current (Fig. 3E–G) and the increase in $[Na^+]_i$ were abolished. Third, no change in a^i_{Cl} was detected with a Cl^--sensitive microelectrode during large currents resulting from bath application of 10^{-3} mol l^{-1} ACh (Fig. 3C,D).

Effects of $[K^+]_o$

The inward current induced by ACh was dependent on the potassium concentration in the perfusing saline. The current was increased when the potassium concentration was lowered to 0.31 mmol l^{-1}, and the magnitude of this effect was unchanged over a range of holding potentials (Fig. 4).

The ACh-induced current sometimes decayed with a single exponential from its peak to the original value, but usually the recovery appeared to consist of two

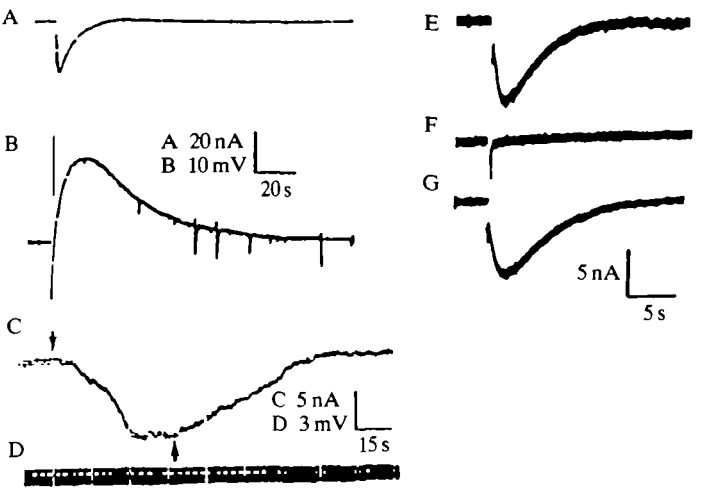

Fig. 3. (A,B) Simultaneous recordings of (A) the current induced by ionophoretically applied acetylcholine (ACh), and (B) intracellular sodium activity a^{i}_{Na}. (C,D) Simultaneous recordings of (C) the current induced by bath-applied ACh (10^{-3} mol l^{-1}) and (D) intracellular chloride activity a^{i}_{Cl}. Arrows show the times at which ACh application began (downwards) and the point at which the cell was again bathed in normal saline (upward). (E,F,G) The effects on the ACh-induced current of Na^{+}-free saline: (E) normal saline; (F) Na^{+}-free saline (Tris-substituted) 5 min; (G) following a 60 min wash in normal saline.

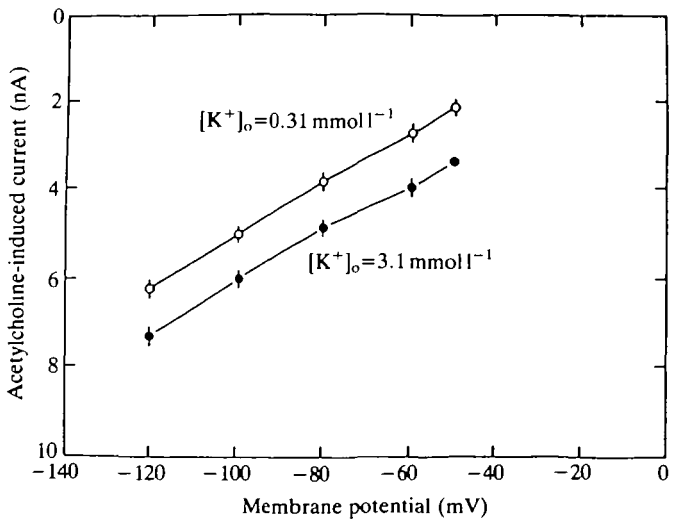

Fig. 4. Dependence on potassium of the ACh-induced current. The relationship between peak ACh-induced inward current and membrane potential in normal (3.1 mmol l^{-1} K^{+}) saline (●) and low-potassium (0.31 mmol l^{-1}) saline (○). Data show mean±S.E.M. for six experiments. The data were normalized to give the same current at −50 mV for each potassium concentration.

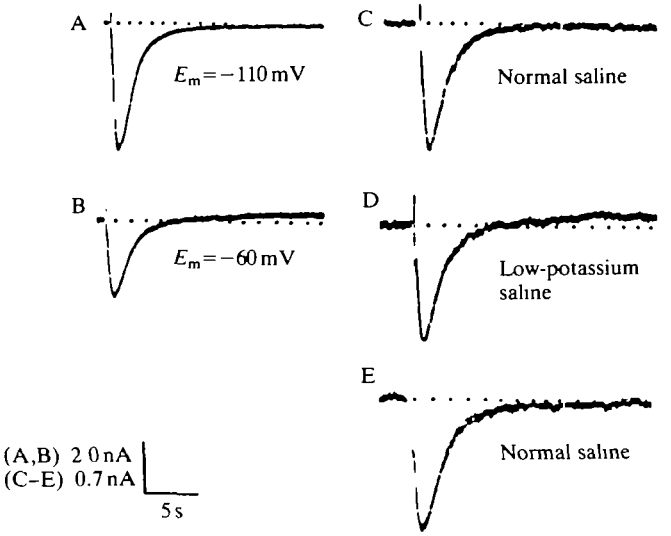

Fig. 5. Response to ionophoretically applied ACh showing a large inward current and a smaller, delayed outward current. This delayed current was inwardly directed at a membrane potential (E_m) of −110 mV (A) and outwardly directed at −60 mV (B). The dependence of the delayed component on $[K^+]_o$ for a cell clamped at −78 mV is also shown: (C) normal saline (K^+, 3.1 mmol l^{-1}); (D) low-K^+ saline (K^+, 0.31 mmol l^{-1}); (E) normal saline, 15 min. The dotted line shows the level of the holding current before the application of ACh.

exponents with the slowest component often overshooting in an outward direction for up to several seconds (Fig. 5B). This delayed outward component was reduced as the membrane potential was moved in a negative direction and disappeared when the cell was clamped at E_K (approximately −97 mV). At more negative holding potentials the delayed component was reversed and became inwardly directed (Fig. 5A). Perfusing the cell with low-K^+ (0.31 mmol l^{-1}) saline had a similar effect on the delayed outward component to that produced by depolarizing the membrane (Fig. 5C–E). These results suggest that the delayed response to ACh is carried by K^+.

Effects of prolonged ACh applications

An increase in the duration of the ACh application, together with an increase in the total ionophoretic dose, could reduce the peak inward current and increase the delayed outward current (Fig. 6A,B). This suggests that the outward current is developing during the inward phase of the response.

The electrogenic sodium pump could be stimulated to some extent by the rise in $[Na^+]_i$ resulting from ACh application (cf. Figs 1, 3). Since the D_f neurone cell body possesses a high resting permeability to K^+, any increase in $[K^+]_i$ would result in an increase in outward current. The after-hyperpolarization following application of ACh to smooth muscle has been attributed entirely to such a

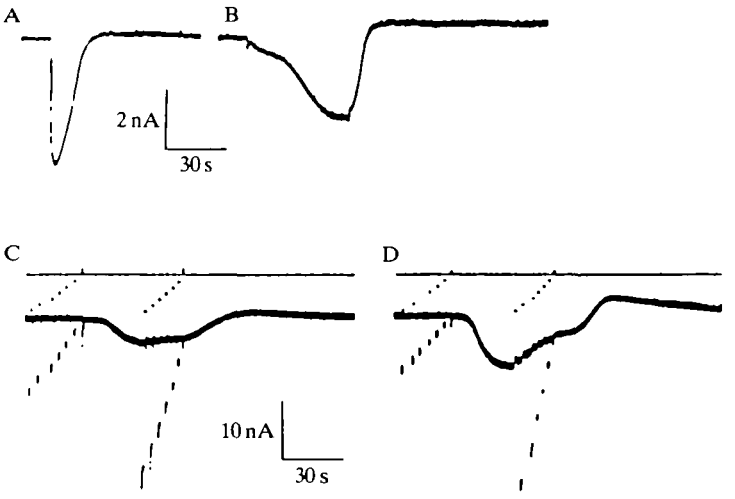

Fig. 6. Current responses to prolonged doses of ACh. (A) Ionophoretic dose 400 nA, 500 ms; (B) ionophoretic dose 50 nA, 48 s (same preparation as A). Holding potential −70 mV; vertical scale bar 2 nA. (C) Ionophoretic dose 45 nA, 48 s. Cell in stationary, oxygenated saline. (D) Same dose as C, but with the cell in saline perfused at 2 ml min^{-1}. (C,D) Upper traces, voltage recordings; lower traces, current recordings. Holding potential −60 mV. Voltage jumps; 500 ms hyperpolarizing pulses in 10 mV steps both before and during ACh application.

mechanism (Bolton, 1973). Both the ACh-induced inward current and the delayed outward current were considerably lower in stationary oxygenated saline (Fig. 6C) than under continuous perfusion (Fig. 6D). However, stimulating the sodium pump could increase the transmembrane gradient not only for K^+ but also for Na^+ and probably Ca^{2+} through a Na^+/Ca^{2+} exchange process (see Discussion).

To determine the effects of prolonged agonist doses over a range of membrane potentials, experiments were performed in which ACh was applied continuously by ionophoresis until a steady-state plateau response was obtained (usually after 10–30 s). Both before the application of ACh, and during the steady-state response, a series of 500 ms rectangular command pulses was applied by means of which the membrane potential was jumped from −60 to −120 mV in 10 mV steps (e.g. Fig. 6C,D). The ACh-induced current at each membrane potential was determined by subtracting the control current (determined in normal saline) from the current in the presence of ACh (cf. David and Sattelle, 1984). The ACh-induced currents obtained using such steady-state applications appear to have a more negative equilibrium potential than currents induced by single 500 ms pulses of ACh with the cell clamped for 2 min at each potential (Fig. 7).

No evidence of an outward current was obtained during prolonged ACh applications in low-Na^+ (50 mmol l^{-1}) saline in which Tris was substituted for Na^+. Indeed, the inward component of the response was suppressed to well below the level expected from the theoretical change in E_{Na}. This effect was not due to Tris

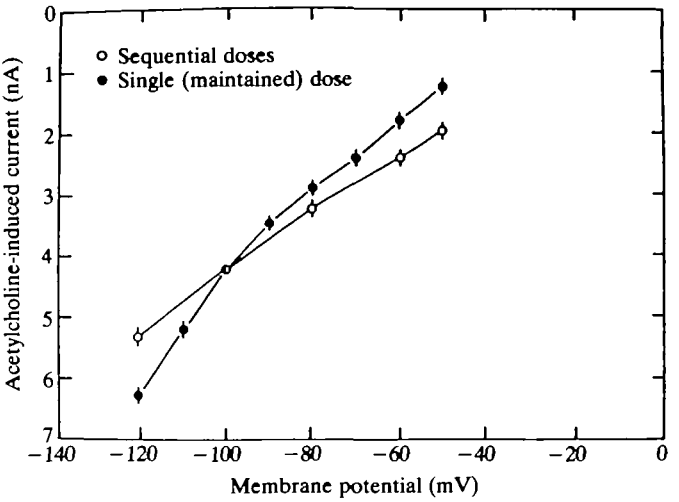

Fig. 7. The relationship between ACh-induced current and membrane potential recorded under voltage-clamp. Open circles show the response to brief (500 ms) pulses of ACh with the cells clamped for 2 min at each membrane potential. Data represent mean±S.E.M. for six preparations. Closed circles show the response to prolonged (20–40s) pulses of ACh with the cells clamped at −50 mV and jumped for 1 s to each membrane potential. Data represent mean±S.E.M. for 10 preparations and were normalized to give the same current at −100 mV.

binding to sites within the ACh-activated channel (Fiekers and Henderson, 1982), since similar results were produced by low-Na^+ (50 mmol l^{-1}) saline containing sucrose as a sodium substitute (Fig. 8). After washing in normal saline for 30 min, the ACh-induced inward current was only partially restored and the delayed outward current was much reduced. Since the ACh-induced current was suppressed by intracellular Ca^{2+} (see below), the observed attenuation of the response in low-Na^+ saline could be explained by a reduced Na^+/Ca^{2+} exchange mechanism leading to an increase in $[Ca^{2+}]_i$.

Effects of changing $[Ca^{2+}]_i$ and $[Ca^{2+}]_o$

The effect of a reduction in $[Ca^{2+}]_i$ upon the ACh-induced current was examined by injecting the calcium chelator BAPTA. Depolarization of the cell body membrane of motor neurone D_f has been shown to increase its permeability to Ca^{2+} which in turn activates a K^+ conductance (I_C) (Thomas, 1984). Although this neurone is normally electrically inexcitable, membrane depolarization produces Ca^{2+}-dependent action potentials when I_C is eliminated by lowering $[Ca^{2+}]_i$ (Pitman, 1979). In the present experiments, BAPTA was injected until membrane depolarization produced all-or-none action potentials. These spikes were blocked by externally applied Cd^{2+} (1.0 mmol l^{-1}) and were therefore probably the result of Ca^{2+} entry. Following BAPTA injection, the ACh-induced inward current was increased over a range of membrane potentials (−60 to −120 mV) (Fig. 9).

Potentiation of the inward current component was detected at membrane potentials more negative than E_K (where the potassium current would be inwardly directed), and was therefore unlikely to result from the elimination of I_C. The delayed outward current was reduced by BAPTA injection but was not eliminated.

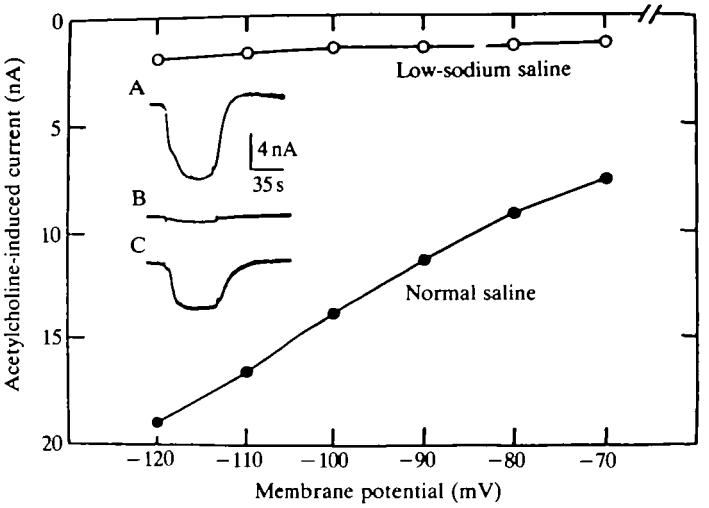

Fig. 8. Effects of low-Na^+ saline on membrane current in response to prolonged (39 s, 60 nA) ionophoretic doses of ACh. Insets show current: (A) in normal saline; (B) in low-Na^+ (50 mmol l^{-1}), 60 min; (C) following a wash in normal saline, 20 min. Holding potential, −64 mV. Graph shows the ACh-induced current–voltage relationship in normal saline (●) and in low-Na^+ (sucrose-substituted) saline (○).

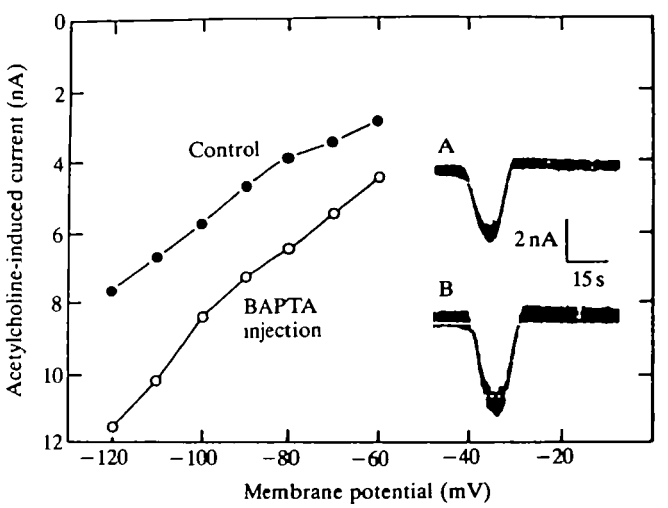

Fig. 9. Effects of intracellular injection of BAPTA on the ACh-induced current. Insets show currents recorded (A) before and (B) after injection of BAPTA. Graph shows the ACh-induced current–voltage relationship for a neurone before (●) and after (○) BAPTA injection.

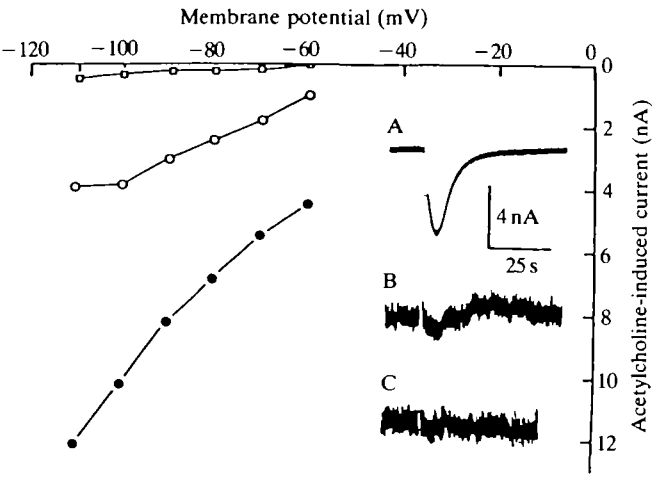

Fig. 10. Effects of high-Ca^{2+} saline on the ACh-induced current. Insets show ACh-induced currents recorded (A) in normal saline; (B) in high-Ca^{2+} (27 mmol l^{-1}) saline, 40 min; (C) in high-Ca^{2+} saline, 55 min. Graph shows the ACh-induced current over a range of membrane potentials in normal saline (●); high-Ca^{2+} saline, 20 min (○); high-Ca^{2+} saline, 50 min (□).

If the observed potentiation of the inward component of the response was due to more Ca^{2+} entering, as a result of an increase in the transmembrane calcium gradient, then raising $[Ca^{2+}]_o$ might be expected to have the same effect on the inward current as BAPTA injection. This was not found to be the case. In high-Ca^{2+} (27 mmol l^{-1}) saline the ACh-induced inward current was rapidly suppressed, the induced outward current was increased in amplitude, and the current–voltage relationship appeared to be shifted in a negative direction (Fig. 10). In this experiment ACh application was without effect after 50 min in high-Ca^{2+} saline over membrane potentials in the range −60 to −110 mV.

To determine the effects of 0 mmol l^{-1} $[Ca^{2+}]_o$, Ca^{2+} in the saline was replaced by the organic cation L-lysine, a poorly permeant ion at the frog end-plate (Dwyer *et al.* 1980). EGTA (1.0 mmol l^{-1}) was added to this saline in an attempt to reduce $[Ca^{2+}]_o$ in the vicinity of the cell membrane to below 10^{-6} mol l^{-1}. Within 30 min in Ca^{2+}-free saline the ACh-induced inward current was considerably increased over the membrane potential range −50 to −120 mV (Fig. 11). The delayed outward current was also potentiated, probably as a result of sodium pump stimulation. However, since it is unlikely that all Ca^{2+} was removed from around the membrane within this period (Thomas, 1984), some contribution from I_C can be expected.

Discussion

By application of ion-sensitive electrodes to the cell body of the fast coxal depressor motor neurone (D_f) of the cockroach (*Periplaneta americana*), the

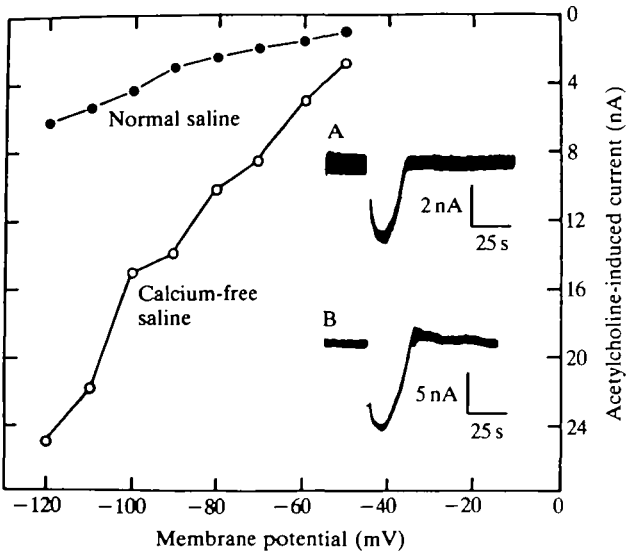

Fig. 11. Effects of zero-Ca^{2+} saline on the ACh-induced current. Insets show ACh-induced current recorded in (A) normal saline, and (B) zero-Ca^{2+}, 30 min. Graph shows ACh-induced current over a range of membrane potentials in normal saline (●), and in zero-Ca^{2+} saline (○), 90 min.

following intracellular ion concentrations are estimated (in mmol l^{-1}): Na^+, 9±1; K^+, 144±3; Cl^-, 7±1. In stationary, oxygenated saline a mean resting potential of −63.0±0.9 mV is recorded. Resting potential is largely maintained by the membrane permeability to K^+ and to a lesser extent to Na^+. The 42 mV membrane potential change accompanying a 10-fold change in $[K^+]_o$ is similar to values reported for giant axons of *Periplaneta americana* (Yamasaki and Narahashi, 1959; Treherne *et al.* 1970), axons of *Manduca sexta* (Pichon *et al.* 1972) and cell bodies of *Carausius morosus* (Treherne and Maddrell, 1967). When motor neurone D_f was bathed in low-Na^+ (50 mmol l^{-1}) or zero-Na^+ saline, $[Na^+]_i$ was reduced and the membrane hyperpolarized, usually to E_K. This hyperpolarization may have been entirely due to the elimination of Na^+ entry. However, since there is evidence to suggest that $[Ca^{2+}]_i$ increases in low-Na^+ saline (see below), I_C may also have increased. There does not appear to be a contribution of Ca^{2+} to the resting potential, but Ca^{2+} is indirectly active in regulating the permeability to Na^+ and K^+. Zero-Ca^{2+} saline results in membrane depolarization and is accompanied by a reduction in input resistance, effects to be expected from an increase in Na^+ permeability. Increasing $[Ca^{2+}]_o$ produces membrane hyperpolarization and a reduction in input resistance, results consistent with an increase in K^+ permeability.

The presence of an electrogenic sodium pump is indicated by the effects of temperature, ouabain and Na^+ injection. The resting potential is increased by approximately 13 mV in continuously perfused saline, presumably due to stimu-

lation of the electrogenic sodium pump by elevation of the partial pressure of oxygen. Similar results have been observed for the neuronal cell bodies of other invertebrates: *Aplysia californica* (Chalazonitis and Gola, 1965); *Helix aspersa* (Moreton, 1969; Kerkut and York, 1969); *Lymnaea stagnalis* (Sattelle, 1974).

Fast responses of neurones to ACh are normally mediated by an increase in membrane permeability to ions such as Na^+, K^+, Cl^- or some combination of these species. To date the most detailed studies on invertebrates have been performed on the cell bodies of molluscan neurones. In *Aplysia californica*, Kehoe (1972*a*,*b*,*c*) characterized an excitatory (depolarizing) response associated with a Na^+ channel ($E_{ACh}=+20\,mV$), and a fast inhibitory (hyperpolarizing) response linked to a Cl^- channel ($E_{ACh}=-80\,mV$). A third type of ACh receptor is linked to a K^+ channel ($E_{ACh}=-90\,mV$). Each exhibits distinct pharmacological characteristics. Both rapid responses were blocked by *d*-tubocurarine, whereas only the depolarizing response was blocked by α-bungarotoxin. In *Planorbarius corneus*, the slow K^+-dependent ACh response is sensitive to muscarinic antagonists such as benzilylcholine mustard (Ger and Zeimal, 1977). In *Helix aspersa*, R. J. Walker and collaborators have obtained similar results to those reported for *Aplysia* and showed decamethonium to be an agonist at ACh receptors linked to Cl^- channels (Yavari *et al.* 1979) on cells such as E4. Decamethonium is without effect on ACh excitation mediated by Na^+ channels in neurone F1.

For motor neurone D_f of the cockroach, a detailed pharmacological profile of the response to ACh has been obtained using a range of nicotinic and muscarinic agents (David and Sattelle, 1984). Of these, nicotinic receptor ligands are particularly active, but none of those tested is observed to block only part of the ACh-induced response. Thus, it appears that a single population of receptors is involved. Estimated reversal potentials (E_{ACh}) are in the range previously reported for insect neurones (0 to $-30\,mV$), though, because of the difficulties of directly reversing the response, all values reported to date are no more than suggestive of the ionic mechanisms involved. In the present study, direct evidence has been obtained for a major contribution of Na^+ to the inward current activated by ACh application. Externally applied ACh also appears to result in an outward flow of K^+ since, when the driving force on K^+ is increased by reducing $[K^+]_o$, the amplitude of the ACh-induced inward current is reduced and the current–voltage relationship shifts in a negative direction. The amplitude of the ACh-induced outward current increases with increasing durations of agonist application and, initially, with increasing $[Ca^{2+}]_o$ and persists for longer than the inward component of the response. This delayed outward component is probably due to a combination of (i) Ca^{2+} entering the cell and activating I_C and (ii) Na^+ entering and stimulating the Na^+/K^+ pump. Such a dual action is consistent with the effects observed following BAPTA injection, where the delayed outward current is significantly reduced but not eliminated. Agonist-induced Ca^{2+}-activated K^+ currents have also been observed in *Aplysia* neurones (Ascher *et al.* 1978) and exocrine glands (Trautmann and Marty, 1984; Findlay, 1984). The extent of the involvement of the sodium pump in the delayed response to ACh is difficult to

measure since inhibiting the sodium pump (by application of 10^{-4} mol l^{-1} ouabain) results in a rapid decline in the Na^+ and K^+ gradients and, as a consequence, probably the Ca^{2+} gradient, owing to a reduced Na^+/Ca^{2+} exchange process. The ACh-induced response would be reduced by the fall in these ion gradients as well as by the secondary effects of an elevated $[Ca^{2+}]_i$. The reverse would be expected following saline perfusion which could account for the observed increase in the ACh-induced inward current (Fig. 6C,D). Thus, ACh appears to induce an increase in membrane permeability to Na^+, Ca^{2+} and K^+ mediated by a population of neuronal nicotinic ACh receptors.

The suppression of the ACh-induced current by high $[Ca^{2+}]_o$ and its potentiation by low $[Ca^{2+}]_o$ (see also Adams, 1977; Adams and Sakmann, 1978; Ascher *et al.* 1978) requires further consideration. Attenuation of the inward component in high-calcium saline is detected at membrane potentials more negative than E_K and is therefore unlikely to result from a calcium-activated potassium current. This attenuation could be due to competition between ions as they pass through the channel (Cohen and Van der Kloot, 1978; Lewis, 1979). Surface charges on the external surface of the membrane would also affect the local concentration of ACh (Van der Kloot and Cohen, 1979). This could explain the potentiation produced by zero-Ca^{2+} saline shown in Fig. 11, since if this is caused by a greater sodium component, E_{ACh} will be shifted in a positive direction.

Alternatively, since this cell is relatively permeable to Ca^{2+}, increasing (or decreasing) $[Ca^{2+}]_o$ would result in both sides of the membrane gaining (or losing) Ca^{2+}. The reduction of the ACh-induced response seen in high $[Ca^{2+}]_o$ may therefore have been the result of an increase in $[Ca^{2+}]_i$. The finding that this suppression is accelerated by prolonged ACh application lends support to this explanation. Alternatively, the ACh receptor might be inactivated by Ca^{2+} binding to an internal receptor site or through some Ca^{2+}-dependent second messenger.

The sensitivity of the ACh-induced current to $[Ca^{2+}]_i$ might explain the dramatic reduction or elimination of the ACh-induced current seen in low-Na^+ or zero-Na^+ saline. A reduction in $[Na^+]_o$ has been shown to produce a rise in $[Ca^{2+}]_i$ in a number of tissues; for example, squid giant axon (Baker *et al.* 1971); guinea pig smooth muscle (Tomita and Watanabe, 1973) and *Helix* neurones (Meech, 1974). Since the resting membrane of motor neurone D_f appears to have a significant permeability to Ca^{2+}, some mechanism must exist by which Ca^{2+} is removed from the cell. One way in which Ca^{2+} could be extracted would be by a Na^+/Ca^{2+} exchange process, the energy for which would be supplied by the electrochemical gradient of Na^+ across the membrane. Reducing $[Na^+]_o$ would therefore be expected to lead to a rise in $[Ca^{2+}]_i$ and consequent suppression of the ACh-induced response. Such a mechanism may also explain the slow recovery of the ACh-induced current upon return to normal-sodium saline, and the reduction in membrane resistance produced by the application of ouabain. In the latter case, the rise in $[Na^+]_i$ would reduce the transmembrane Na^+ gradient, resulting in a rise in $[Ca^{2+}]_i$ and consequent increase in K^+ permeability. The coupling of a rise

in $[Na^+]_i$ to a rise in $[Ca^{2+}]_i$ has been attributed to such an exchange process in a number of tissues (e.g. Blaustein and Nelson, 1982; Deitmer and Schlue, 1983).

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