Journal of Physiology (1988), **399**, *pp.* 519–535
With 9 text-figures
Printed in Great Britain

INDIRECT EFFECTS OF ACETYLCHOLINE ON THE ELECTROGENIC SODIUM PUMP IN BULL-FROG ATRIAL MUSCLE FIBRES

BY H. HASUO*, K. KOKETSU* AND S. MINOTA†

*From the *Department of Physiology, Kurume University School of Medicine, Kurume 830, Japan and the †Department of Physiology, Saga Medical School, Saga 840-01, Japan*

(*Received 4 August 1987*)

SUMMARY

1. Effects of acetylcholine (ACh) on the activity of electrogenic Na^+ pump in bull-frog atrial muscle fibres were examined using the single sucrose-gap voltage clamp technique.

2. In the K^+-free solution, 10 μM-ACh induced a large outward current (ACh-induced current) with an increase in the membrane conductance.

3. The amplitude of the ACh-induced current decreased to 15% of the control 10 min after application of 1 μM-ouabain, suggesting the contribution of electrogenic Na^+ pump to the ACh-induced current. The remaining ACh-induced current was not affected even if the concentration of ouabain was increased ten times.

4. The K^+-activated current induced by an activation of the electrogenic Na^+ pump was suppressed or reversed its direction during the course of the ACh-induced current.

5. The ACh-induced current was completely inhibited by applications of either atropine or barium ions while the K^+-activated current was not affected.

6. Both ouabain-sensitive and -insensitive ACh-induced currents were decreased when the membrane was hyperpolarized and eliminated around −95 mV.

7. The ouabain-sensitive component was decreased by increasing the external K^+ concentration $[K^+]_o$; the proportions of this current to ACh-induced current in 0·5, 0·75, 1 and 2 mM $[K^+]_o$ were 54, 42, 34 and 14%, respectively.

8. The current–voltage (*i–v*) relation obtained in 2 or 4 mM $[K^+]_o$, where the currents carried by Na^+ and Ca^{2+} were blocked by application of 1 μM-TTX and 1 mM-Cd^{2+}, exhibits marked inward-going rectification but does not show a clear N-shaped feature. Ba^{2+} (1 mM) induced an inward current at the holding potential (−80 mV) and eliminated the inward-going rectification of the membrane.

9. These results suggest that the increase in the K^+ permeability by ACh increases the concentration of K^+ immediately outside of the membrane, which in turn stimulates the electrogenic Na^+ pump mechanism. The physiological significance of the action of ACh on the electrogenic Na^+ pump in bull-frog atrium is discussed in relation to the background K^+ current ($I_{K,1}$).

† To whom correspondence should be addressed.

INTRODUCTION

In cardiac muscles the functional importance of the Na^+ pump appears to be greater than that in most other excitable tissues (for review, see Glitsch, 1982; Gadsby, 1984). Since the Na^+–K^+ pump would be electrogenic (Page & Storm, 1965; Glitsch, 1972; Isenberg & Trautwein, 1974; Noma & Irisawa, 1975; Glitsch, Grabowski & Thielen, 1978; Eisner & Lederer, 1980; Daut & Rudel, 1982), it may influence not only the membrane potential but also the frequency and shape of the action potential (Gadsby, 1984). It is, therefore, interesting to know how the electrogenic Na^+ pump activity is modulated physiologically by the action of neurotransmitters, especially catecholamines and ACh, as both types of transmitters are released from nerve terminals innervating the cardiac muscle fibres and regulate the cardiac muscle activities.

The activation of the electrogenic Na^+ pump by catecholamines was suggested quite some time ago by Dudel & Trautwein (1956), Trautwein & Schmidt (1960) and Vassalle & Barnabei (1971). The mechanism underlying the action of adrenaline in bull-frog atrium has been suggested to be an increase in the rate of Na^+ extrusion without affecting both the Na^+/K^+ coupling ratio and the total number of pumping sites (Akasu, Ohta & Koketsu, 1978). Gadsby (1983) showed, however, that β-adrenergic agonists such as isoprenaline increase resting membrane K^+ conductance which may in turn stimulate the Na^+–K^+ pump.

On the other hand, the effect of ACh on the electrogenic Na^+ pump has not been fully investigated. ACh increases the K^+ conductance (Harris & Hutter, 1956; Trautwein & Dudel, 1958; Ten Eick, Nawrath, McDonald & Trautwein, 1976; Giles & Noble, 1976; Garnier, Nargeot, Ojeda & Rougier, 1978; Noma & Trautwein, 1978; Carmeliet & Ramon, 1980; Ojeda, Rougier & Tourneur, 1981; Argibay, Dutey, Ildefonse, Ojeda, Rougier & Tourneur, 1983; Mubagwa & Carmeliet, 1983; Sakmann, Noma & Trautwein, 1983; Soejima & Noma, 1984; Carmeliet & Mubagwa, 1986*a*) and it may secondarily cause an accumulation of K^+ in the outer surface of the membranes (Garnier *et al.* 1978; Colatsky & Tsien, 1979). In the bull-frog isolated heart muscle fibres, the inwardly going rectification channel ($I_{K,1}$ channel) is particularly absent in the pacemaker cells (Giles & Shibata, 1985) and much less present in the atrial cells compared to other preparations (Hume & Giles, 1983). Accordingly, it is expected that in those cells the K^+ accumulation caused by ACh in the extracellular space would be greater than that in other preparations and in turn stimulates indirectly the electrogenic Na^+ pump since the activity of this pump is dependent on the $[K^+]_o$ (Noma & Irisawa, 1974, 1975; Akasu *et al.* 1978; Glitsch *et al.* 1978; Eisner & Lederer, 1980; Gadsby, 1980; Kurachi, Noma & Irisawa, 1981). We examined this possibility in bull-frog atria and showed that ACh indeed accelerates indirectly the electrogenic Na^+ pump, especially in the solution containing less than 1 mM-K^+. The physiological significance of the indirect activation of the electrogenic Na^+ pump by ACh in bull-frog atrium will be discussed in relation to the i–v relationship of $I_{K,1}$ channels. A preliminary communication of some of the results has been published (Minota & Koketsu, 1979).

METHODS

The current and voltage clamp experiments were performed at room temperature (20–23 °C), using a single sucrose-gap technique (Beeler & Reuter, 1970; Akasu *et al.* 1978).

The preparation consisted of thin trabeculae (300–500 μm in diameter, 5–7 mm in length), isolated from the bull-frog (*Rana catesbeiana*) atrium.

The bath was separated into three compartments by two thin rubber membranes with holes through which the preparation was pulled; the middle compartment (2 mm in width) was perfused with sucrose solution. The right- and left-side outer compartments were perfused with the test and Ringer solutions, respectively, and platinum electrodes were placed in both compartments for applying clamp currents using the voltage clamp amplifier (Nihon Kohden CEZ-1100) across the sucrose gap. The membrane potential was measured between two electrodes; one filled with 3 M-KCl (40–50 MΩ) was impaled into a fibre and the other was Ag–AgCl indifferential electrode placed in the test compartment. The changes of both voltage and current were continuously recorded by using a pen-writing recorder (Nihon Kohden RJG-4024, flat response up to 100 Hz) and stored on FM tape (Sony NFR-3515W, 1 kHz). For the construction of the dose–response curve, the results were fitted to the Hill equation using the non-linear least-squares fitting method calculated by the computer (NEC 9801). The complicated equivalent circuit of the muscle and the use of electrode systems may limit the achievement of a complete space clamping (Trautwein, 1973). Accordingly, our results will have to be considered as only semi-quantitative, because the true membrane current is contaminated by an unknown proportion of other current such as leakage current.

The composition of the Ringer solution used was (mM): NaCl, 112; KCl, 2·0; $CaCl_2$, 0·1; $MgCl_2$, 6; $NaHCO_3$, 2·4; and glucose, 2·5; pH 7·4. A lower calcium and higher magnesium solution is used to reduce contraction and facilitate the prolonged maintenance of the microelectrode in individual cells. Under these conditions, the Na^+–Ca^{2+} exchange system which might be electrogenic (Kimura, Noma & Irisawa, 1986; Mechmann & Pott, 1986) should be deactivated or reduced. The solutions containing various concentrations of K^+ were made by substituting NaCl for KCl. The isotonic sucrose solution used for sucrose gap was 240·7 mM.

The drugs used were ACh chloride (Wako, Japan), atropine sulphate (Tokyo Kasei, Japan), ouabain (Merck) and tetrodotoxin (TTX, Sankyo). ACh was used at the concentration of 10 μM in most experiments, since at this concentration the ACh-induced response would be saturated for a longer ACh application (see Fig. 2*C* in Garnier *et al.* 1978).

RESULTS

ACh-induced current in the K^+-free solution

The muscle fibres were first equilibrated in the K^+-free solution for 1 h in order to load Na^+ in the intracellular compartment by inhibiting the Na^+–K^+ pump mechanisms. Under these conditions, the membrane was gradually depolarized and reached a stable potential level of $-46{\cdot}6 \pm 1{\cdot}8$ mV (mean ± S.E.M., $n = 56$). As seen in Fig. 1*A*, 10 μM-ACh when applied to the atrial muscle fibres caused a large membrane hyperpolarization. The peak amplitude of this hyperpolarization, which occurred within 30 s after application of ACh, was $24{\cdot}8 \pm 2{\cdot}9$ mV ($n = 14$). When the membrane was clamped at the resting potential by using the voltage clamp circuit, a large outward current was generated in the presence of ACh (Fig. 1*B*). The peak amplitude of ACh-induced current recorded at -50 mV was $1{\cdot}60 \pm 0{\cdot}15$ μA ($n = 28$). The increase of membrane conductance was observed during ACh application when small brief command pulses (500 ms in duration, 8 mV in amplitude) were applied at an interval of 4 s through the membrane (Fig. 1*B*). Figure 1*C* shows the relative changes in the membrane conductance before, during and after application of ACh. The relative increase of conductance 30 s after application of ACh was $76{\cdot}9 \pm 9{\cdot}3$ %

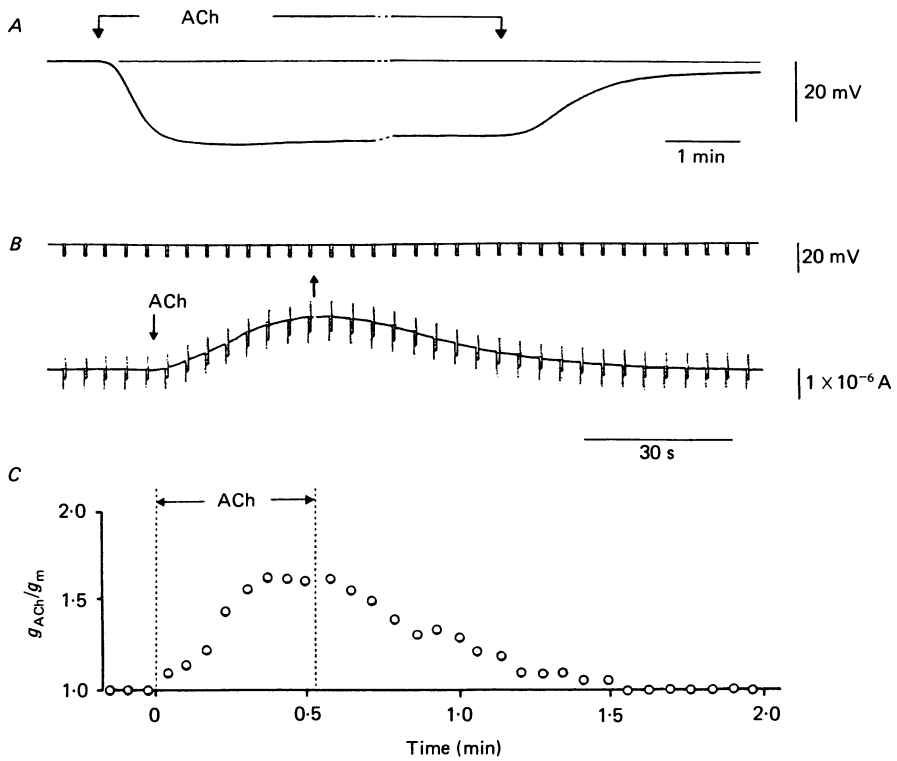

Fig. 1. Effects of 10 μM-ACh on the atrial muscle fibres. *A*, ACh-induced hyperpolarization in the K^+-free solution. Ten micromolar ACh was applied for 20 min. The dotted line in the record showed a period of 15 min. The resting membrane potential was −49 mV. *B*, ACh-induced outward current obtained by the voltage clamp technique and the changes in the membrane conductance. Upper trace, voltage recording; lower trace, membrane current. The holding potential was −50 mV. ACh was applied for 30 s and then washed out as indicated by downward and upward arrows, respectively. In order to measure the changes in the membrane conductance, hyperpolarizing pulses (500 ms in duration, 8 mV in amplitude) were continuously applied at an interval of 4 s. *C*, the relative changes in the membrane conductance by ACh (g_{ACh}) were calculated from the data in *B* and plotted against time. The mean membrane conductance (g_m) before application of ACh was normalized as 1·0, $n = 14$.

($n = 14$). This value is similar to that recorded in the solution containing 2 mM-K^+ (Giles & Noble, 1976).

The effect of ouabain on ACh-induced current

It is widely accepted that ACh increases the K^+ conductance of the membrane of cardiac muscle fibres by activating the muscarinic receptor–channel complex. The membrane hyperpolarization caused by ACh would, therefore, be explained simply by the acceleration of K^+ conductance. It was, however, found that the ACh-induced current in the K^+-free solution was greatly sensitive to the cardiac glycoside, ouabain, (Fig. 2). The amplitude of the ACh-induced current reduced to 15·7 ± 4·8% ($n = 9$) of the control within 15 min after application of 1 μM-ouabain. This effect of ouabain on ACh-induced current was partially reversed 30 min after its

withdrawal. The remaining part of ACh-induced current in ouabain was not changed even when the concentration of ouabain was raised to 10 μM. The membrane conductance did not alter appreciably in the presence of ouabain. These results indicate that the ACh-induced current consists of two different components, namely ouabain-sensitive and ouabain-insensitive ones.

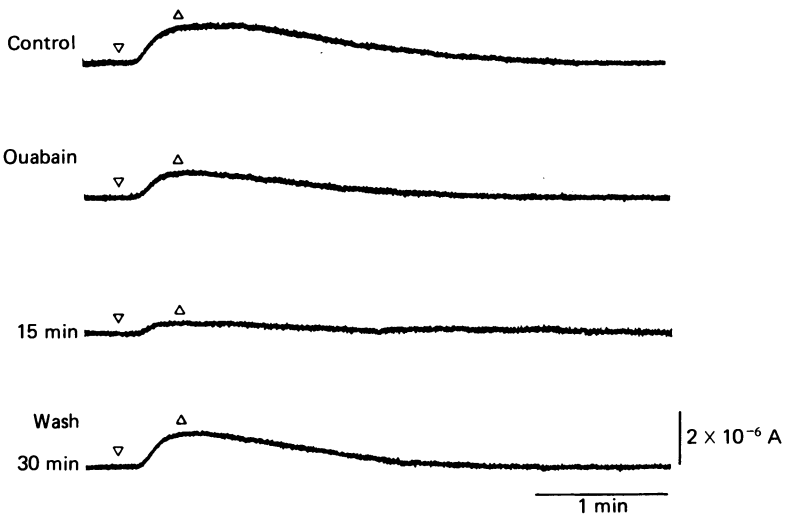

Fig. 2. Effect of 1 μM-ouabain on the ACh-induced current in the K^+-free solution. Ten micromolar ACh was applied between downward and upward arrow-heads as indicated in each trace. The holding potential was −50 mV.

Comparison between ACh-induced current and K^+-activated current

The reduction of the ACh-induced current by ouabain raises the possibility that the ouabain-sensitive component may represent an increase in the electrogenic Na^+ pump activity caused by ACh, since ouabain is known to be a specific inhibitor of the Na^+–K^+ pump mechanism (for review, see Akera & Brody, 1982). In order to examine this possibility, we studied the nature of ACh-induced current as compared with that of the K^+-activated current, which is the membrane outward current produced mainly by an activation of the electrogenic Na^+ pump when the perfusate is changed from the K^+-free to Ringer (2 mM-K^+) solutions (Fig. 7 and see also Noma & Irisawa, 1974, 1975; Akasu *et al.* 1978; Gadsby, 1980; Kurachi *et al.* 1981; Shibata, Momose & Giles, 1983).

As seen in Fig. 3, the large outward current was induced when the muscle fibres were perfused briefly with the 2 mM-K^+-containing solution after exposure to zero $[K^+]_o$. The peak amplitude of the K^+-activated current at −50 mV was $1{\cdot}51 \pm 0{\cdot}13$ μA ($n = 32$). The shape of K^+-activated current varied in different cells examined. One type of response reached peak value and then gradually declined (Fig. 3*A*), whereas the other showed no reduction of the amplitude (Fig. 3*B*). During the perfusion of K^+, the electrogenic Na^+ pump current (outward direction) and the current caused by a passive movement of K^+ (inward direction) occurred

simultaneously. The inward current caused by a passive movement of K^+ could be observed after inhibition of pump component in the presence of ouabain. This was shown in Fig. 7. Therefore, the shape of K^+-activated current would be determined by the rate of change of two current components with time. The K^+-activated current was reduced (Fig. 3*A*, twelve cells), or eliminated (five cells) and even reversed its direction (Fig. 3*B*, five cells) in different cells during the generation of ACh-induced current. These results strongly suggest that the activity of the electrogenic Na^+ pump was already increased in the presence of ACh.

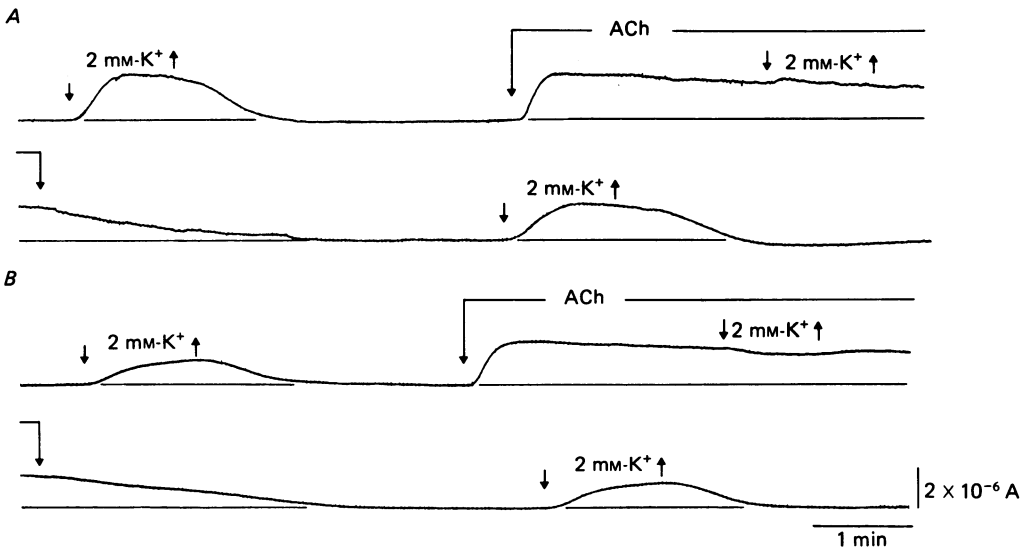

Fig. 3. Comparison between the ACh-induced and K^+-activated currents. The K^+-activated current was induced when the perfusate was changed from the K^+-free solution to a solution containing 2 mM-K^+. Note, the K^+-activated current was reduced (*A*) or reversed its direction (*B*) during applications of 10 μM-ACh. *A* and *B* were obtained from different fibres. Upper and lower traces in *A* and *B* are continuous tracings. The holding potentials in *A* and *B* are -50 mV.

Effect of atropine on ACh-induced and K^+-activated currents

The ACh-induced current was completely blocked within 3 min by atropine, a muscarinic blocking agent, at the concentration of 1·5 μM (Fig. 4), while the K^+-activated current was not affected even if the concentration of atropine was increased to 15 μM (five cells). Furthermore, the K^+-activated current was still generated during a prolonged application of ACh plus atropine (Fig. 4). The membrane conductance did not change in the presence of atropine.

Effect of barium ions on the ACh-induced and K^+-activated currents

The muscarinic ACh-induced current in mammalian preparations is blocked by application of Ba^{2+} at low concentration (Mubagwa & Carmeliet, 1983; Carmeliet & Mubagwa, 1986*b*). This blockade is thought to be due to the binding of Ba^{2+} to sites in the muscarinic ACh channel (Carmeliet & Mubagwa, 1986*b*). Therefore, if the

increase in the K^+ efflux caused by ACh is necessary for an activation of the electrogenic Na^+ pump, Ba^{2+} will block this activation.

Figure 5 shows the effects of Ba^{2+} on the ACh-induced and K^+-activated currents obtained from the same preparation. The amplitude of the ACh-induced current was completely blocked 10 min after application of 1 mM-Ba^{2+} (Fig. 5*D*). On the other

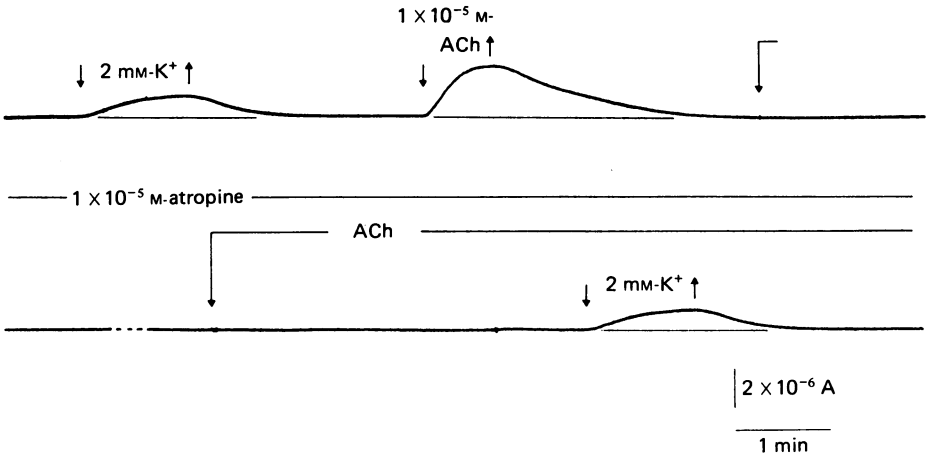

Fig. 4. Effects of atropine (10 μM) on the ACh-induced and K^+-activated currents. Upper and lower traces are continuous recordings. The dotted line in the lower record indicates a period of 5 min. The holding potential was −50 mV. Note, atropine blocked the ACh-induced current without affecting the K^+-activated current.

hand, the K^+-activated current induced by 2 mM-K^+ was not significantly changed in the solution containing not only Ba^{2+} but also both Ba^{2+} and ACh (Fig. 5*C* and *D*). The membrane conductance was slightly decreased (94·3±4·5% of the control, $n = 5$) in the presence of Ba^{2+} (Fig. 5*A* and *C*). The ACh-induced current reappeared 5 min after wash-out of Ba^{2+} (Fig. 5*F*). A similar observation was made in all other preparations examined.

These results indicate that the electrogenic Na^+ pump mechanism is not interfered with by the action of Ba^{2+} and suggest that the activation of this pump by ACh might be related to an increase in the K^+ permeability.

Potential dependence of ACh-induced current

Figure 6*A* shows the effect of membrane potential on the ACh-induced current in the absence or presence of 1 μM-ouabain. The peak amplitude of ACh-induced and ouabain-insensitive currents decreased when the membrane was hyperpolarized. The subtraction of the ouabain-insensitive current (Fig. 6*B*, ●) from the ACh-induced current (○) yielded the ouabain-sensitive current (△). It was clear that the amount of this current was changed non-linearly in relation to the changes in membrane potential, while the ouabain-insensitive current was changed almost linearly with membrane potential. The linear line intersected at −112 mV in this preparation when the extrapolation was applied to zero current of the ouabain-

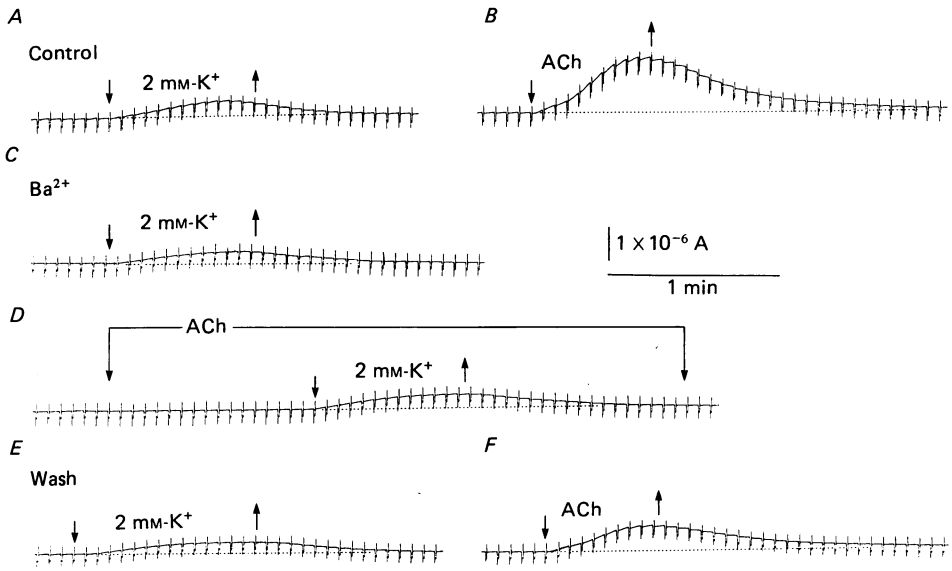

Fig. 5. Effects of 1 mM-Ba^{2+} on the ACh-induced and K^+-activated currents. *A* and *B* are control responses without Ba^{2+} at a holding potential of −50 mV. *C* and *D* are taken 5 min after application of Ba^{2+}. *E* and *F* are obtained 10 min after removal of Ba^{2+}. In order to measure the changes in the membrane conductance, the hyperpolarizing command pulses (500 ms in duration, 8 mV in amplitude) were continuously applied at an interval of 4 s.

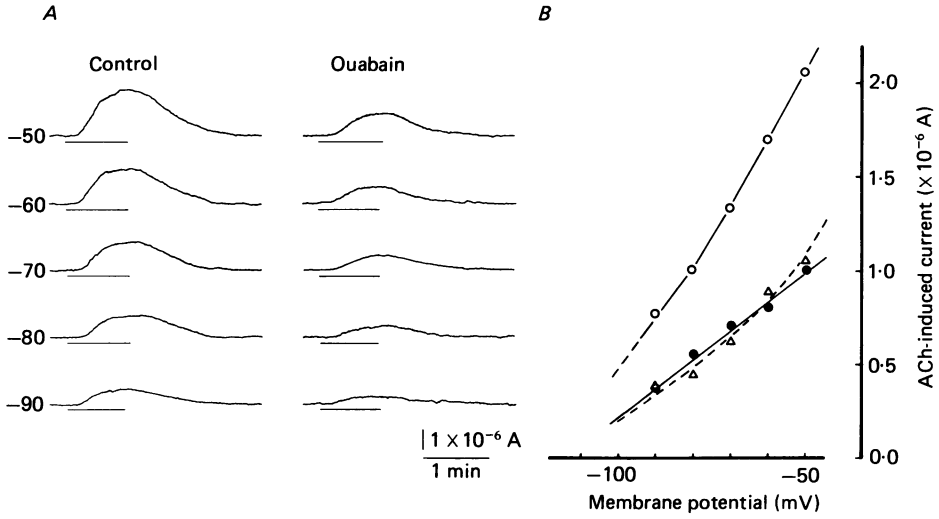

Fig. 6. The potential dependence of the ACh-induced current. *A*, ACh-induced currents at different holding potentials with or without 1 μM-ouabain. The holding potentials are indicated beside each trace in millivolts. The bars under each record show the period of application of 10 μM-ACh. *B*, the relationship of the amplitude of ACh-induced current against the membrane potential. ○ and ● are obtained before and during application of 1 μM-ouabain as shown in *A*. △ indicate the Na^+ pump currents which are obtained by subtracting the current in ouabain (●) from the total current (○). Both continuous and dashed lines were drawn by eye.

insensitive components. The mean potential was $-95{\cdot}3 \pm 4{\cdot}1$ mV ($n = 7$). This potential might be the K^+ equilibrium potential. The ouabain-sensitive current was also greatly reduced at this potential level.

The electrogenic Na^+ pump activity

In order to know the dependence of the electrogenic Na^+ pump activity on extracellular K^+, the relationship between the amplitude of Na^+ pump currents and various $[K^+]_o$ was examined in the K^+-free solution containing 2 μM-TTX. The K^+-activated current measured at a holding potential of -50 mV was increased by increasing $[K^+]_o$ (Fig. 7*A* and ○ in *B*). After treatment of 10 μM-ouabain for 20 min, however, the K^+-activated current reversed its direction and also its amplitude was increased by increasing $[K^+]_o$ (Fig. 7*A* and △ in *B*). The actual electrogenic Na^+ pump current, then, would be estimated from the difference between currents that were obtained in the presence or absence of ouabain. The amplitude of the electrogenic Na^+ pump currents induced by various $[K^+]_o$ was plotted against each $[K^+]_o$ (Fig. 7*C*). If the Hill equation was applied to this relation using the non-linear least-squares fitting method, there was a good fit between the experimental result and theoretical curve (dotted line). According to the computer analysis the maximum pump current was estimated to be 5·19 μA. This was 1·95 times larger than that induced by 2 mM-K^+ (normal Ringer solution). The apparent dissociation constant (K_m) for the Na^+ pumping sites was 2·15 mM and the Hill coefficient was 1·25. These results are consistent with that reported by Akasu *et al.* (1978) and suggest that the Na^+ pump mechanism is not fully activated in the normal Ringer solution (2 mM-K^+). It is, therefore, expected that ACh can activate indirectly the electrogenic Na^+ pump even in the K^+-containing solution.

ACh-induced current in the K^+-containing solution

The contribution of the electrogenic Na^+ pump current underlying the ACh-induced current was examined by using ouabain in the solution containing various $[K^+]_o$. When the $[K^+]_o$ was increased from 0 to 2 mM, the resting membrane potential was gradually changed to more negative values and reached the stable level of about -75 mV. The mean values of the resting membrane potential obtained 1 h after the perfusion of the solution containing 0, 0·5, 0·75, 1·0 and 2·0 mM-K^+ were $-46{\cdot}6 \pm 1{\cdot}8$ (mV, mean ± S.E.M., $n = 58$), $-50{\cdot}4 \pm 1{\cdot}6$ ($n = 23$), $-55{\cdot}0 \pm 4{\cdot}5$ ($n = 5$), $-77{\cdot}4 \pm 2{\cdot}3$ ($n = 22$) and $-77{\cdot}2 \pm 2{\cdot}4$ ($n = 22$), respectively. The increase in the membrane potential would be due to activation of the electrogenic Na^+ pump, since the Na^+–K^+ pump mechanism would be activated partially under these conditions (see Fig. 7). The amplitude of ACh-induced currents obtained at -50 mV was reduced dose dependently in the solution containing 0, 0·5, 0·75, 1·0 and 2·0 mM-K^+ (Fig. 8*B*, ○), the values being $1{\cdot}60 \pm 0{\cdot}06$ (μA, mean ± S.E.M., $n = 28$), $1{\cdot}44 \pm 0{\cdot}05$ ($n = 6$), $1{\cdot}38 \pm 0{\cdot}05$ ($n = 4$), $0{\cdot}71 \pm 0{\cdot}11$ ($n = 7$) and $0{\cdot}52 \pm 0{\cdot}06$ ($n = 14$), respectively. The ouabain sensitivity of the ACh-induced current was also reduced when $[K^+]_o$ was increased. Examples obtained in the 0·75 and 2·0 mM-K^+ solutions are shown in Fig. 8*A*. The amount of ouabain-sensitive component of the ACh-induced current in 0, 0·5, 0·75, 1·0 and 2·0 mM-K^+ solution was plotted and shown in Fig. 8*B* (●), the values being $1{\cdot}33 \pm 0{\cdot}07$ (μA, mean ± S.E.M., $n = 8$), $0{\cdot}79 \pm 0{\cdot}06$ ($n = 5$), $0{\cdot}58 \pm 0{\cdot}09$

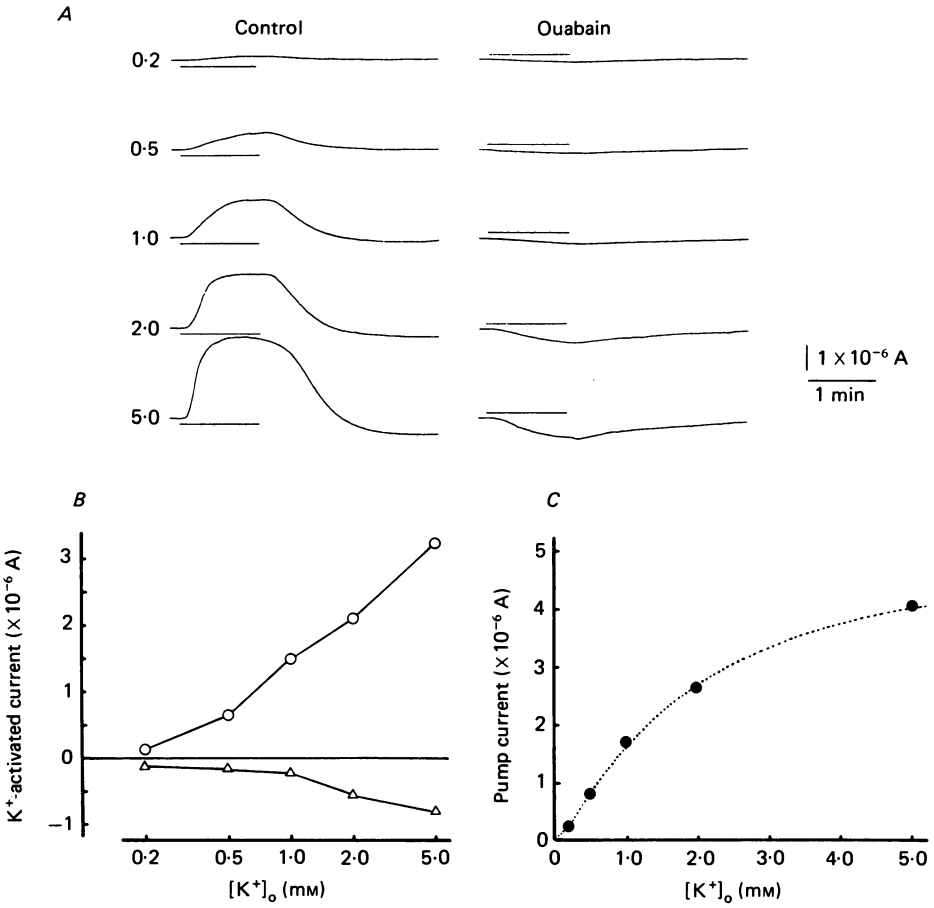

Fig. 7. Activities of the electrogenic Na^+ pump in various $[K^+]_o$. *A*, K^+-activated currents produced by different $[K^+]_o$ in the presence or absence of 10 μM-ouabain. The holding potential was −50 mV. The number to the left of each trace indicates K^+ concentration in millimolar. The K^+-containing solution was changed from the K^+-free solution at an interval of 5 min. The bars under each trace show a period of perfusion of K^+-containing solution. Note: the direction of K^+-activated current is reversed in the presence of ouabain. *B*, the amplitude of K^+-activated currents plotted against $[K^+]_o$. ○ and △ are obtained before and during application of ouabain, respectively. *C*, the dose–response relationship of the K^+-activated current. The data were fitted to the Hill equation by computer using a least-squares fitting method (dotted line). The Hill number, apparent dissociation constant (K_m) and maximum response are calculated to be 1·25, 2·15 mM and 5·19 μA, respectively.

($n = 4$), $0{\cdot}25 \pm 0{\cdot}04$ ($n = 4$) and $0{\cdot}07 \pm 0{\cdot}02$ ($n = 10$), respectively. These results suggest that ACh can activate the electrogenic Na^+ pump even in the presence of extracellular K^+.

The i–v relations in the K^+-containing solution

It has been reported that the inward-rectifying K^+ channel ($I_{K,1}$ channel) is much less present in frog atrial cells compared to ventricular cells (Hume & Giles, 1983).

The lack or less presence of $I_{K,1}$ channels provides the possibility that the K^+ accumulation in the cleft between cells will easily occur when the muscarinic ACh channels are opened by ACh. Since the activity of the electrogenic Na^+ pump is dependent on $[K^+]_o$, the large amount of K^+ accumulation results in the large Na^+ pump activation. This possibility was examined by analysing a current–voltage relationship obtained in the solution containing K^+. The inward currents carried by Na^+ and Ca^{2+} were blocked with pre-treatment by both 1 μM-TTX and 1 mM-Cd^{2+} for over 40 min.

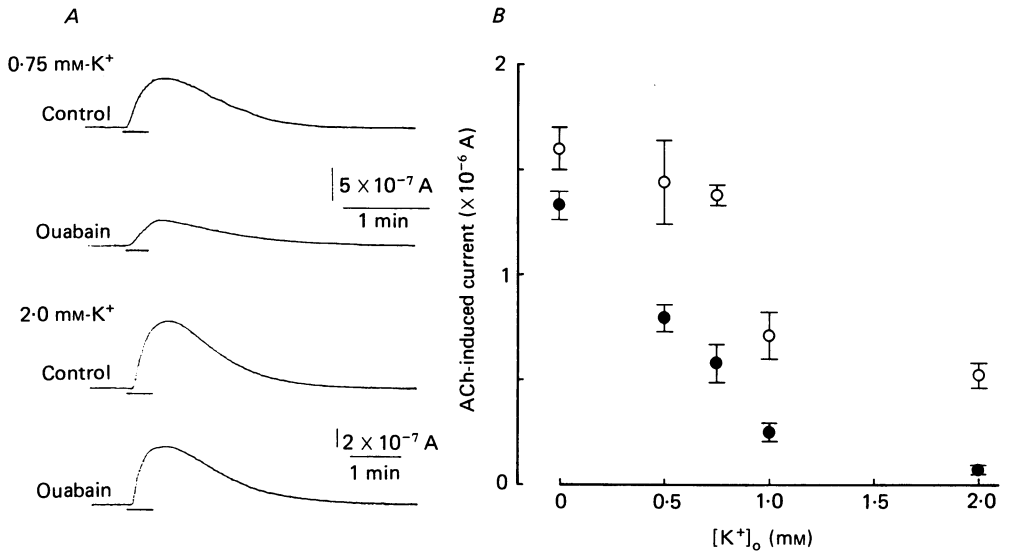

Fig. 8. Effects of 1 μM-ouabain on the ACh-induced current in various $[K^+]_o$. *A*, effects of ouabain on the ACh-induced currents produced in solutions containing 0·75 and 2·0 mM-K^+. In both cases, the effects of ouabain were measured 20 min after application of 1 μM-ouabain. Ten micromolar ACh was applied during a period as indicated by short bars under each trace. *B*, the amplitudes of the total ACh-induced current (○) and ouabain (1 μM)-sensitive current (●) were plotted against $[K^+]_o$. The holding potential in each solution containing different $[K^+]_o$ was adjusted to be −50 mV. The experimental procedures were the same as in *A*. Vertical bars indicate S.E.M. Each value was calculated from four to ten experiments.

In order to construct the *i–v* curve, the cell membranes were hyperpolarized or depolarized from the holding potential (−80 mV) by applying different magnitudes of the rectangular pulse (500 ms in duration) at intervals of 7 s. The currents caused by these pulses of 30 mV amplitude are shown in Fig. 9*A*. The peak amplitude of the current at the end of test pulses was measured and plotted against the voltage (Fig. 9*B*). In 2 mM $[K^+]_o$, the *i–v* relation exhibits marked inward-going rectification at potentials positive to a holding potential of −80 mV (*Ba*, ○). When $[K^+]_o$ was elevated to 4 mM, the current flowed inwardly at the holding potential and its amplitude reached its maximum value within 3 min. The mean value was $1{\cdot}06 \pm 0{\cdot}13$ μA, $n = 6$. The *i–v* relation obtained 6 min after changing a solution exhibits more marked inward-going rectification than that observed in 2 mM $[K^+]_o$

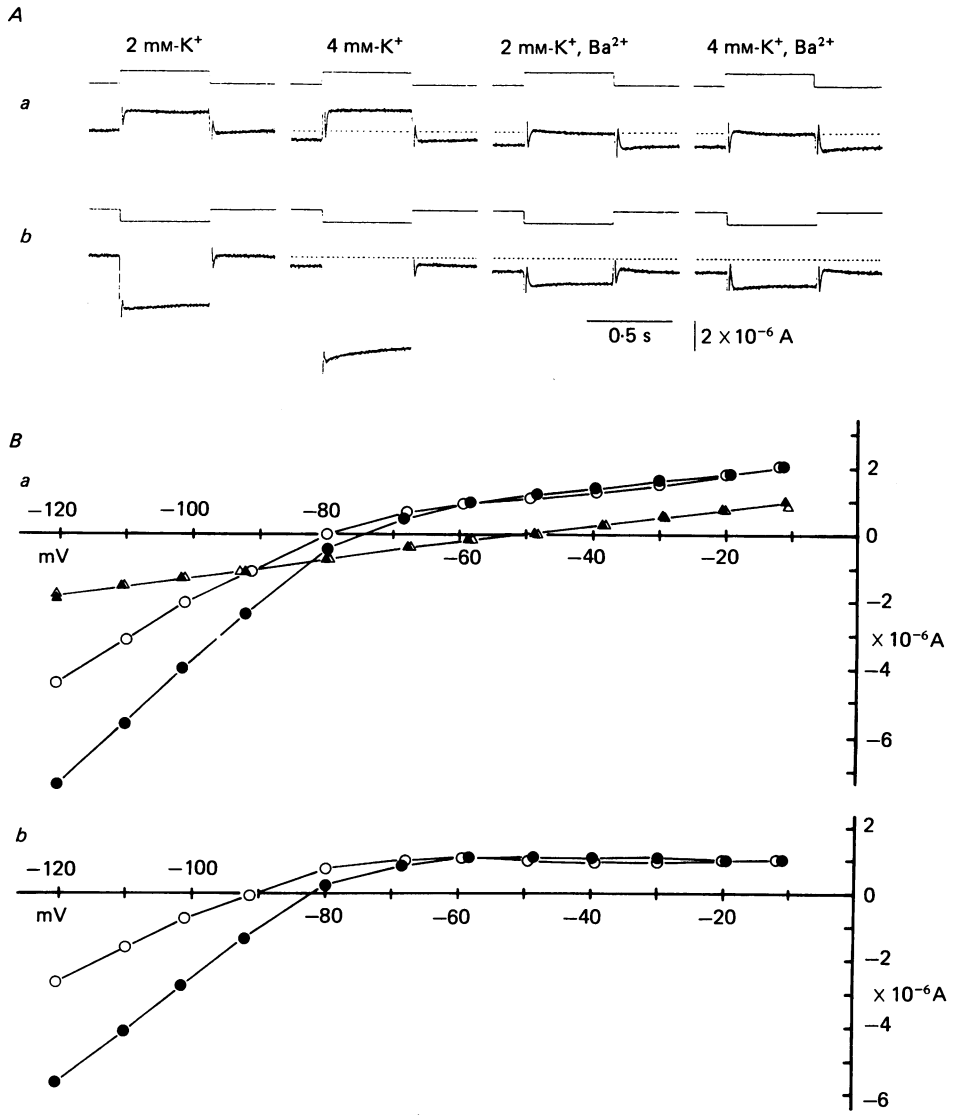

Fig. 9. *A*, currents induced by the depolarizing (*a*) and hyperpolarizing (*b*) voltage pulses in the solution containing either 2 or 4 mM $[K^+]_o$ with or without 1 mM-Ba^{2+}. Upper and lower traces in *a* and *b* indicate the voltage and current recordings, respectively. Depolarizing or hyperpolarizing voltage pulses of 30 mV amplitude were given from a holding potential of −80 mV for 0·5 s. Upward direction of current traces indicates the outward current. The dotted lines show the zero current level obtained in 2 mM $[K^+]_o$. The numbers above indicate $[K^+]_o$ in millimolar. *Ba*, current–voltage relations in 2 and 4 mM $[K^+]_o$ with or without 1 mM-Ba^{2+}. The membrane currents were measured at the end of voltage pulses induced at intervals of 7 s. ○ and △: currents in 2 mM $[K^+]_o$ solution before and after exposure to 1 mM-Ba^{2+}, respectively. ● and ▲: currents in 4 mM $[K^+]_o$ solution before and after exposure to 1 mM-Ba^{2+}, respectively. The measurement in the presence of Ba^{2+} was started 6 min after exposure to Ba^{2+}. *Bb*, Ba^{2+}-sensitive current–voltage relations in 2 (○) and 4 (●) mM $[K^+]_o$ solution. Each point is the difference between currents in the presence or absence of 1 mM-Ba^{2+} as shown in *Ba*.

(*Bb*, ●). Both curves crossed each other around −60 mV. In order to separate a true $I_{K,1}$ from other currents, the *i–v* relations in 2 or 4 mM $[K^+]_o$ were constructed 10 min after application of 1 mM-Ba^{2+}. Ba^{2+} is known to block the $I_{K,1}$ channel at this concentration in bull-frog atrial cells (Momose, Szabo & Giles, 1983) and in mammalian preparations (Osterrieder, Yang & Trautwein, 1982; Kameyama, Kiyosue & Soejima, 1983; Sakmann & Trube, 1984; Carmeliet & Mubagwa, 1986*b*). When the cells were perfused with 1 mM-Ba^{2+}, the inward current was induced at a holding potential of −80 mV in the solution containing either 2 or 4 mM $[K^+]_o$. The amount of current induced by Ba^{2+} in 2 mM $[K^+]_o$ was 1·28±0·19 μA (n = 14), which was larger than that in 4 mM $[K^+]_o$. However, if the net inward current was calculated from the control (zero current level shown by dotted lines in Fig. 9*A*), both values did not differ obviously from each other. This suggests that the $I_{K,1}$ channel is indeed blocked by Ba^{2+} at this concentration. It was clear that in the presence of Ba^{2+}, no inward-going rectification had been observed in the solution containing either 2 (Fig. 9*Ba*, △) or 4 mM $[K^+]_o$ (▲) and seen clearly that there were no significant differences between both relationships. Figure 9*Bb* shows the real *i–v* relationships related to the $I_{K,1}$ channel in 2 and 4 mM $[K^+]_o$, that were constructed from the subtraction of currents obtained in the presence or absence of Ba^{2+}. The reversal potential in 4 mM $[K^+]_o$ was shifted from −90 to −82 mV. At potentials positive to −60 mV, both *i–v* relations are almost similar and exhibit no appreciable negative slope which is a characteristic feature of $I_{K,1}$ channel. These results are consistent with that reported by Hume & Giles (1983) and suggest that the $I_{K,1}$ channel is less developed in frog atrial cells.

In addition, the *i–v* relation obtained in the K^+-free solution did not exhibit the inward-going rectification and Ba^{2+} (1 mM) did not affect obviously this relationship. In some cases, however, a small Ba^{2+}-sensitive inward current could be detected at a holding potential of −50 mV (five out of twelve cells); these results indicate that $I_{K,1}$ channels are inactivated almost completely in the K^+-free solution.

DISCUSSION

The membrane of bull-frog atria was hyperpolarized by ACh in the K^+-free solution. According to the results obtained by the voltage clamp technique, the outward current corresponding to this hyperpolarization is composed of two different components, one which is ouabain-sensitive and the other which is ouabain-insensitive. The large part of the ACh-induced current (more than 80%) is sensitive to ouabain. Since ouabain is known to be a specific blocker of the Na^+–K^+ pump through the inhibition of the Na^+–K^+-ATPase activity, the ouabain-sensitive current would be generated by an activation of the electrogenic Na^+ pump (Glitsch, 1982; Akera & Brody, 1982). This is suggested in experiments where the nature of the ACh-induced current was compared with that of the K^+-activated current (Fig. 3) which was mainly due to activation of the electrogenic Na^+ pump current (Fig. 7). If the ACh-induced current is a simple passive K^+ current induced by the muscarinic action of ACh, the amplitude of the K^+-activated current observed in the presence of ACh must be unchanged at the same potential level because the amount of the electrogenic Na^+ pump current would be determined by the rate of extrusion of

Na^+ from cells, which is related to the changes in both the Na^+/K^+ coupling ratio and number of pumping sites (Akasu *et al.* 1978; Glitsch, 1982). However, the K^+-activated current was not only reduced by more than 90% but also reversed its direction. This strongly suggests that the electrogenic Na^+ pump is already activated during application of ACh and, therefore, the further activation of Na^+ pump by an addition of extracellular K^+ is limited. The reversal of the K^+-activated current observed in some cells may be explained by the idea that the current component involved in the passive distribution of K^+ across the membrane is larger than that of the Na^+ pump activity. The ouabain-insensitive current must have resulted from an increase in K^+ conductance caused by the muscarinic action of ACh because it was accompanied by an increase of membrane conductance and became smaller with membrane hyperpolarization. The reversal potential estimated by the extrapolation in the presence of ouabain is around -95 mV, suggesting the K^+ equilibrium potential (Fig. 6). Finally, the muscarinic blocking agent, atropine, blocked this current completely (Fig. 4).

As shown in Fig. 4, the K^+-activated current was not affected by atropine while the ACh-induced current was completely blocked. This indicates that the activity of the electrogenic Na^+ pump is not interfered with by atropine and that the binding of ACh to the muscarinic receptor is the first step for the activation of this pump. Ba^{2+}, which acts on the channel sites (Momose *et al.* 1983; Carmeliet & Mubagwa, 1986*b*), blocks the ACh-induced current without affecting the K^+-activated current. Thus, the activation of the electrogenic Na^+ pump by ACh appears to be due to increase in the K^+ permeability. Furthermore, the ouabain-sensitive current is greatly reduced at the potential level where ouabain-insensitive current is eliminated (Fig. 6*B*). At this level, the net flux of K^+ would be small or nil. It is, therefore, most likely that the increase in K^+ permeability caused by ACh leads to an accumulation of K^+ immediately outside of the membrane, and the increase of K^+ stimulates the electrogenic Na^+ pump.

The ACh-induced current decreased when the membrane was hyperpolarized (Fig. 6). The ouabain-sensitive current component obtained after subtraction of the ouabain-insensitive current from the ACh-induced current shows a non-linear voltage dependence (Fig. 6*B*) in the range between -50 and -90 mV. The non-linear voltage dependence of the Na^+ pump may be explained as follows: (1) When the membrane was held at the depolarized voltage, a larger amount of K^+ would be accumulated in the extracellular space and produce a larger electrogenic Na^+ pump current. (2) The voltage dependence of the electrogenic Na^+ pump current has been reported by Gadsby, Kimura & Noma (1985) and Hasuo & Koketsu (1985). It declines steadily from a maximum level near 0 mV, becoming very small at -140 mV. These factors would contribute to the non-linearity of the amplitude of Na^+ pump current in relation to the membrane potential.

The pump activity is dependent on the pre-treatment of $[K^+]_o$ (Fig. 8). The resting membrane potential is shifted to a more negative potential with increased $[K^+]_o$. This negative shift of the membrane potential may be due to activation of the Na^+ pump, since the direction must be reversed if the potential is determined by the passive distribution of potassium ions. The half-activation of the Na^+ pump occurred at 2·15 mM-K^+. This value agrees with observations obtained on this preparation

(Akasu *et al.* 1978) and on mammalian preparations (Glitsch *et al.* 1978; Gadsby, 1980). In the presence of a physiological concentration of K^+ (2 mM), the electrogenic Na^+ pump is already, but not fully, activated (Fig. 7). The ouabain sensitivity of the ACh-induced current in 2 mM-K^+ is about 14% (0·073 μA) of the total current. If the effective resistance is assumed to be 30 KΩ, the change of membrane potential is calculated to be 2·2 mV. Therefore, the indirect effects of ACh on the Na^+ pump current seem to be important in regulating the membrane excitability at normal and slightly reduced $[K^+]_o$ in bull-frog atrial cells.

The question arises why our preparations show such a large pump component affected by ACh. If the $I_{K,1}$ channel in bull-frog atrial cells is less developed compared to that of mammal preparations, the increase in K^+ permeability caused by ACh will provide the large accumulation of K^+ in the cleft between cells and in turn act to increase the Na^+ pump activity. The data in Fig. 9 clearly show that the *i–v* relations obtained in the solution containing 2 or 4 mM $[K^+]_o$ exhibit marked inward-going rectification at potentials positive to −80 mV. However, we failed to find the clear N-shape in both *i–v* relations. Hume & Giles (1983) reported that in single isolated bull-frog atrial cells the total time-independent background current ($I_{K,1}$) is very small in healthy, normally polarized cells. Our results are consistent with their observations. Therefore, when the muscarinic ACh channels are opened by ACh the potassium ions may be accumulated rapidly in the external surface of the bull-frog atrium muscle fibres. The excess K^+ in the clefts between cells stimulates the electrogenic Na^+ pump and in turn provides the membrane hyperpolarization due to the low conductance of $I_{K,1}$ channel. In the sino-atrial cells, the $I_{K,1}$ channels are absent almost completely in the bull-frog (Giles & Shibata, 1985) and in mammalian tissue (Noma, Nakayama, Kurachi, & Irisawa, 1984). In those cells, the indirect activation of Na^+ pump by ACh would be more important for a critical regulation of the pacemaker activities than that in other cells.

Carmeliet & Mubagwa (1986*a*) reported that ACh does not activate the Na^+–K^+ pump current in rabbit cardiac purkinje fibres. The difference between their results and our own may be explained as follows. In rabbit cardiac purkinje fibres, a wide intercellular cleft exists (Colatsky & Tsien, 1979) and the $I_{K,1}$ channel is well developed (see Fig. 4 in Carmeliet & Mubagwa 1986*a*). In this case, the K^+ accumulation cannot occur even when the muscarinic ACh channels are opened by ACh, because the excess K^+ may diffuse rapidly into a wide intercellular space and a part of this K^+ may be taken up simultaneously into cells through the $I_{K,1}$ channel.

It may be noted that Ojeda *et al.* (1981) and Argibay *et al.* (1983) suggest that $I_{K,1}$ current and ACh (or carbachol)-induced current are flowing through the same channel. However, the recent reports show that ACh-induced current is different from $I_{K,1}$ currents in mammalian sino-atrial and atrio-ventricular preparations (Noma & Trautwein, 1978; Noma, Peper & Trautwein, 1979; Sakmann *et al.* 1983; Carmeliet & Mubagwa, 1986*b*).

We thank Dr N. J. Dun for his critical reading of the manuscript.

REFERENCES

AKASU, T., OHTA, Y. & KOKETSU, K. (1978). The effect of adrenaline on the electrogenic Na^+ pump in cardiac muscle cells. *Experientia* **34**, 488–490.

AKERA, T. & BRODY, T. M. (1982). Myocardial membranes: regulation and function of the sodium pump. *Annual Review of Physiology* **44**, 375–388.

ARGIBAY, J. A., DUTEY, P., ILDEFONSE, M., OJEDA, C., ROUGIER, O. & TOURNEUR, Y. (1983). Block by Cs of K current i_{k1} and of carbachol induced K current i_{Cch} in frog atrium. *Pflügers Archiv* **397**, 295–299.

BEELER, G. W. & REUTER, H. (1970). Voltage clamp experiments on ventricular myocardial fibres. *Journal of Physiology* **207**, 165–190.

CARMELIET, E. & MUBAGWA, K. (1986*a*). Changes by acetylcholine of membrane currents in rabbit cardiac Purkinje fibres. *Journal of Physiology* **371**, 201–217.

CARMELIET, E. & MUBAGWA, K. (1986*b*). Characterization of the acetylcholine-induced potassium current in rabbit cardiac Purkinje fibres. *Journal of Physiology* **371**, 219–237.

CARMELIET, E. & RAMON, J. (1980). Effect of acetylcholine on time-independent currents in sheep cardiac Purkinje fibers. *Pflügers Archiv* **387**, 207–216.

COLATSKY, T. J. & TSIEN, R. W. (1979). Electrical properties associated with wide intracellular clefts in rabbit Purkinje fibres. *Journal of Physiology* **290**, 227–252.

DAUT, J. & RUDEL, R. (1982). The electrogenic sodium pump in guinea-pig ventricular muscle: inhibition of pump current by cardiac glycosides. *Journal of Physiology* **330**, 243–264.

DUDEL, J. & TRAUTWEIN, W. (1956). Die Wirkung von Adrenalin auf das Ruhepotential von Myokardfasern des Vorhofs. *Experientia* **12**, 396–398.

EISNER, D. A. & LEDERER, J. (1980). Characterization of the electrogenic sodium pump in cardiac Purkinje fibres. *Journal of Physiology* **303**, 441–474.

GADSBY, D. C. (1980). Activation of electrogenic Na^+/K^+ exchange by extracellular K^+ in canine cardiac Purkinje fibers. *Proceedings of the National Academy of Sciences of the U.S.A.* **77**, 4035–4039.

GADSBY, D. C. (1983). β-Adrenoceptor agonists increase membrane K^+ conductance in cardiac Purkinje fibres. *Nature* **306**, 691–693.

GADSBY, D. C. (1984). The Na/K pump of cardiac cells. *Annual Review of Biophysics and Bioengineering* **13**, 373–398.

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

GARNIER, D., NARGEOT, J., OJEDA, C. & ROUGIER, O. (1978). The action of acetylcholine on background conductance in frog atrial trabeculae. *Journal of Physiology* **274**, 381–396.

GILES, W. & NOBLE, S. J. (1976). Changes in membrane currents in bullfrog atrium produced by acetylcholine. *Journal of Physiology* **261**, 103–123.

GILES, W. R. & SHIBATA, E. F. (1985). Voltage clamp of bull-frog cardiac pacemaker cells: a quantitative analysis of potassium currents. *Journal of Physiology* **368**, 265–292.

GLITSCH, H. G. (1972). Activation of the electrogenic sodium pump in guinea-pig auricles by internal sodium ions. *Journal of Physiology* **220**, 565–582.

GLITSCH, H. G. (1982). Electrogenic Na pumping in the heart. *Annual Review of Physiology* **44**, 389–400.

GLITSCH, H. G., GRABOWSKI, W. & THIELEN, J. (1978). Activation of the electrogenic sodium pump in guinea-pig atria by external potassium ions. *Journal of Physiology* **276**, 515–524.

HARRIS, E. J. & HUTTER, O. F. (1956). The action of acetylcholine on the movements of potassium ions in the sinus venosus of the frog. *Journal of Physiology* **133**, 58–59*P*.

HASUO, H. & KOKETSU, K. (1985). Potential dependency of the electrogenic Na^+-pump current in bullfrog atrial muscles. *Japanese Journal of Physiology* **35**, 89–100.

HUME, J. R. & GILES, W. (1983). Ionic currents in single isolated bullfrog atrial cells. *Journal of General Physiology* **81**, 153–194.

ISENBERG, G. & TRAUTWEIN, W. (1974). The effect of dihydro-ouabain and lithium-ions on the outward current in cardiac Purkinje fibers. *Pflügers Archiv* **350**, 41–54.

KAMEYAMA, M., KIYOSUE, T. & SOEJIMA, M. (1983). Single channel analysis of the inward rectifier K current in the rabbit ventricular cells. *Japanese Journal of Physiology* **33**, 1039–1056.

KIMURA, J., NOMA, A. & IRISAWA, H. (1986). Na–Ca exchange current in mammalian heart cells. *Nature* **319**, 596–597.

KURACHI, Y., NOMA, A. & IRISAWA, H. (1981). Electrogenic sodium pump in rabbit atrio-ventricular node cell. *Pflügers Archiv* **391**, 261–266.

MECHMANN, S. & POTT, L. (1986). Identification of Na–Ca exchange current in single cardiac myocytes. *Nature* **319**, 597–599.

MINOTA, S. & KOKETSU, K. (1979). Activation of the electrogenic Na-pump of cardiac muscle fibres by ACh in K-free solutions. *Experientia* **35**, 772–773.

MOMOSE, Y., SZABO, G. & GILES, W. (1983). An inwardly rectifying K^+ current in bullfrog atrial cells. *Biophysical Journal* **41**, 311a.

MUBAGWA, K. & CARMELIET, E. (1983). Effects of acetylcholine on electrophysiological properties of rabbit cardiac purkinje fibers. *Circulation Research* **53**, 740–751.

NOMA, A. & IRISAWA, H. (1974). Electrogenic sodium pump in rabbit sinoatrial node cell. *Pflügers Archiv* **351**, 177–182.

NOMA, A. & IRISAWA, H. (1975). Contribution of an electrogenic sodium pump to the membrane potential in rabbit sinoatrial node cells. *Pflügers Archiv* **358**, 289–301.

NOMA, A., NAKAYAMA, T., KURACHI, Y. & IRISAWA, H. (1984). Resting K conductances in pacemaker and non-pacemaker heart cells of the rabbit. *Japanese Journal of Physiology* **34**, 245–254.

NOMA, A., PEPER, K. & TRAUTWEIN, W. (1979). Acetylcholine-induced potassium current fluctuations in the rabbit sino-atrial node. *Pflügers Archiv* **381**, 255–262.

NOMA, A. & TRAUTWEIN, W. (1978). Relaxation of the ACh-induced potassium current in the rabbit sinoatrial node cell. *Pflügers Archiv* **377**, 193–200.

OJEDA, C., ROUGIER, O. & TOURNEUR, Y. (1981). Effects of Cs on acetylcholine induced current. Is i_{K1} increased by acetylcholine in frog atrium? *Pflügers Archiv* **391**, 57–59.

OSTERRIEDER, W., YANG, Q.-F. & TRAUTWEIN, W. (1982). Effects of barium on the membrane currents in the rabbit S–A node. *Pflügers Archiv* **394**, 78–84.

PAGE, E. & STORM, S. R. (1965). Cat heart muscle *in vitro* VIII. Active transport of sodium in papillary muscles. *Journal of General Physiology* **48**, 957–972.

SAKMANN, B., NOMA, A. & TRAUTWEIN, W. (1983). Acetylcholine activation of single muscarinic K^+ channels in isolated pacemaker cells of the mammalian heart. *Nature* **303**, 250–253.

SAKMANN, B. & TRUBE, G. (1984). Voltage-dependent inactivation of inward-rectifying single-channel currents in the guinea-pig heart cell membrane. *Journal of Physiology* **347**, 659–683.

SHIBATA, E. F., MOMOSE, Y. & GILES, W. (1983). Measurement of an electrogenic Na^+/K^+ pump current in individual bullfrog atrial myocytes. *Proceedings of the International Union of Physiological Sciences* **15**, 51.

SOEJIMA, M. & NOMA, A. (1984). Mode of regulation of the ACh-sensitive K-channel by the muscarinic receptor in rabbit atrial cells. *Pflügers Archiv* **400**, 424–431.

TEN EICK, R.,, NAWRATH, H., MCDONALD, T. & TRAUTWEIN, W. (1976). On the mechanism of the negative inotropic effect of acetylcholine. *Pflügers Archiv* **361**, 207–213.

TRAUTWEIN, W. (1973). Membrane currents in cardiac muscle fibers. *Physiological Reviews* **53**, 793–835.

TRAUTWEIN, W. & DUDEL, J. (1958). Zum Mechanismus der Membranwirkung des Acetylcholin an der Herzmuskelfaser. *Pflügers Archiv* **266**, 324–334.

TRAUTWEIN, W. & SCHMIDT, R. F. (1960). Zur Membranwirkung des Adrenalins an der Herzmuskelfaser. *Pflügers Archiv* **271**, 715–726.

VASSALLE, M. & BARNABEI, P. (1971). Norepinephrine and potassium fluxes in cardiac Purkinje fibers. *Pflügers Archiv* **322**, 287–303.