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CHANGES IN MEMBRANE CURRENTS IN BULLFROG ATRIUM PRODUCED BY ACETYLCHOLINE

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SUMMARY

1. A double sucrose-gap voltage-clamp technique has been used to study the effects of acetylcholine on the membrane currents in atrial trabeculae of the bullfrog, *Rana catesbeiana*.

2. The second, or slow inward (Ca^{2+}/Na^{+}) current, was found to be markedly reduced by concentrations of acetylcholine greater than approximately $2{\cdot}0 \times 10^{-8}$ M. The resulting decrease in net calcium entry provides a straightforward explanation for the negative inotropic action of acetylcholine in atrial muscle.

3. Measurements of membrane resistance near the resting potential showed that relatively high doses of acetylcholine (approximately 10^{-7} M) decrease membrane resistance by about twofold. This effect is shown to be the result of an increase in a time-independent background current which appears to be carried mainly by potassium ions.

4. Using appropriate pharmacological techniques, it has been possible to demonstrate: (i) that the peak slow inward current is reduced to about half its initial value before any significant increase in background current occurs; (ii) that even when a sufficient dose of acetylcholine to produce an increase in background current is used, the background current shows inward-going rectification and cannot account for the observed reduction in the slow inward current.

5. No consistent change was observed in the degree of activation of the time-dependent outward membrane currents after application of concentrations of acetylcholine which produced large decreases in the peak slow inward current.

6. These results are discussed in relation to previous electro-physiological and radioisotope studies of the mechanism of the negative inotropic effect of acetylcholine in cardiac muscle.

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INTRODUCTION

Application of acetylcholine, or stimulation of the vagus nerve, produces a number of characteristic changes in the electrical and mechanical activity of atrial muscle. These include: (i) a small hyperpolarization (2–5 mV) of the maximum diastolic potential; (ii) a marked decrease in the height and duration of the action potential; (iii) a significant decrease in twitch tension, the negative inotropic effect. The classical explanation for all of these changes is based on the finding that acetylcholine selectively increases the membrane permeability to potassium ions (for reviews see Hutter, 1961; Trautwein, 1963; Krnjević, 1974). The resulting increase in potassium background current is thought to cause a hyperpolarization toward the potassium equilibrium potential, as well as the decrease in the height and duration of the action potential. The negative inotropic effect of acetylcholine is considered to be a consequence of the smaller and shorter action potential (Burgen & Terroux, 1953).

This hypothesis is based on the results of tracer experiments which showed that acetylcholine produced increases in the rate of ^{42}K efflux from tortoise and frog sinus venosus tissue (Harris & Hutter, 1956; Hutter, 1961) and on measurements which showed that the reversal potential of acetylcholine response was very close to the predicted potassium equilibrium potential (Trautwein & Dodd, 1958).

The object of the present experiments was to test the possibility that acetylcholine may be changing one or more of the time-dependent ionic currents which underlie the action potential in frog atrium. An unexpected result was that low concentrations of acetylcholine produce a large reduction in the peak slow inward current. Some of these results have previously been published as part of a Ph.D. thesis (Giles, 1974) and as communications to the Physiological Society (Giles & Tsien, 1975; Blood, Giles & Noble, 1976).

METHODS

A double sucrose-gap technique has been used to polarize and voltage clamp fine strips of muscle from the atrium of the bullfrog *Rana catesbeiana*. The perfusion bath, the method for the dissection of the preparation (Brown & Noble, 1969), and the electronic apparatus (Brown, Clark & Noble, 1976*a*) have previously been described in detail. Only important changes or additions in the technique will be described in this paper.

Preparation. Fine strips, or trabeculae, of atrial muscle were taken from the endocardial surface of the wall of the atrium, or from the interatrial septum. These preparations were approximately 150–200 μm in diameter and 3–4 mm in length. After a short healing-over period (approx. 15 min) in normal Ringer solution the resting potential of each preparation was about -80 mV (see *Gap potential*) and a normal action potential could be elicited (see Fig. 1).

Solutions. Normal Ringer solution: NaCl, 90 mM; $NaHCO_3$, 20 mM; KCl, 2 mM;

$MgCl_2$, 1 mM; $CaCl_2$, 1·1 mM; glucose, 2 g/l.; equilibrated with a mixture of 5% CO_2 and 95% O_2. High K^+ Ringer solution: KCl, 92 mM; $KHCO_3$, 20 mM; $MgCl_2$, 1 mM; $CaCl_2$, 1·1 mM; glucose, 2 g/l.; equilibrated with a mixture of 5% CO_2 and 95% O_2. Isotonic sucrose: sucrose, 146 g/l. (B.D.H. 'Analar' grade); glucose, 2 g/l.

Drugs. In all voltage-clamp experiments tetrodotoxin (TTX, obtained from Sigma Ltd) was added to the normal Ringer solution to a final concentration of 2·0 or $4{\cdot}0 \times 10^{-3}$ g/l. ($6{\cdot}4$–$12{\cdot}8 \times 10^{-6}$ M).

Acetylcholine and carbachol were added as the chloride salts. These drugs as well as atropine, were prepared as stock solutions and were kept refrigerated until shortly before being diluted with Ringer solution to the final concentration.

Solutions flowed by gravity into all compartments of the perfusion bath at 1·5–2 ml/min. At concentrations greater than approximately $2{\cdot}0 \times 10^{-8}$ M, acetylcholine produced changes in the action potential (see Fig. 1) within 1 min. However, voltage-clamp experiments were not started until the drug-containing solutions had been in contact with the preparation for about 10 min. A typical voltage-clamp experiment lasted 1–1·5 hr.

Gap potential. Before each voltage-clamp experiment an estimate of the average resting potential of the trabeculum was obtained. This was done by perfusing both end compartments of the perfusion bath with high potassium (112 mM-K^+) Ringer solution (see Brown & Noble, 1969). The resulting negative voltage deflexion averaged $77{\cdot}3 \pm 5$ mV (mean $\pm$ S.D. of observation) in these experiments. This 'gap potential' compares favourably with micro-electrode resting potential measurements from frog atrium (Glitch, Haas & Trautwein, 1965, $75 \pm 4{\cdot}6$; Maughan, 1973, $-80{\cdot}5 \pm 4{\cdot}1$).

Uniformity of the voltage clamp. Johnson & Lieberman (1971) have criticized the double sucrose-gap technique for voltage clamping frog atrial muscle. A major point in their criticism is that the potential in the test compartment is unlikely to be constant in either the axial or the radial direction during the activation of the fast inward (Na^+) current. In the present study, tetrodotoxin (TTX, $2{\cdot}0 \times 10^{-3}$ g/l.) was applied before each voltage-clamp experiment in order to block the fast inward current selectively.

Tarr & Trank (1974) have recently reported that non-uniformity artifacts are also present during the activation of the slow inward (Ca^{2+}/Na^+) current. It is important to note, however, that Tarr & Trank have assumed that the fast inward (Na^+) current is *completely abolished* by $2{\cdot}0 \times 10^{-7}$ M ($6{\cdot}4 \times 10^{-5}$ g/l.) TTX. This TTX concentration (which is about thirty times less than was used in the present experiments) has been reported to markedly reduce the rate of the initial, regenerative depolarization in frog ventricle (Hagiwara & Nakajima, 1966) but it fails to completely block the rapid inward current in frog atrium (Giles, 1974; Connor, Barr & Jakobsson, 1975). Thus, it is possible that the micro-electrode measurements of Tarr & Trank which seemed to show non-uniformity artifacts during the activation of *only* the slow (Ca^{2+}/Na^+) inward current, in fact were made during the activation of *both* inward currents.

Since a closed perfusion system was used in the present experiments it was not possible to insert a micro-electrode into the centre compartment in order to test directly for non-uniformity during the activation of the slow inward current. However, this kind of experiment has been performed during single sucrose-gap experiments on trabeculae from mammalian ventricle. The results indicate that the slow inward current can be quantitatively studied in that tissue (New & Trautwein, 1972; Reuter, 1973). Our computer calculations based on the cable equations and equivalent circuit given by Brown, Noble & Noble (1976*c*) also indicate that axial non-uniformity is not a serious problem in situations where the conductance increase is of

the approximate size measured during activation of the slow inward (Ca^{2+}/Na^{+}) current in these experiments (see Appendix).

For double sucrose-gap voltage-clamp experiments, transgap leakage currents can cause large errors in the measurement of actual membrane currents (see Appendix by Tsien & McGuigan, in McGuigan, 1974). This kind of artifact is minimal when the length of the centre or test compartment is greater than approximately half the resting space constant (λ) of the preparation, i.e. when

$$\frac{1}{\lambda} < 0{\cdot}50.$$

The resting space constant of typical atrial trabeculae from *Rana catesbeiana* is approximately 690 μm (Brown *et al.* 1976*c*). The width of the centre compartment of the perfusion bath was 400 μm. Therefore, in the present experiments

$$\frac{1}{\lambda} = \frac{400}{690} = 0{\cdot}58.$$

RESULTS

Atrial action potentials in acetylcholine

Fig. 1 shows the results of an experiment which was designed to study the effects of a low dose ($5{\cdot}0 \times 10^{-8}$ M) of acetylcholine on the action potential in bullfrog atrium. From Fig. 1*A* it is apparent that this concentration of acetylcholine produced a marked shortening of the action potential, and a significant decrease in its peak height. A hyperpolarization, which is a characteristic response of frog sinus venous tissue to acetylcholine, was not observed in the present experiments unless a larger concentration of acetylcholine (approximately 10^{-7} M) was applied. This point will be pursued further in relation to Fig. 6 and Fig. 7, and in the Discussion.

Fig. 1*B* illustrates the initial depolarization phases of the same action potentials which are shown in Fig. 1*A*. The final, or 'secondary depolarization phase' of the upstroke appears to be selectively reduced by this dose of acetylcholine. This result has previously been observed in a microelectrode study of the effect of localized application of acetylcholine to rabbit atrium (Paes de Carvahlo, Hoffman & Paula de Carvahlo, 1969). It is well known that the regenerative depolarization of the action potential is diphasic in both frog and mammalian atrium. The magnitude of the secondary depolarization phase has been shown to be dependent on the external calcium concentration (for summary of evidence see Paes de Carvahlo *et al.* 1969). Therefore, a possible interpretation of the results in Fig. 1*B* is that acetylcholine decreases the slow inward (Ca^{2+}/Na^{+}) current.

Some additional indirect evidence for this hypothesis is illustrated in frames *C* and *D* of Fig. 1. In this part of the experiment the same preparation was perfused with a Ringer solution containing TTX at a concentration of $2{\cdot}0 \times 10^{-3}$ g/l. Under these conditions, the regenerative

depolarization was monophasic and its threshold was approximately −40 mV (see Fig. 1*D*). The maximum rate of depolarization and the peak height of such action potentials are thought to be directly related to the magnitude of the slow inward (Ca^{2+}/Na^{+}) current (Rougier, Vassort, Garner, Gargouil & Coraboeuf, 1969). Fig. 1*C* shows that application of acetyl-

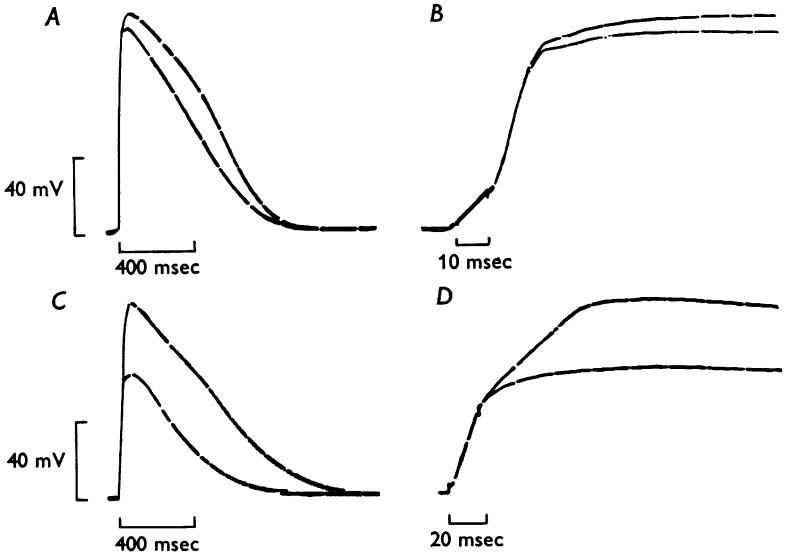

Fig. 1. Membrane action potentials recorded from a bullfrog atrial trabeculum. *A*, larger action potential obtained in normal Ringer solution; smaller, shorter action potential recorded after the steady-state effect of acetylcholine ($5{\cdot}0 \times 10^{-8}$ M) had been reached. The 10 msec stimuli of 1·1 times threshold strength were applied at a rate of 0·2 Hz. *B*, same results as in *A*, displayed at a twenty times faster sweep speed. *C*, both action potentials were recorded after the trabeculum had been perfused with Ringer solution containing TTX ($2{\cdot}0 \times 10^{-3}$ g/l.). The larger action potential was the control, and the smaller, shorter action potential was obtained in acetylcholine ($5{\cdot}0 \times 10^{-8}$ M). The 16 msec, 1·1 times threshold stimuli were applied at 0·2 Hz. *D*, same results as in *C*, recorded at a ten times faster sweep speed. The data in *B* and *D* indicate that secondary depolarization phase of the action potential is reduced by acetylcholine. For further explanation, see text.

choline ($5{\cdot}0 \times 10^{-8}$ M) produces a large decrease in both the peak height and the duration of the 'Ca^{2+} dependent action potential'; and from Fig. 1*D* it is quite clear that the regenerative depolarization has been virtually abolished by acetylcholine.

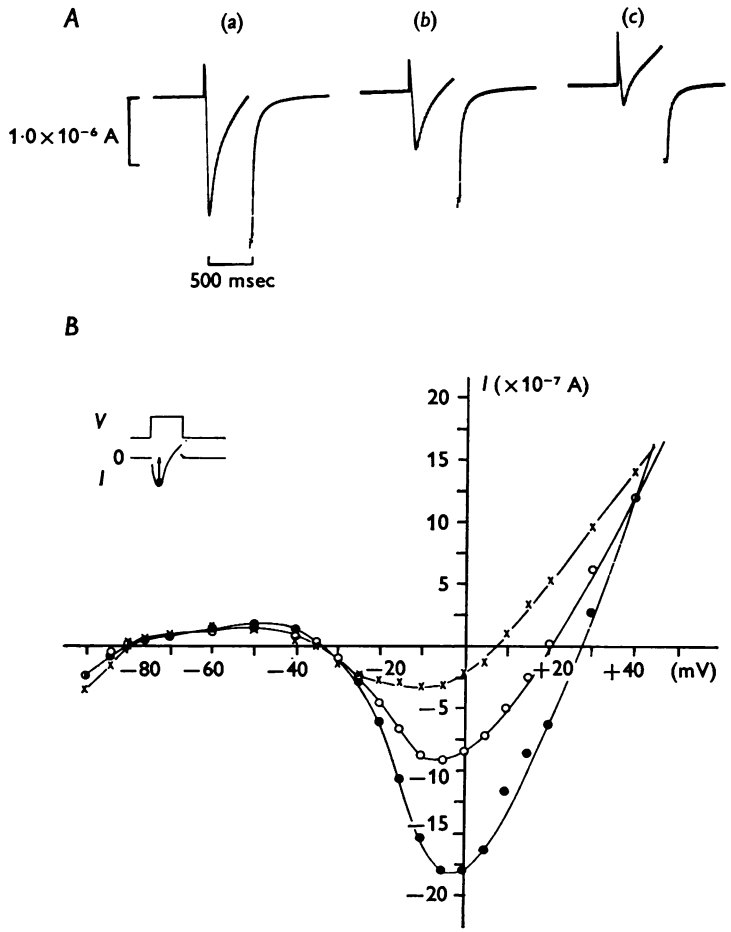

Fig. 2. Effect of acetylcholine on the slow inward (Ca^{2+}/Na^{+}) current. The preparation was continuously superfused by TTX ($2{\cdot}0 \times 10^{-3}$ g/l.) Ringer solution. *A*, shows pen-recorder tracings of the slow inward current in response to 500 msec, 80 mV depolarization from the resting potential (−80 mV). (*a*) is the control, and (*b*) and (*c*) were recorded after application of $3{\cdot}0 \times 10^{-8}$ M and $1{\cdot}2 \times 10^{-7}$ M acetylcholine respectively. In each frame the capacitative transient and the onset of the inward current have been retouched for photography. In *B* the peak inward or minimum outward current in response to each rectangular voltage-clamp pulse was measured as shown in the inset. Conventional current–voltage relations were then constructed without making corrections for leakage currents. The filled circles (●) represent the control current–voltage relation (TTX $2{\cdot}0 \times 10^{-6}$ g/ml.), the open circles (○) the current–voltage relation obtained during application of $3{\cdot}0 \times 10^{-8}$ M acetylcholine and the crosses (×) the current–voltage relation obtained in $1{\cdot}2 \times 10^{-7}$ M acetylcholine.

Effect of acetylcholine on the slow inward current

Figure 2 shows the results of a voltage-clamp experiment which was designed to test the hypothesis that acetylcholine reduces the slow inward (Ca^{2+}/Na^{+}) current in frog atrium.

The preparation was perfused with TTX Ringer, and 500 msec rectangular voltage-clamp pulses were applied from the resting potential (−80 mV) at a frequency of approximately 0·2 Hz. The peak inward or minimum outward current in response to each clamp pulse was measured and plotted as a conventional current-voltage relation, without correction for leakage currents.

After the control current–voltage relation had been obtained, a low concentration of acetylcholine ($3{\cdot}0 \times 10^{-8}$ M) was applied. This caused a marked reduction in the net slow inward current: its maximum magnitude being reduced to about half the control value. In separate experiments which measured the peak twitch height in atrial trabeculae, the first negative inotropic effect was observed when $1{\cdot}0 \times 10^{-9}$ M acetylcholine was applied; and $1{\cdot}0 \times 10^{-8}$ M acetylcholine reduced the twitch height to between 0·5 and 0·6 of the control value (B. E. Blood, unpublished communication).

Fig. 2 also shows that the acetylcholine effect is dose-dependent. Increasing the concentration approximately fourfold (from $3{\cdot}0 \times 10^{-8}$ M to $1{\cdot}2 \times 10^{-7}$ M) caused a further decrease in the slow inward current. Furthermore, the acetylcholine effect appeared to be rather selective for the slow inward current. No significant changes in the current–voltage relation negative to the threshold for the slow inward current were observed, and the three current–voltage relations intersected close to the estimated reversal potential for the slow inward current.

The reduction of the slow inward current by acetylcholine appears to be a typical muscarinic response. Thus, carbachol is approximately ten times more effective than acetylcholine in producing the effect, and the changes produced by both acetylcholine and carbachol can be blocked by atropine (W. Giles and S. J. Noble, unpublished).

However, atropine does not always produce a straightforward blockade of the acetylcholine-induced reduction on the slow inward current in frog atrium. Fig. 3 illustrates one such result, obtained in an experiment similar to the one described in conjunction with Fig. 2. In this case, $5{\cdot}0 \times 10^{-8}$ M acetylcholine completely abolished the net slow inward current. Subsequent application of atropine ($1{\cdot}0 \times 10^{-7}$ M) in the presence of acetylcholine ($5{\cdot}0 \times 10^{-8}$ M) resulted in a very considerable increase in the slow inward current. An increase in the slow inward current has also been observed following the application of atropine (10^{-5} to 10^{-8} M) alone. In

separate experiments, similar doses of atropine produced an increase in twitch tension (Blood, Giles & Noble, 1976). The most obvious way in which atropine could produce an increase in the slow inward current, and a positive inotropic effect, is by blocking the muscarinic action of endogenous acetylcholine. However, further experiments are needed to establish whether this is its primary mode of action.

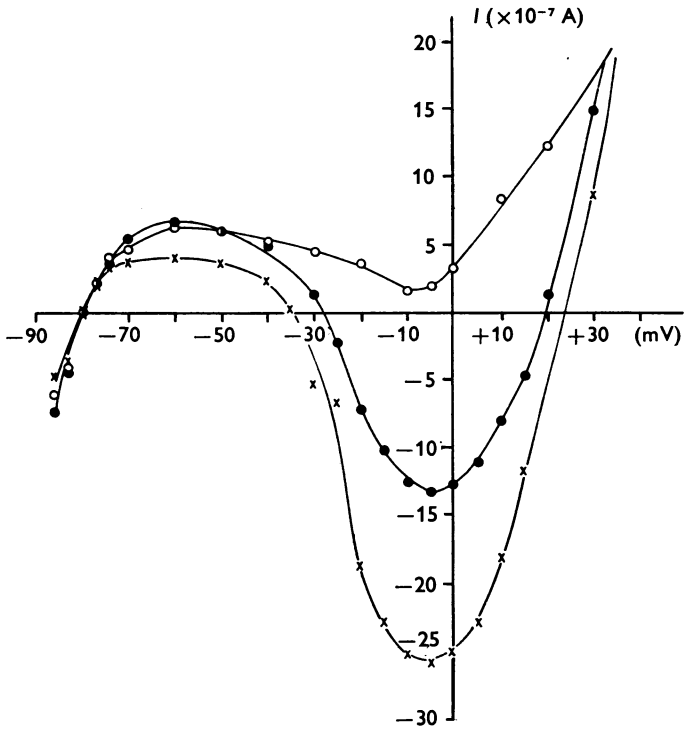

Fig. 3. Effects of acetylcholine and atropine on the slow inward (Ca^{2+}/Na^{+}) current. The protocol for this experiment, and the method of measuring and plotting the peak currents were the same as those described in the legend of Fig. 2. After the control current–voltage relation (●) was obtained (TTX $2{\cdot}0 \times 10^{-6}$ g/ml.), acetylcholine at $5{\cdot}0 \times 10^{-8}$ M (○) was applied. Finally, atropine at $1{\cdot}0 \times 10^{-7}$ M (×) was added in the presence of the acetylcholine. For further explanations see text.

Outward currents in acetylcholine

Careful inspection of Fig. 2 in the range of potentials negative to the resting potential shows that $1{\cdot}2 \times 10^{-7}$ M acetylcholine produced a small increase in inward current in that region of the current–voltage relation. Woodbury & Crill (1961), by replacing chloride with sulphate, have shown that in frog atrium the contribution of chloride ions to the increase in

membrane conductance produced by acetylcholine (approximately $5{\cdot}0 \times 10^{-6}$ M) is very small. Moreover, impalements in frog atrium with chloride-sensitive micro-electrodes indicate that the equilibrium potential for chloride ions is approximately -35 mV (Ladle & Walker, 1975). Therefore the most likely cause of the increased inward current is an increase in a

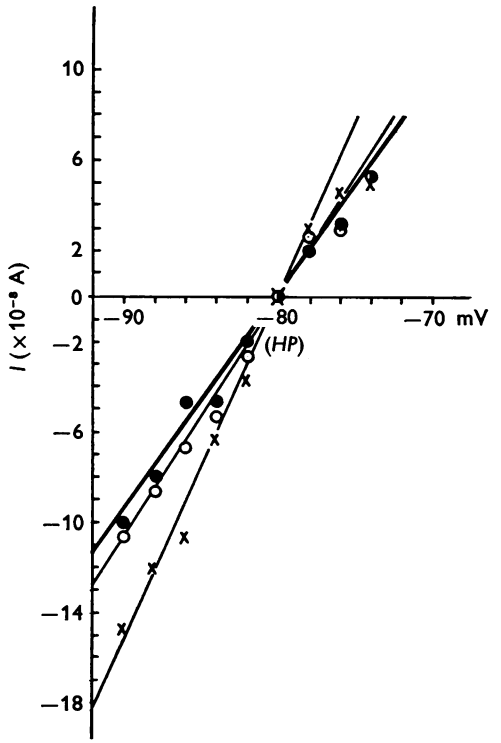

Fig. 4. Effect of acetylcholine on membrane conductance, measured near the resting potential (-80 mV). Small (2 mV steps), short (500 msec) rectangular clamp pulses were applied from -80 mV and the minimum inward or outward currents were recorded and plotted. The holding potential (HP) was -80 mV. No data positive to -75 mV were included because marked inward rectification was observed positive to that potential. Each set of points has been fitted with a straight line by eye. If the slope conductance is taken as 1 for the control (●), it is 1·13 in $6{\cdot}0 \times 10^{-8}$ M acetylcholine (○) and 1·62 in $1{\cdot}2 \times 10^{-7}$ M acetylcholine (×).

potassium current which has its reversal potential very close to the resting potential. Evidence for an increased potassium permeability in response to acetylcholine has been obtained from ^{42}K tracer experiments (Hutter, 1957). These results are the basis of the classical theory of cholinergic inhibition in cardiac muscle. We therefore attempted to

study the effects of acetylcholine on the time-independent (background) and time-dependent (delayed rectifier) outward currents.

Fig. 4 shows an experiment in which the membrane slope conductance was used as an indirect measure of the time-independent (background) current. The membrane potential was held at the resting potential and small rectangular voltage-clamp steps were applied. The current changes in

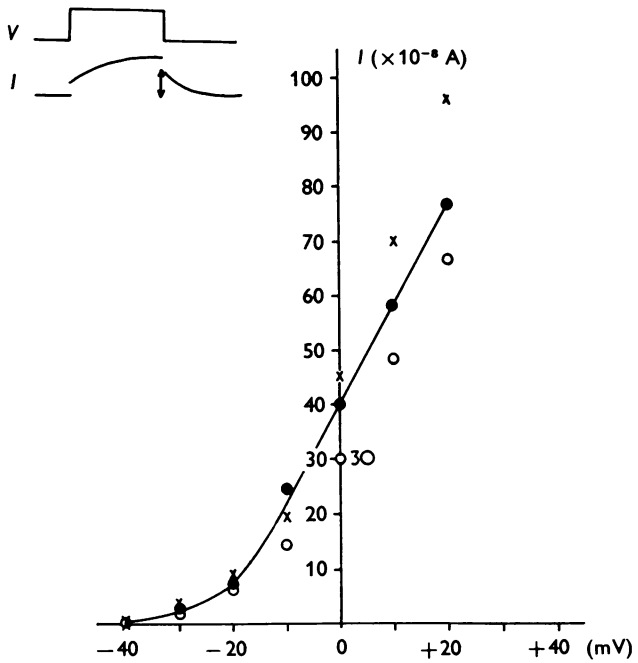

Fig. 5. Effect of acetylcholine on the time-dependent activation of outward current. As shown in the inset, long rectangular depolarizations were applied and the peak amplitude of the positive decay tail was plotted against the level of depolarization. The curve was fitted to the control (●) points by eye. Corresponding points in acetylcholine ($5{\cdot}0 \times 10^{-8}$ M), and in acetylcholine ($5{\cdot}0 \times 10^{-8}$ M) plus atropine ($1{\cdot}0 \times 10^{-8}$ M), are shown as open circles (○) and crosses (×) respectively. See text for details of the experiment.

response to each clamp step were measured at approximately 100 msec after the onset of the step and were plotted against voltage. Each set of data was fitted by a straight line to give the slope conductance. No slope conductance data were obtained positive to −75 mV because of marked inward rectification in that range of potentials. If the control slope conductance is taken as 1·0, then the corresponding values in $6{\cdot}0 \times 10^{-8}$ M and $1{\cdot}2 \times 10^{-7}$ M acetylcholine were 1·13 and 1·62 respectively. When the

data in Fig. 2 are considered in conjunction with Fig. 4, it is quite clear that the slow inward current is more sensitive to acetylcholine than is the background current. Thus, while $3{\cdot}0 \times 10^{-8}$ M acetylcholine produced a substantial reduction of the peak net slow inward current (Fig. 2), $6{\cdot}0 \times 10^{-8}$ M acetylcholine produced only a very small increase in background current (Fig. 4).

Since the time-dependent outward currents (which are carried mainly by potassium ions) are thought to generate repolarization in frog atrium (Giles, 1974; Brown, Clark & Noble, 1976*a*, *b*), it is possible that an increase in these membrane currents is responsible for the observed decrease in duration of the action potential (see Fig. 1). In Fig. 5, an experiment which measured the amount of activation of the time-dependent outward currents is shown. The data were obtained by measuring the peak amplitudes of the positive decay tails which followed long, rectangular depolarizations from the resting potential.

Changes in the reversal potential of these currents, caused by extracellular potassium accumulation, prevent a steady-state activation curve from being obtained (see Noble, 1976). The data in Fig. 5 represent 'composite activation curves'. Each point represents the largest decay tail observed in response to a given level of depolarization.

Acetylcholine ($5{\cdot}0 \times 10^{-8}$ M, which again markedly reduced the slow inward current), produced a small decrease in the amount of activation of time-dependent outward current in the plateau range of potentials. The size of this decrease in activation showed some variability, but a *decrease* in the activation of outward current cannot account for the shortening of the action potential by acetylcholine.

The results therefore suggest that acetylcholine produces two physiologically important changes in membrane currents in frog atrium: a decrease in the slow inward current; and a small increase in the time-independent background current. Fig. 6 shows an experiment in which these two changes were studied simultaneously. A current–voltage relation for the slow inward current was first obtained by a protocol identical to that described in conjunction with Fig. 2. The slow inward current was then completely blocked by application of 4 mM manganese. The difference, obtained by subtraction, between these two current–voltage relations represents the magnitude of the slow inward current and is plotted as (○) in Fig. 6*B*.

Kass & Tsien (1975) have reported that Mn^{2+} is not an ideal blocker of the slow inward current in sheep Purkinje fibres since it also alters the time-independent and time-dependent outward currents. In frog atrium, 4 mM-Mn^{2+} does not appear to have these side-effects, although other pharmacological agents, for example D-600 and verapamil, certainly do (W. Giles and S. J. Noble, unpublished).

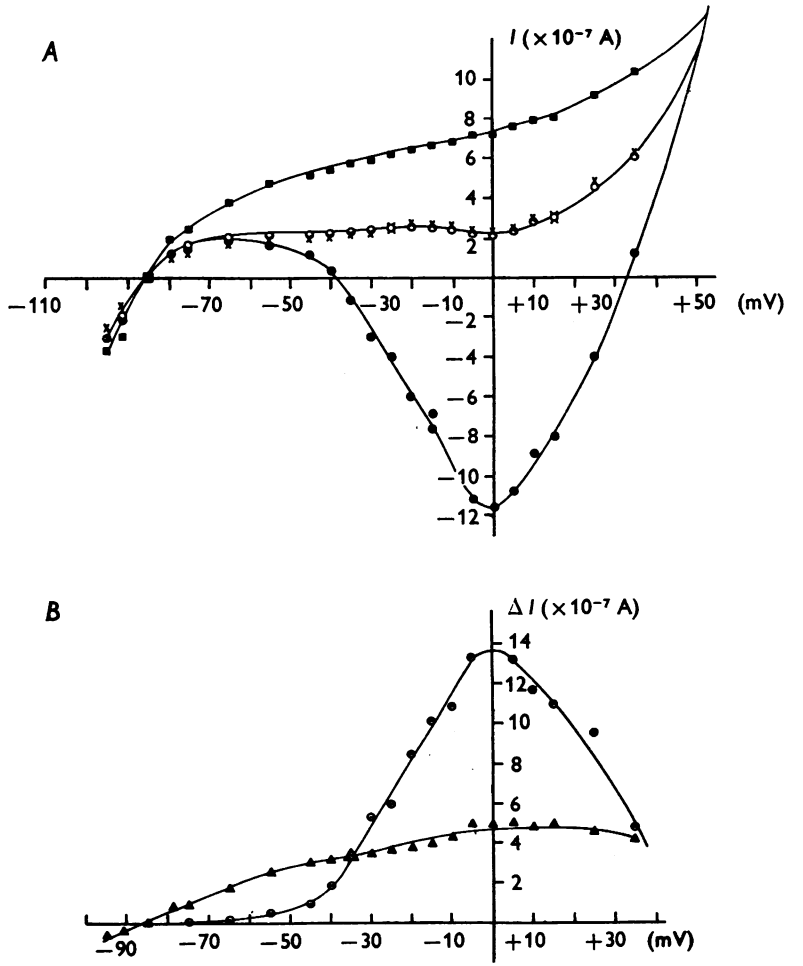

Fig. 6. Comparison of the current–voltage relations of the slow inward (Ca^{2+}/Na^{+}) current, and the outward (potassium) background current which is recorded after application of a relatively high concentration ($1{\cdot}0 \times 10^{-7}$ M) of acetylcholine. The data, obtained in TTX ($2{\cdot}0 \times 10^{-3}$ g/l.) Ringer, which are shown in *A*, represent: (i) the control (●) current–voltage relation for the slow inward current; (ii) the current–voltage relation obtained after the slow inward current was completely blocked by 4 mM-Mn^{2+} (○); (iii) the subsequent change in (ii) after $1{\cdot}0 \times 10^{-7}$ M ACh (■) was added; (iv) the blockage of the acetylcholine-induced outward current by atropine at $1{\cdot}0 \times 10^{-5}$ M (×). *B*, shows a direct comparison of the *I–V* relations for the slow inward current (shown as ⊙, and obtained by subtracting (ii) from (i) in *A*); and the acetylcholine-induced outward current (shown as ▲, and obtained by subtracting (iii) from (iv) in *A*). Note that the ordinate in this part of the Figure is current change (ΔI) rather than absolute units of current. For further description and explanation of this experiment see text.

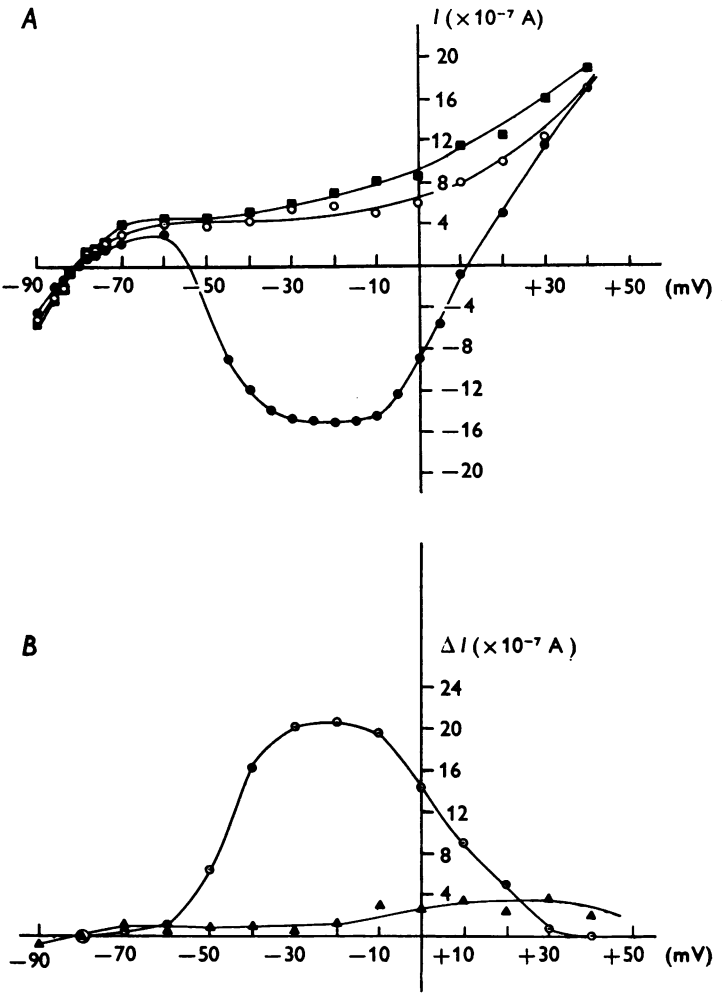

Fig. 7. Comparison of the current–voltage relations of the slow inward (Ca^{2+}/Na^{+}) current, and the outward (potassium) background current which is recorded after $5{\cdot}0 \times 10^{-8}$ M acetylcholine. As in Fig. 6, the experiment was run in TTX ($2{\cdot}0 \times 10^{-3}$ g/l.) Ringer, and the data in *A* show: (i) the control (●) current–voltage relation for the slow inward current; (ii) the current–voltage relation (○) obtained after the slow inward current was blocked by 5 mM-Mn^{2+}; (iii) the subsequent change in (ii) after $5{\cdot}0 \times 10^{-8}$ M acetylcholine was added, shown by (■). As in Fig. 6*B*, Fig. 7*B* shows a comparison of the current–voltage relations for the slow inward current (⊙), and the outward background current produced by $5{\cdot}0 \times 10^{-8}$ M acetylcholine (▲).

A rather large concentration of acetylcholine ($1{\cdot}0 \times 10^{-7}$ M) was then applied in the presence of the Mn^{2+}, and the currents in response to each depolarization were measured approximately 100 msec after the onset of the pulse. The filled squares in Fig. 6*A* show these responses and indicate that a significant increase in time-independent (background) outward current was observed. Finally, the current–voltage relation for this background current was obtained by taking the difference between the 'Mn^{2+} current–voltage relation' and the one obtained in the presence of acetylcholine. It has been plotted as filled triangles (▲) in Fig. 6*B*.

Fig. 6*B* shows that the acetylcholine-induced background current inwardly rectifies and that it has a value of zero at approximately -85 mV, suggesting that it is mainly carried by potassium ions. From this analysis, it is also quite clear that even relatively large doses of acetylcholine ($1{\cdot}0 \times 10^{-7}$ M) do not produce enough increase in outward background current to account for the observed reduction in net slow inward current.

In experiments similar to the one shown in Fig. 6, but in which smaller doses of acetylcholine were applied, only very small increases in background current were recorded. An example of such an experiment is shown in Fig. 7. These results support the indirect slope conductance measurements in Fig. 4, by showing that $5{\cdot}0 \times 10^{-8}$ M acetylcholine produces little increase in the time-independent outward currents in frog atrium. Furthermore, they are in agreement with the results of Trautwein, Kuffler & Edwards (1956) which showed that the lowest concentration of acetylcholine which would significantly decrease the space constant of quiescent frog atrium was approximately $5{\cdot}0 \times 10^{-7}$ M.

DISCUSSION

The main conclusion from these experiments is that low concentrations of acetylcholine cause a selective and marked decrease in the magnitude of the slow inward (Ca^{2+}/Na^{+}) current in frog atrial muscle. A number of previous radioisotope and electrophysiological experiments provide support for this finding.

Grossman & Furchgott (1964) and Hoditz & Lüllman (1964) independently reported that application of acetylcholine to guinea-pig atria produced a significant decrease in the component of ^{45}Ca uptake which was related to the mechanical activity of the preparation. In addition, Paes de Carvalho *et al.* (1969) found that acetylcholine decreased the calcium-sensitive secondary depolarization in rabbit atrium. Recently, Prokopczuk, Lewartowski & Czarnecka (1973) have shown that acetylcholine and manganese ions have very similar and additive effects on the electrical and mechanical activity of rabbit atrium, and have hypothesized

that a reduction in transmembrane calcium influx may be an important factor in the negative inotropic effect of acetylcholine.

Our results provide the first direct evidence for this hypothesis. Since they were reported (Giles, 1974; Giles & Tsien, 1975), somewhat similar findings have been published by Ikemoto & Goto (1975).

A reduction in the slow inward (Ca^{2+}/Na^{+}) current would be expected to have a negative inotropic effect in atrial muscle. Marked reductions in net slow inward (Ca^{2+}/Na^{+}) current and in peak twitch height were observed at acetylcholine doses greater than approximately $2{\cdot}0 \times 10^{-8}$ M in the present experiments. Vassort & Rougier (1972) and Einwächter, Haas & Kern (1972) have shown that the influx of calcium and sodium during the activation of the slow inward current triggers and controls the phasic component of tension development in frog atrium. Our results therefore provide a straightforward explanation for the negative inotropic action of acetylcholine in the atrium.

This explanation, however, may not be applicable to the tonic component of mechanical activity in atrial muscle. The development of tonic tension is not directly related to the activation of the slow inward current (Einwächter *et al.* 1972). Therefore, acetylcholine may not produce a reduction in tension under certain experimental conditions. For example, activation of contraction by repetitive stimuli, or by maintained depolarizations, both favour tonic tension development and may be insensitive to acetylcholine (see Antoni & Rotmann, 1968; Ikemoto & Goto, 1975).

A second important result from the present experiments is the demonstration that the slow inward (Ca^{2+}/Na^{+}) current is markedly reduced by concentrations of acetylcholine (approximately $5{\cdot}0 \times 10^{-8}$ M) which do not significantly increase the background (potassium) current. Furthermore, even when relatively high doses of acetylcholine (greater than 10^{-7} M) are applied, the current–voltage relation of the acetylcholine-sensitive background current inwardly rectifies. Therefore relatively little net outward current is added in the plateau range of potentials. This indicates that the reduction in net slow inward (Ca^{2+}/Na^{+}) current probably is an important factor in the shortening of the atrial action potential which is produced by acetylcholine.

It is important to note that rather high concentrations of acetylcholine have been applied in most previous reports of cholinergic effects on the rate of ^{42}K loss from loaded cardiac muscle. Harris & Hutter (1956) and O. F. Hutter (1957, 1961 and personal communication) applied between $2{\cdot}0 \times 10^{-7}$ M and $1{\cdot}0 \times 10^{-6}$ M acetylcholine. Although significant changes (approximately threefold) occurred in the rate of ^{42}K loss from *sinus* tissue, only very small rate changes (approximately 1·3 times the control) were observed in frog and tortoise *atria* (Hutter, 1957). Rayner &

Weatherall (1959) did similar ^{42}K wash-out experiments on rabbit atria and reported that even $2{\cdot}0 \times 10^{-5}$ M acetylcholine produced only very small increases (1·2–1·4 times the control rate) in the rate of ^{42}K loss. These radioisotope results therefore agree with our voltage-clamp measurements in indicating that even large doses of acetylcholine produce only very small changes in background (potassium) current.

It is interesting that the two autonomic transmitters in the frog, adrenaline and acetylcholine, appear to have exactly opposite actions on the slow inward (Ca^{2+}/Na^{+}) current. Vassort, Rougier, Garnier, Sauvait, Coraboeuf & Gargouil (1969) have previously shown that adrenaline produces a significant increase in the slow inward (Ca^{2+}/Na^{+}) current in frog atria. However, the effects of the two transmitters on the time-dependent outward currents in frog atrium do not show such a clearcut contrast. Thus, although Brown, McNaughton, Noble & Noble (1975) have shown that adrenaline produces a marked increase in time-dependent outward current, the present results indicate that acetylcholine produces only small and somewhat variable changes (see Fig. 5 and related text).

Even high doses of acetylcholine produce only a very small hyperpolarization (2–5 mV) of atrial muscle (Fig. 1*A*). The magnitude of this hyperpolarization is presumably limited by $(E_m - E_K)$. In frog atrium a recent measurement of $(E_m - E_K)$ made by using potassium-selective micro-electrodes indicates that this difference is very small, approximately 3 mV (Walker & Ladle, 1973). However, Walker & Ladle have also shown that $(E_m - E_K)$ in frog sinus venosus is much larger than in the atrium, approximately 33 mV. This result is in accordance with two well known responses of sinus tissue to vagal stimulation: (1) a rather large hyperpolarization (del Castillo & Katz, 1955; Hutter & Trautwein, 1956), and (2) a two- to threefold increase in the rate of ^{42}K efflux (Harris & Hutter, 1956). It is therefore quite possible that the negative, *chronotropic* response of the frog heart is produced by a selective increase in sinus potassium permeability. The question of whether or not the slow inward (Ca^{2+}/Na^{+}) current is also reduced by acetylcholine in the frog sinus venosus cannot be answered until it is possible to do appropriate voltage-clamp experiments with sinus tissue.

Note added in proof

Since this paper was submitted, Ten Eick, Nawrath, McDonald & Trautwein (1976) have reported a single sucrose-gap study of the effects of acetylcholine on membrane currents and twitch tension in a variety of mammalian atria. Several of their results disagree with those of the present study. They have found that approximately 10^{-7} M acetylcholine increases the time-independent background current, but has no effect on the slow

inward current, or on tension elicited by voltage-clamp depolarizations. Higher doses of acetylcholine (about $5{\cdot}1 \times 10^{-6}$ M) were needed to reduce the slow inward current and the twitch tension.

The reasons for the discrepancies between these results and our own are not entirely clear. Two points are, however, worth noting.

In frog atrium, $5{\cdot}0 \times 10^{-8}$ M acetylcholine markedly reduces the secondary, 'Ca^{2+} – dependent', depolarization phase of the action potential. A similar result has been reported for rabbit atrium by Paes de Carvahlo *et al.* (1969). Ten Eick *et al.* report that their preparations never regeneratively depolarize beyond − 10 mV. Their control action potentials therefore have no conventional plateau phase, and may have an abnormally small slow inward (Ca^{2+}/Na^{+}) current.

In order to obtain an estimate of the peak of slow inward current as a function of membrane potential, Ten Eick *et al.* use an extrapolation procedure (see their Fig. 2). However, in the absence of a control experiment in which the slow inward current is selectively abolished and the outward time-independent (background) current is studied in isolation; it is very difficult to distinguish between a *decrease* in slow inward current, and an *increase* in the outward background current (see their Fig. 3). Furthermore, a substantial increase in background current would *not* be expected on the basis of radioisotope (^{42}K) data in mammalian atria. Rayner & Weatherall (1959) showed that even $2{\cdot}0 \times 10^{-5}$ M acetylcholine produced only very small (1·2–1·4 times) increases in the rate of ^{42}K efflux from rabbit atria.

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APPENDIX

The influence of non-uniformity on the analysis of the slow inward current

The purpose of this Appendix is to determine whether the current–voltage relations described in this paper are likely to be significantly distorted by the voltage non-uniformities that must occur between the voltage-recording and current-passing ends of the test-gap region. To do this we used a computer program based on that used in a previous paper to analyse the influence of non-uniformity on the outward potassium currents (Brown *et al.* 1976*c*). As shown in that paper, the test gap is taken to be

half a space constant in length. The program solves the cable differential equation:

$$\frac{1}{r_a}\frac{\partial^2 V}{\partial x^2} = C\frac{\partial V}{\partial t} + i_1. \tag{1}$$

where r_a is the axial resistance per unit length of cable, V is the potential, x is distance, t is time, C is the capacitance per unit length and i_1 is the ionic current per unit length.

The latter is the sum of two components:

$$i_1 = i_{K_1} + i\,(V, t), \tag{2}$$

where i_{K_1} is the background potassium current. This was represented by a polynomial expression that reproduces the mixture of inward and outward going rectification displayed by i_{K_1}:

$$i_{K_1} = g_m(V - 3V^2 + 2{\cdot}5V^3), \tag{3}$$

where g_m is the resting membrane conductance.

The unit of V is taken to be 100 mV. $i(V, t)$ is a time and voltage dependent current given by equations of the Hodgkin–Huxley type:

$$i(V, t) = \bar{g}\, y\, (E - E_{rev}),$$

$$\frac{dy}{dt} = \alpha\,(1-y) - \beta\, y,$$

where

$$\alpha = 0{\cdot}005 \exp\,((V-75)/20),$$

and

$$\beta = 0{\cdot}005 \exp\,(-(V-75)/20).$$

In the previous paper for analysing potassium currents, E_{rev} was set to 0 (the resting potential). In this paper, to reproduce inward currents we set E_{rev} to +120 mV. If the resting potential is taken to be −80 mV this gives a reversal potential of +40 mV for the Ca^{2+}/Na^{+} current.

Calculations of the peak currents in response to voltage-clamp steps were then performed for various values of $\bar{g}$. The resulting current–voltage relations are shown in Fig. 8. The values of $\bar{g}$ shown are expressed with respect to g_m, e.g. a value of $\bar{g} = 5$ means that the maximum value of the inward current conductance is five times the resting conductance. It can be seen that the curves show relatively little distortion even when $\bar{g} = 10$. This value of $\bar{g}$ is larger than that corresponding to any of the results shown in the present paper. The maximum distortion is a 10 mV shift in the peak current potential (from 0 mV at $\bar{g} = 1{\cdot}25$ to +10 mV at $\bar{g} = 10$). The maximum degree of non-uniformity (at the peak inward current value) was 21 mV ($\bar{g} = 10$), 9 mV ($\bar{g} = 5$), 4 mV ($\bar{g} = 2{\cdot}5$), 1·5 mV ($\bar{g} = 1{\cdot}25$). The set of curves is clearly very similar to those obtained in Figs. 2 and 3, where $\bar{g}$ is reduced or increased by acetylcholine or atropine. It is

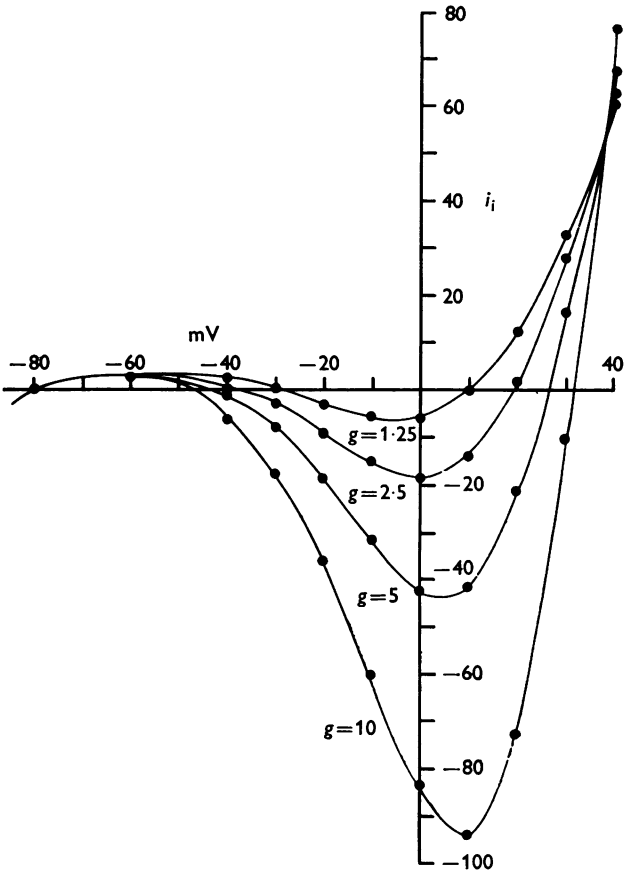

Fig. 8. Plots of computed current–voltage relations for the Ca^{2+}/Na^{+} current corresponding to four different values of ($\bar{g}$). Note that, in the case of $\bar{g} = 10$, the ratio between the maximum outward current developed between −80 and −40 mV and the maximum inward current at +10 mV is 30 to 1. The corresponding values for the control current–voltage curve in Fig. 2 is 7·4 to 1, and in Fig. 3 it is 2 to 1. The current units are arbitrary in this Figure.

interesting to note that the extent of axial non-uniformity produced by *inward* current flow (9 mV at $\bar{g} = 5$) is substantially less than that obtained for the *outward* currents (60 mV at $\bar{g} = 5$ see Brown *et al.* 1976*c*).

In conclusion, it is unlikely that the current–voltage relations described in this paper are significantly distorted by axial voltage non-uniformities. Distortion by radial non-uniformity, however, cannot be assessed using this computer program, since this would require knowledge of the access resistance which is not yet available.

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