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# ELECTROMECHANICAL STUDIES ON THE INOTROPIC EFFECTS OF ACETYLSTROPHANTHIDIN IN VENTRICULAR MUSCLE

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SUMMARY

1. Three phases in the inotropic response of acetyl strophanthidin (AcS) on the electromechnical activity of the frog ventricular myocardium were identified and studied using a single sucrose voltage-clamp technique and other conventional electrophysiological methods.

2. The positive inotropic response of the drug was accompanied by a shift in tension–voltage relation, so that more tension developed with every depolarization step above the mechanical threshold (−50 mV). Only at higher drug concentrations or with long exposure times did the mechanical threshold shift to more negative membrane potentials (−60 to −70 mV).

3. In tetrodotoxin-treated muscles AcS produced marked potentiation of twitch tension and an appropriate shift in the tension–voltage relation.

4. The positive inotropic response of the drug was not related to the magnitude of the direction of the fast or slow Na current.

5. In tetrodotoxin-treated ventricular strips the direction or the magnitude of the secondary inward current ($I_{Ca}$ or $I_{Na}$) were not related to the inotropic effect of AcS.

6. AcS shortens the action potential markedly during the later stages of its positive inotropic response. When $Ca^{2+}$ is omitted from the bathing solution AcS not only fails to shorten the action potential, but often prolongs it.

7. The shortening of the action potential in the presence of AcS is accompanied by an increase in the 'instantaneous' membrane conductance both at rest and during the time course of the plateau.

8. The decline in the positive inotropic response of the drug was accompanied by the shortening of the action potential. Electrical or chemical prolongation of the action potential restored the full positive inotropic response if the membrane had not depolarized.

9. Membrane depolarization and the development of diastolic tension

always occurred at later stages of drug action. Elevation of $[Mg^{+2}]_o$ to 5 or 10 mM prevented or suppressed the membrane depolarization and the diastolic tension.

10. KCl-induced contractures were potentiated throughout the duration of drug exposure. The tonic component of the contracture tension was markedly elevated especially at later stages of drug action.

11. The experimental evidence suggests that no unitary mechanism could account for multiple actions of acetyl strophanthidin. However, the contributions of the Na pump, the $Ca^{+2}$ sequestering system, and the K-efflux system to the various stages of drug action are discussed.

## INTRODUCTION

The similarity in the effects of digitalis and calcium on the contraction of heart muscle (Loewi, 1917; Wilbrandt, 1958) and the enhancement of calcium influx by digitalis (Holland & Sekul, 1961; Grossman & Furchgott, 1964) have led to the suggestion that the inotropic action of digitalis is mediated through alterations of the excitation–contraction coupling processes. Two major possibilities have been considered to explain how such alterations can produce the inotropic effect of the drug on cardiac muscle: (1) interaction of digitalis with the surface membrane producing (*a*) direct increase in the calcium current found to be responsible for activation of contraction (Beeler & Reuter, 1970*a*; Vassort & Rougier, 1972); (*b*) indirect increase in Ca influx by modification of intracellular $Na^+$ concentration as a result of inhibition of the $Na^+$ pump (Langer & Serena, 1970; Langer, 1968) or alterations in the membrane conformation or the affinities of $Na^+$ pump (Schwartz, 1974); (2) interaction of digitalis with the relaxing systems causing an altered rate of activator uptake or release, and in turn potentiating the contractile state (Lee & Choi, 1966; Klaus & Lee, 1969; Lee & Klaus, 1971; Stam, Weglicki, Gertz & Sonnenblick, 1973).

The experiments to be described in this report were designed to examine the extent to which any of the above postulated processes contribute to the inotropic effect of the drug. Frog ventricular muscle was used for its well-documented lack of t-tubular system, fairly sparse sarcoplasmic reticulum (Staley & Benson, 1968; Page & Niedergerke, 1972) and for the observation that the electrical activity of the surface membrane directly controls the contractile response (Antoni, Jacob & Kaufmann, 1968; Morad & Orkand, 1971). Acetyl strophanthidin, a rapidly acting digitalis analogue (onset of action 30–60 sec), was used in these experiments.

A preliminary report of this work has been presented (Morad & Greenspan, 1973).

## METHODS

*Preparation.* Quiescent ventricular strips, 0·4–0·7 mm in diameter and 3·0–5·0 mm in length, were dissected from frogs (*Rana pipiens*) maintained under ultraviolet light at room temperature. In some experiments, matched ventricular strips were obtained from the same circular portion of an excised ventricle. Thinner strips (0·3–0·4 mm in diameter) were found to yield larger and more reproducible contracture tensions when exposed to solutions containing 100 mM-KCl. All experiments were conducted at 22–24° C. In the voltage-clamp experiments, strips ranging in diameter from 0·3–0·5 mm, and shorter than 0·5 mm in length were used.

*Solutions.* The main solutions used had the following composition in m-mole/l: (1) *normal Ringer:* NaCl 116, $NaHCO_3$ 2·0, KCl 3·0, $CaCl_2$ 0·2–1·0, pH 7·4–7·6; (2) *KCl-Ringer:* NaCl 116, $NaHCO_3$ 2·0, KCl 100, $CaCl_2$ 0·2–1·0. This solution was made hypertonic to maintain a constant $Na^+$ concentration, and to avoid the complications arising from variation of the $[Ca]_o/[Na]_o^2$ (Lüttgau & Niedergerke, 1958); (3) *zero-Ca Ringers:* NaCl 116, $NaHCO_3$ 2·0, KCl 3·0. Ca concentration in this solution was determined spectrophotometrically to be $1·0 \times 10^{-6}$ M. Mg was omitted from all of these solutions. In experiments where the effect of magnesium ions was examined, $MgCl_2$, 1·0–10·0 m-mole, was added to a litre of prepared Ringer solution without correcting for changes in osmolarity.

*Experimental set-up.* Ventricular strips were suspended in a 0·3 ml. Lucite chamber adapted for rapid perfusion. The strips were either pinned to the base of the chamber, e.g. in the 0-Ca experiments where intracellular electrical measurements were made, or were attached to an isometric tension transducer and perfused at a constant rate of 0·5 ml./min. Perfusion rates were increased to 8–10 ml./min during KCl contracture experiments. Stimulating current was applied between two large Ag/AgCl electrodes connected to the output of a Grass stimulator (model S44). The preparation was stimulated uniformly throughout the experiment at a constant frequency (6 or 12 shocks/min) with pulses 1–4 msec in duration and about 1·5 times threshold. All ventricular strips were equilibrated under these conditions for 1·0 hr in normal Ringer solution before any experimental manipulations. Isometric tension was recorded with a force transducer (Endovco model S8701–2). Transmembrane potential was measured with a standard 3·0 M-KCl-filled glass micro-electrode connected to the input of a negative capacitance preamplifier (Zeltex 132Z). The output of the tension transducer and cathode follower were displayed on a storage oscilloscope (Tektronix model 564). Tension traces were also displayed on a pen recorder (Brush model 220). Voltage-clamp studies were carried out using a 'hybrid' sucrose-gap technique, essentially similar to that described by Morad & Orkand (1971).

### *Criteria and precautions*

(1) *Mechanical measurements.* Ventricular strips showing spontaneous activity, fluctuating base-line tension, and an abnormally long relaxation phase were considered unsuitable for these studies. In experiments where the muscle was exposed to high concentrations of KCl, a number of factors known to alter the rate of development and amplitude of contracture tension were closely controlled (e.g. stimulus frequency was maintained at 6/min, Ca concentration 1·0 mM, perfusion rate 8–10 ml./min). Thicker strips, 0·7–1·0 mm in diameter, produced small contractures relative to twitch tension (contracture/twitch = 0·33–0·50). Thinner strips 0·3–0·4 mm in diameter, yielded much larger contractures (contracture/twitch = 0·8–1·2). It was felt that the thinner strips allow for more rapid and thorough access of KCl to the individual muscle fibres.

(2) *Electrical measurements.* A ventricular strip was considered experimentally suitable when the membrane resting potential was more negative than $-75$ mV, and the stimulated action potential had an overshoot potential of $+20$ to $+30$ mV and a duration of 500–800 msec. Whenever possible, continuous impalement of a single cell was maintained. This was often difficult due to the marked increase in contraction of the muscle when exposed to AcS. In such cases, multiple impalements in various parts of the preparation were made to assure the validity of the measurements.

(3) *Voltage-clamp experiments.* In experiments where the membrane potential of a ventricular strip was electrically controlled, stringent criteria were set to assure spacial and temporal homogeneity during a voltage-clamp step. These criteria are essentially similar to those described by Morad & Orkand (1971). Some of the problems encountered which would lead to artifacts if not corrected were: (*a*) persisting activation of tension in the muscle segment bathed in sucrose; (*b*) leakage of current in extracellular pathways across the sucrose gap; (*c*) inhomogeneous voltage distribution in the segment of the ventricular strip in the test gap. These problems were dealt with according to the guidelines set by Morad & Orkand (1971). Extra care was taken to use thinner and shorter preparations than those generally considered acceptable to improve the clamping capabilities. For a more detailed discussion of the problems encountered in voltage clamping with the hybrid sucrose-gap technique, consult Morad & Trautwein, 1968; Morad & Orkand, 1971; Johnson & Lieberman, 1971; Reuter, 1973; Y. Goldman & M. Morad, 1975 (to be published).

*Drugs.* The following drugs were used in these experiments: (1) acetyl strophanthidin (AcS, E. Lilly), $5{\cdot}0 \times 10^{-6}$ to $1{\cdot}0 \times 10^{-4}$ M; (2) tetrodotoxin (TTX), Sigma, $1{\cdot}0 \times 10^{-6}$ M/l.; (3) tetraethylammonium chloride (TEA, Eastman), 5·0–20·0 mM; (4) acetylcholine (Sigma), $1{\cdot}0 \times 10^{-6}$ M. Atropine sulphate (Schwarz/Mann), $1{\cdot}0 \times 10^{-5}$ M and propranolol hydrochloride (Sigma), $1{\cdot}0 \times 10^{-6}$ M were added in a number of experiments to block cholinergic and adrenergic neurotransmitter activity.

## RESULTS

### *Electromechanical effects of acetyl strophanthidin*

*Time-dependent effects of acetyl strophanthidin.* A continuous recording of developed tension from a beating frog ventricular strip exposed to acetyl strophanthidin ($5 \times 10^{-6}$ M) is shown in Fig. 1. Three empirical phases of drug action can be described. The duration of each phase was found to be dose-dependent, and varied slightly from preparation to preparation at a given dose. For the phases of drug action described below, the drug concentrations were $5 \times 10^{-6}$ to $1{\cdot}0 \times 10^{-5}$ M. Higher concentrations generally shortened the duration of each phase of the drug action.

During the first 10–30 min of drug exposure, the rate of development and peak amplitude of contraction are increased (Fig. 1, trace *B*), and the time-to-peak of tension is unchanged or slightly prolonged. The rate of relaxation at this stage of the drug action is not significantly altered and the resting tension remains unchanged. No change in the transmembrane resting or overshoot potential is seen in this initial phase. However, the configuration of the action potential is altered, such that the average

plateau potential is decreased, despite the absence of any significant change in action potential duration. Occasionally, a small transient increase in the action potential duration is observed.

From 15 to 80 min of drug exposure the rate of development of tension remains elevated, but the amplitude and time-to-peak, as well as the rate of relaxation of twitch tension decreases. Resting tension begins to increase

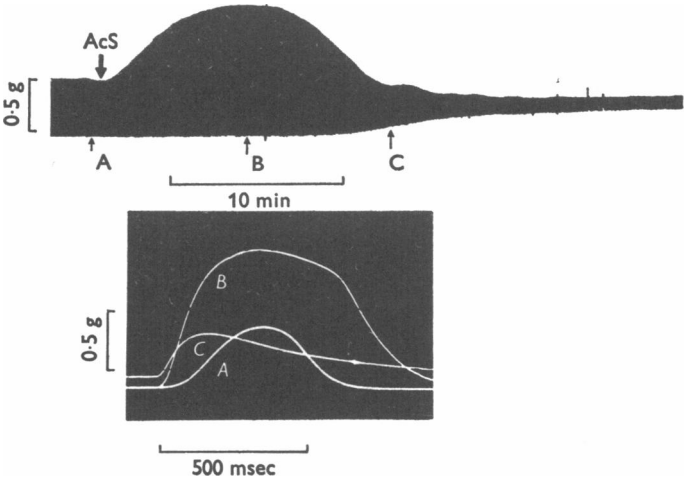


Fig. 1. Contractile response of a ventricular strip to AcS. Shortly after exposure of strip to AcS ($5 \times 10^{-6}$ M, heavy arrow), contractile force increases. Selective records are taken at points *A* (control), *B* (peak of positive inotropic effect), *C* (toxic state) and are superimposed oscilloscopically. Temperature 23° C. Strip diameter 0·5 mm; $[Ca]_o = 1$ mM.

as the twitch amplitude declines (Fig. 1*C*). Resting and overshoot potential decline as the action potential duration shortens markedly (Fig. 2*B*). The changes in the plateau configuration mentioned previously progress as the action potential duration shortens (Fig. 2*C*). Similar changes with other digitalis analogues have been previously reported (Woodbury & Hecht, 1952).

Exposure of the ventricular strip for longer than 80 min causes a decrease in the rate of development and amplitude of contraction. The resting potential and action potential duration progressively decrease, eventually producing action potentials 50–60 mV in amplitude and 100 msec in duration (Fig. 2*C*). In Fig. 3, time-to-peak, amplitude, and rate of relaxation of twitch tension are all plotted as functions of the duration of drug exposure. The onset and time course of the decline of the three parameters are similar. With short exposures of ventricular strips to AcS, the foregoing effects are partly reversible. However, long exposure

times seem to cause irreversible alterations in the excitation-contraction coupling processes of the myocardium.

*Calcium-dependent effects of AcS.* The development and decline of the positive inotropic response to ACS is 2–5 times more rapid in strips bathed

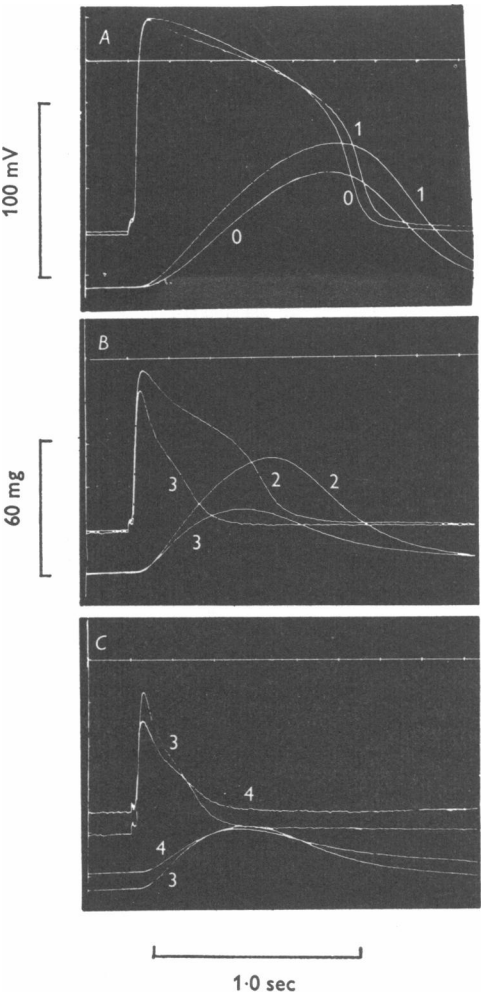


Fig. 2. Contractions and action potentials recorded from a ventricular strip in normal Ringer (traces, 0) and after 5 min of exposure to AcS, $5 \times 10^{-6}$ M (traces 1, panel *A*); after 25–35 min (panel *B*); and after 60–80 min (panel *C*, traces 3 and 4). The shortening of action potential occurs long before the decrease of $dp/dt$ seen in panel *C*. Membrane depolarization and development of diastolic tension, as well as a decrease in relaxation rate, can be seen in panel *C*. Temperature 23° C. $[Ca]_o$ = 1 mM. Corresponding contractions and action potentials have been appropriately numbered.

in Ringer solution containing 1·0 mM-$Ca^{2+}$ than in 0·2 mM-$Ca^{2+}$. In strips bathed in Ringer containing 1·0 mM-$Ca^{2+}$ the percentage increase in the rate of development, amplitude, and rate of relaxation of contraction during the inotropic effect was less than in strips equilibrated in 0·2 mM-$Ca^{2+}$. However, the percentage increase in resting tension was greater in 1·0 mM-$Ca^{2+}$ than in 0·2 mM-$Ca^{2+}$. In this range of $[Ca]_o$, no appreciable differences in the effect of AcS on the rate of repolarization of the plateau was observed.

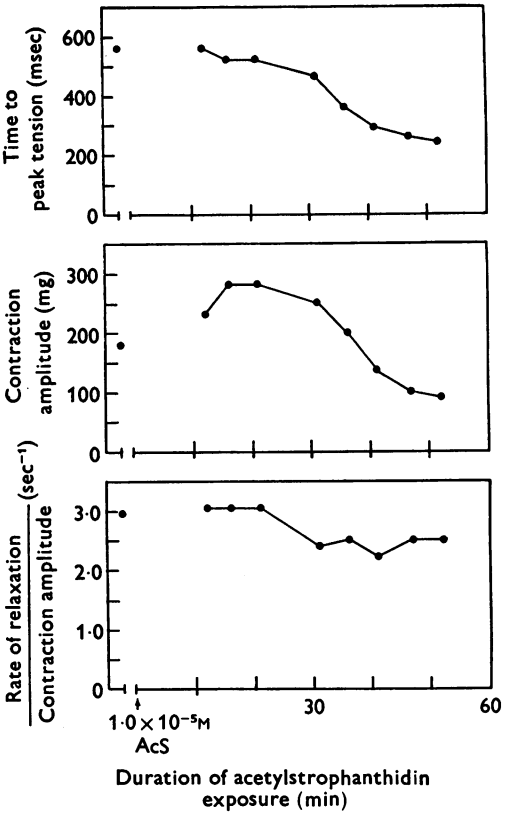


Fig. 3. The effect of AcS ($1·0 \times 10^{-5}$ M) on three parameters of contractile activity in a frog ventricular strip. Time to peak tension in this preparation is directly related to the duration of action potential. The decrease in the rate of relaxation of tension (normalized for contraction amplitude) occurs after the positive inotropic effect of the drug. Temperature 22° C. $[Ca]_o$ = 1 mM.

The marked shortening of the action potential duration occurring at the later stages of drug action was consistently observed in 0·2 or 1·0 mM-$Ca^{2+}$ containing solutions. In contrast, the action potential shortening effect of

AcS was not observed in ventricular strips equilibrated in 'zero-Ca' Ringer. It has been reported that perfusion of frog ventricular strips with 'zero-Ca' solutions causes marked prolongation of the action potential (Harris & Morad, 1971; Garnier, Rougier, Gargouil & Coraboeuf, 1969; Harris & Morad, 1975). When such a preparation was exposed to high concentrations of AcS ($5 \times 10^{-6}$ to $1{\cdot}0 \times 10^{-5}$ M) for prolonged periods, the action potential failed to shorten (Fig. 4). In fact, AcS often prolonged the action potential in 'zero Ca' solutions (Figs. 4, 5). In Fig. 5, the action potential duration of nine ventricular strips perfused with 'zero Ca' Ringer and exposed to AcS were plotted versus the stimulus frequency. It can be seen that AcS not only fails to shorten the action potential at any frequency tested, but seems to prolong the action potential particularly at lower frequencies.

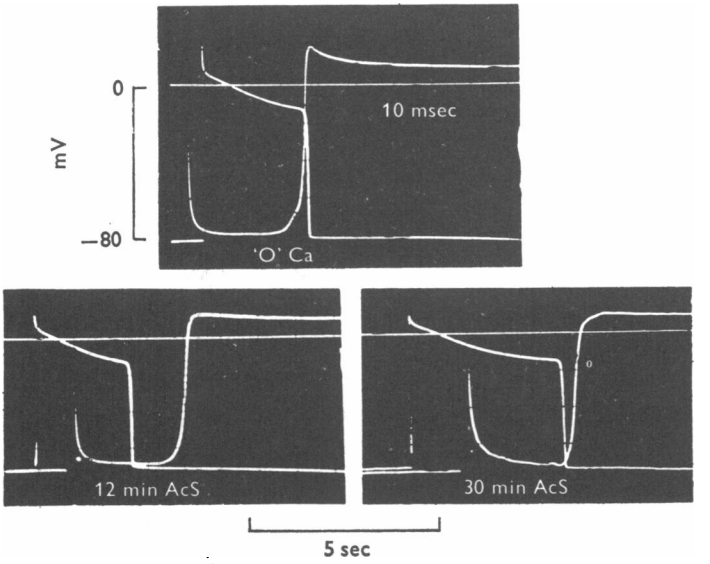


Fig. 4. Intracellular recording of transmembrane potential obtained from a frog ventricular strip continuously perfused with 'zero 'Ca Ringer for 2 hr at 23° C, and then exposed to AcS ($10^{-5}$ M). Action potential, and on expanded time scale, the upstroke of the action potential are recorded after various times of exposure (as indicated on each panel) to the drug. Note that the typically long 'zero Ca' action potentials is at first little affected, but later prolonged in the presence of AcS (compare with Fig. 2, effect of AcS in presence of $Ca^{2+}$). Frequency of stimulation 6/min.

*Experiments to separate the three stages of drug action*

The effects of AcS on the myocardium shown in Figs. 1 and 2 were divided arbitrarily into three phases. In the section to follow, we shall describe experiments in justification of such a separation of the drug action.

*Membrane depolarization and the development of resting tension.* Exposure of ventricular strips to normal Ringer solution containing $5{\cdot}0 \times 10^{-6}$ M to $1{\cdot}0 \times 10^{-5}$ M AcS for longer than 30 min always induced depolarization of the membrane and the development of diastolic tension. Fig. 6 (open circles) shows the effect of AcS on the resting potential of ventricular

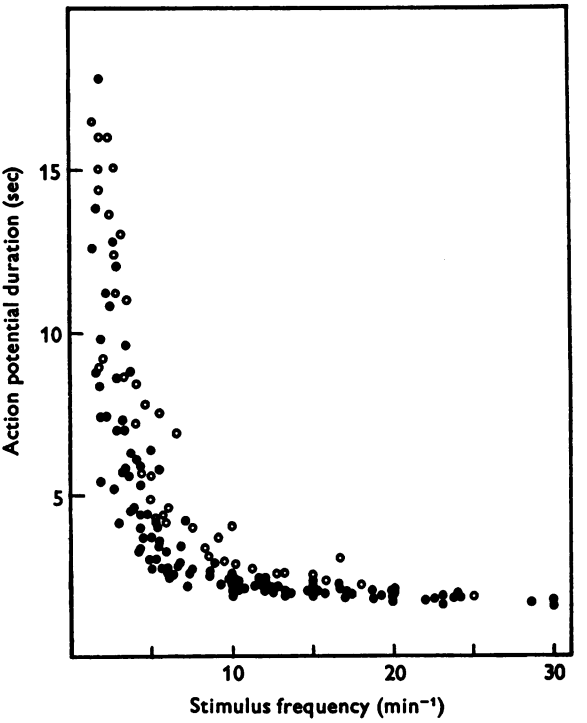


Fig. 5. Comparison of the effect of stimulus frequency on the action potential duration in the presence (open circles) and absence of AcS (filled circles) obtained from ventricular strips bathed in 'zero Ca' Ringer solution for 2–3 hr. Data obtained from nine experiments are summarized in the graph. Note that the action potential duration is consistently prolonged in presence of AcS, the effect being minimal at high frequencies. The duration of action potential was measured by convention from the onset of depolarization to the point where a line drawn tangent to the inflexion of fast repolarization intersected the resting potential (Niedergerke & Orkand, 1966). Temperature 23° C. [AcS] = $10^{-5}$ M.

strips bathed in normal Ringer solution. After 100 min of drug exposure, the resting potential decreased from $-80$ to $-60$ mV in ten ventricular strips. When ventricular strips were equilibrated in Ringer solution containing 5–10 mM-$MgCl_2$ and then exposed to AcS, the preparation failed to depolarize significantly (from $-85$ to $-80$ mV over 100 min in seven

ventricular strips). Removal of $MgCl_2$ from the bathing solution caused the membrane to depolarize rapidly to around $-60$ mV in seven ventricular strips (Fig. 6, filled circles). The marked shortening of the action potential seen in the toxic stages of the drug action was not influenced by the concentration of Mg ions in the bathing medium.

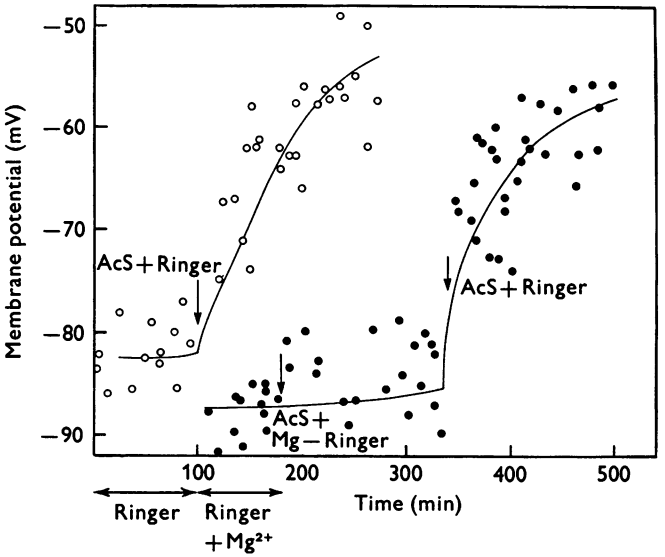


Fig. 6. Resting membrane potentials measured from four ventricular strips, two of which were equilibrated in normal Ringer solution (open circles) and two others in Ringer containing 10 mM-$Mg^{2+}$ (filled circles), both before and during the first 160 min of exposure to AcS ($5 \times 10^{-6}$ M). The muscle chamber contained two matched strips from the same ventricle and impalements were alternated between them. The experimenter worked 'blind' (i.e. he did not know the sequence of solution changes). Multiple impalements were made in each strip and each point on the graph represents the results from a single stable impalement. The curves were drawn by eye. Temperature 24° C, $[Ca^{2+}] = 1$ mM, rate of stimulation 12/min throughout.

In normal Ringer solution containing AcS ($10^{-5}$ M) membrane depolarization always preceded the development of resting tension. Prevention of depolarization by either increasing $[Mg]_o$ or by applying anodal current suppressed the development of slow maintained resting tension. The effect of $Mg^{2+}$ on the development of the steady tension in a pair of matched ventricular strips bathed in Ringer containing AcS is demonstrated in Fig. 7. One of the matched strips was incubated in normal Ringer solution (upper panel), and the other was equilibrated in Ringer containing 5·0 mM-$MgCl_2$ (lower panel). The positive inotropic effect of the

drug and the decline of twitch tension were followed by the development and maintenance of marked steady tension in the strip exposed to normal Ringer solution. The matched strip exposed to Mg-containing Ringer failed to develop significant resting tension even after 2·5 hr drug exposure. In a few preparations a small resting tension with greater delay in its onset could be measured despite the presence of $Mg^{2+}$ in the bathing medium.

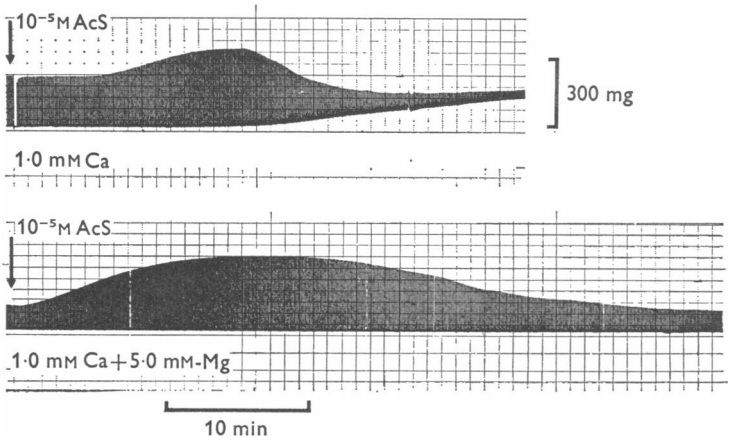


Fig. 7. Continuous recording of contractile activity from two matched ventricular strips – one equilibrated in $Mg^{2+}$-free solution and the other in Ringer containing 5 mM-$Mg^{2+}$ – when exposed to AcS ($10^{-5}$ M). Note that diastolic tension increases following the (+) inotropic response of the drug in $Mg^{2+}$-free solution, while diastolic tension remains constant in the presence of $Mg^{2+}$, despite the fall in twitch tension.

*KCl-induced contractures and relaxation.* The effect of AcS on relaxation processes was studied by measuring the ability of the myocardium to sustain steady-state tension when stressed with KCl depolarization. After two or three control exposures to hypertonic KCl Ringer solution and measurement of time course and amplitude of contracture, AcS was added to the bathing medium. The muscle was then subjected for 2 min test periods to KCl-Ringer, at selected intervals during the time course of the drug action. Fig. 8 illustrates the results of one such experiment as contractures were performed before AcS addition (panel *A*); at the peak of the positive inotropic effect (panel *B*); and as contraction declined during the later phase of the drug action (panel *C*). The rate of development and amplitude of both the peak and steady-state contracture tension is elevated during the time course of the drug action shown (7–22 min). Such experiments consistently showed that during the development of the inotropic effect of the drug, the ability of the muscle to resist $Ca^{2+}$ overload decreases. That is, the relaxation of tension from the peak of contracture is

slowed. The decrease in the rate of relaxation of twitch tension, at later stages of drug action, was also considered as an indicator of the same process.

*Shortening of the action potential and the decline of contraction in the presence of AcS.* The decline of twitch tension after the drug produces its

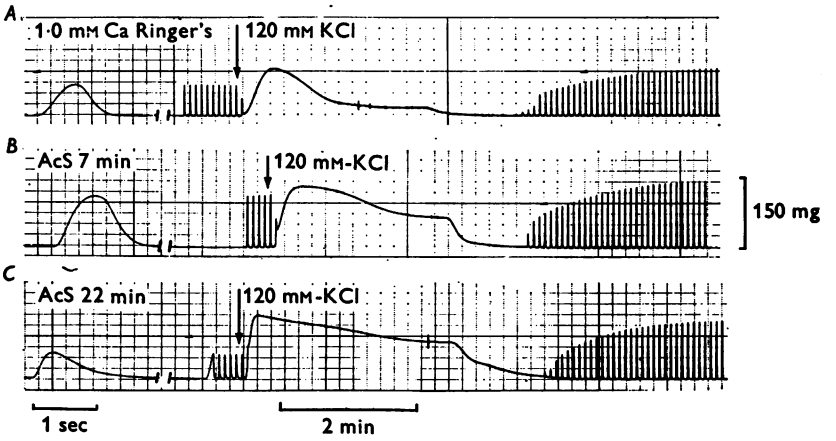


Fig. 8. Effect of AcS ($5 \times 10^{-6}$ M) on contraction and KCl-induced contracture at various stages of drug action. Addition of KCl-Ringer induces development of tension which is considerably enhanced and is better maintained in the presence of AcS. Panel *C* shows that although the (+) inotropic effect of the drug is no longer manifested in the amplitude of twitch tension, the contracture tension remains elevated and shows slower relaxation (compare with panels *A* and *B*). Temperature 22° C.

peak inotropic response, such as that illustrated in Fig. 1, is always accompanied by shortening of the action potential and time-to-peak tension (see Fig. 2). At this stage of drug action, however, depolarization of the myocardium with KCl-Ringer produces large contracture tensions compared to those generated before administration of the drug (see Fig. 8 and Otsuka & Nonomura (1963) for a similar effect of ouabain). These observations suggest that the decline of twitch tension may be partly due to the shortening action potential brought about by the drug. To test such a possibility, two procedures were used to prolong the action potential duration: (1) by clamping the membrane potential to the plateau level and (2) by bathing the muscle in solutions containing tetraethylammonium.

Fig. 9 shows the effect of clamping the membrane potential for 600 msec at the plateau potential (+18 mV) at various stages of the drug action. After 45 min of exposure of the strip to AcS, the action potential is markedly shortened, membrane is depolarized and resting tension has developed (Fig. 9*C*). Application of the voltage-clamp step from a holding

potential equal to the initial resting potential not only reduces the resting tension, but also restores developed tension. After 80–100 min of drug exposure (with the myocardium partially depolarized), prolongation of the action potential fails to restore the positive inotropic effect of the drug. Fig. 10 compares the developed tension for various duration clamps at 15 and 90 min of drug exposure. The ability of the muscle to produce tension higher than the control state seems to be impaired with prolonged drug exposure.

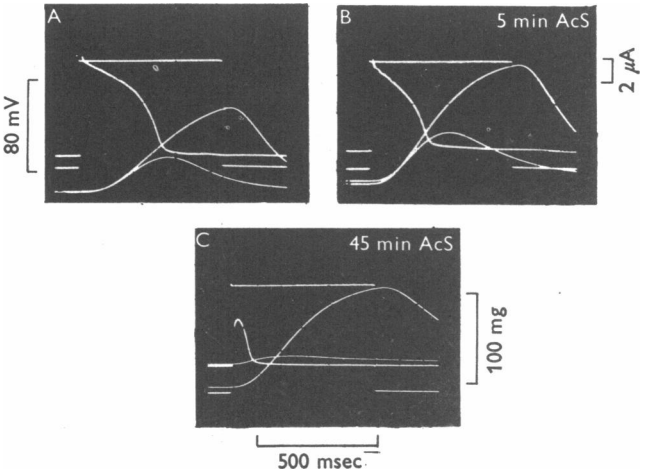


Fig. 9. Superimposed recordings of voltage-clamp steps ($-78$ to $+10$ mV and 600 msec duration) and action potentials, and their accompanying contractions obtained from frog ventricular strips bathed in normal Ringer (panel *A*) and after 5 and 45 min of addition of AcS (panels *B* and *C*). The tension accompanying the clamp steps remains potentiated both at 5 and 45 min of drug exposure, despite reduced contraction associated with the shortened action potential (panel *C*). Current records associated with the clamp steps have been removed to minimize confusion arising from multiple superimposed tracings. Temperature, 23° C. Strip diameter in test gap, 0·3 mm and length 0·5 mm, $[Ca]_o = 0{\cdot}2$ mM. $(AcS]_o = 5 \times 10^{-6}$ M.

Since chemical agents such as the quaternary ammonium ions are known to prolong action potential duration (Hille, 1967; Armstrong, 1969), tetraethylammonium chloride (TEA) in concentrations of 5–20 mM was used to determine whether the decrease in twitch tension brought about by AcS exposure could be reversed. Application of TEA to frog ventricular strips equilibrated in normal Ringer solution causes a slight and transient increase in the rate of development, amplitude, and time-to-peak of contraction, and a similar increase in action potential duration. The time course of these effects is from 10 to 15 min. The effect of TEA on strips exposed for long periods to AcS is more striking. In six experiments, TEA

consistently produced a prolongation of the action potential and a simultaneous increase in twitch tension. In preparations where membrane depolarization and resting tension are suppressed by the presence of $Mg^{2+}$ in the bathing medium, TEA restores the positive inotropic effect of AcS for up to 2 hr. However, if the strip is not pre-treated with $Mg^{2+}$ containing

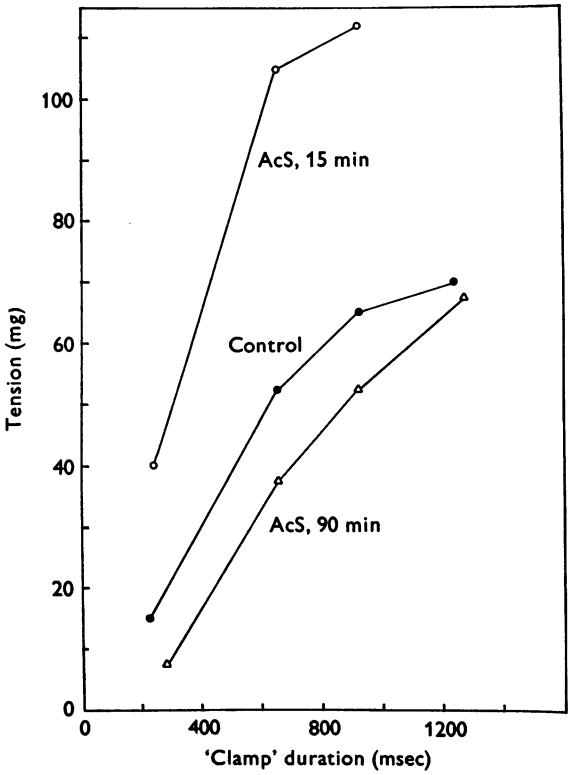


Fig. 10. Comparison of contractile force generated by various duration clamp pulses of similar amplitude (from −80 to +15 mV) in normal Ringer (control) and 15 and 90 min of AcS exposure. Note that after 90 min of drug addition the muscle produces less tension than that obtained in normal Ringer. At this stage of drug action the recorded action potential (not shown in Figure) is 100–150 msec in duration and 40–50 mV in amplitude. Ventricular strip diameter in the test chamber is 0·3 mm and 0·45 mm in length. $[Ca]_o = 0{\cdot}2$ mM. $[AcS]_o = 5 \times 10^{-6}$ M. Temperature 23° C.

solutions, TEA only transiently prolongs the action potential and enhances the developed tension. Fig. 11 shows the effect of application of TEA on a ventricular strip pre-treated with $Mg^{2+}$ and AcS. The potentiation of the twitch tension occurred simultaneously with the prolongation of twitch tension and action potential duration (see Fig. 11, traces *C* and *D*).

*Voltage-clamp experiments in the presence of AcS*

Experiments described thus far have outlined the time course of drug action on the mechanical and electrical properties of the frog ventricle. The results suggest that the time course of drug action could be separated into three stages, and selectively manipulated by altering the ionic environment or by controlling the membrane potential. A single sucrose-gap voltage-clamp technique was used to quantitate the potential dependence of the inotropic effect of acetyl strophanthidin and to investigate whether the inotropic effect of the drug was accompanied by significant changes in amplitude or time course of ionic currents.

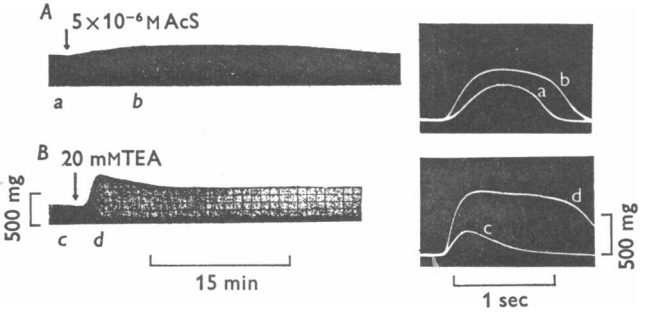


Fig. 11. Continuous recording of twitch tension from a ventricular strip equilibrated in normal Ringer containing 10 mM-$Mg^{2+}$, and exposed to AcS ($5 \times 10^{-6}$ M) and 20 mM TEA, indicated by downward arrows. Individual contractions sampled at different times (points *a–d*) and recorded with faster time base are superimposed in the two side panels. After 60–70 min of AcS exposure, twitch tension decreased to about 50% of control. Addition of TEA at this point restores the contractile force and maintains it for 1–2 hr. Strip diameter 0·75 mm. $[Ca]_o = 1$ mM. Temperature 23° C.

*Voltage–tension relations in the presence of AcS.* It has already been established that in frog ventricular muscle, membrane potential directly controls the development of tension (Antoni *et al.* 1968; Morad & Orkand, 1971). The relation between membrane potential and developed tension is characterized by a mechanical threshold around $-50$ to $-40$ mV, a monotonically increasing tension in the range of $-10$ to $+80$ mV and a plateau level at higher clamp potentials. Fig. 12 confirms such a finding and shows also the effect of AcS on the voltage–tension relation. Test-clamp pulses of 600 msec duration were applied at regular intervals, first in 0·2 mM-$Ca^{2+}$ Ringer and again after administration of a high dose of acetylstrophanthidin ($5 \times 10^{-6}$ M). The marked decrease in the mechanical threshold (toward more negative potential) seen in Fig. 2 is characteristic of higher doses or later stages of drug exposure. Lower doses of AcS or

shorter drug-exposure times produce no significant change in mechanical threshold but produce marked potentiation of tension at each test potential (see Fig. 14). In Ringer solution containing 1·0 mM-$Ca^{+2}$, the voltage–tension relation in the presence of the drug often plateaus at +40 to +60 mV. However in no experiment, even with larger membrane depolarization, was a maxima in voltage–tension relation or a decrease in developed tension seen.

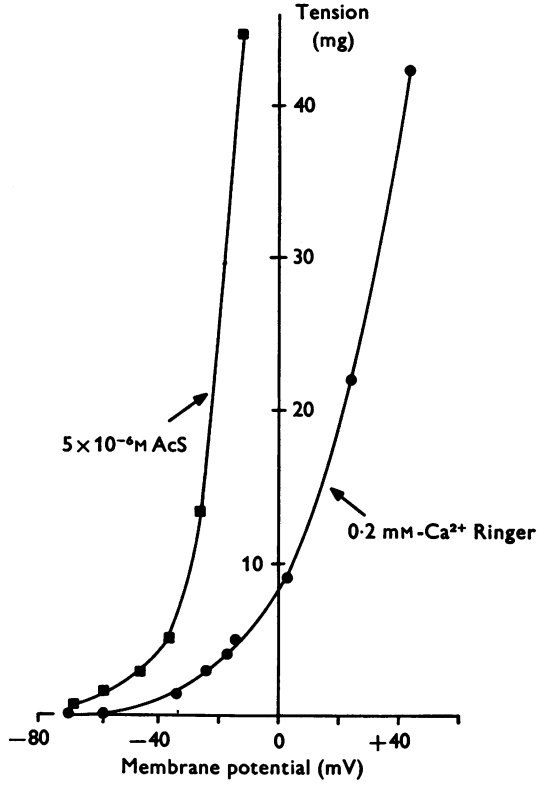


Fig. 12. Voltage–tension relation of a ventricular strip in presence (squares) and absence of AcS (circles). Square clamp pulses (600 msec in duration) were applied to the muscle and developed tension measured. Tension–voltage relation in the presence of AcS was measured late in the time course of drug action, when action potential had markedly shortened. Note that the mechanical threshold is considerably reduced and more tension is generated at each membrane potential in the presence of AcS. Temperature 23° C. $[Ca]_o = 0{\cdot}2$ mM. $[AcS]_o = 5 \times 10^{-6}$ M. Strip diameter in test chamber 0·4 mm and 0·5 mm in length.

*Inward ionic currents and the inotropic effect of acetylstrophanthidin.* Tetrodotoxin in concentrations of $1{\cdot}0 \times 10^{-6}$ to $5 \times 10^{-6}$ M is known to block the upstroke of the frog ventricular myocardium without altering

the shape or duration of the plateau, if the membrane is depolarized to +20 mV with a strong but brief transgap current pulse (Hagiwara & Nakajima, 1966). Fig. 13 shows that AcS is still capable of producing its positive inotropic effect in a ventricular strip which has been pretreated with tetrodotoxin. The positive inotropic effect of AcS in tetrodotoxin-treated ventricular strips could be demonstrated over a wide range of

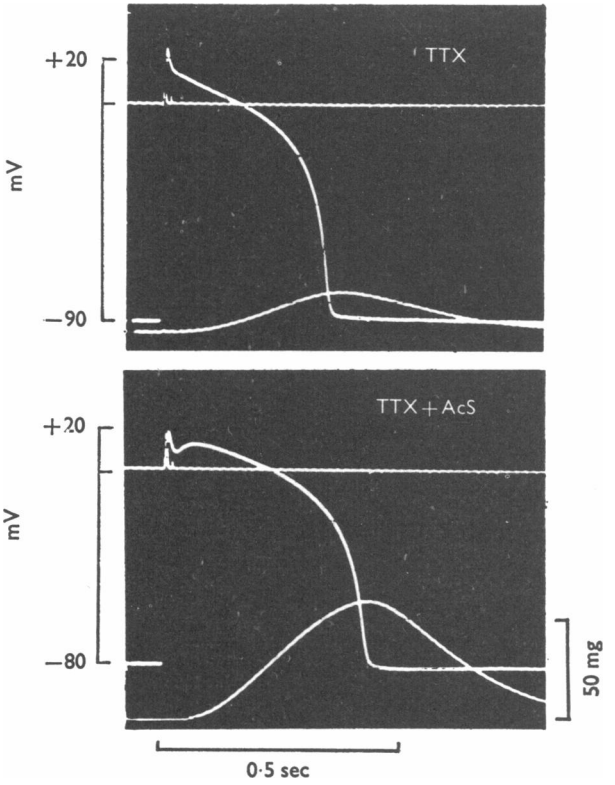


Fig. 13. Effect of AcS on action potential and contraction of a ventricular strip pretreated with tetrodotoxin (TTX). $10^{-6}$ M TTX used in this experiment completely suppressed the action potential upstroke. Action potentials were generated by application of strong transgap current to depolarize the muscle to plateau potentials. Note that the positive inotropic effect of the drug is still present in such a muscle. $[Ca]_o = 0{\cdot}2$ mM. Temperature 24° C. $[AcS]_o = 2 \times 10^{-6}$ mM.

membrane potentials (Fig. 14). This Figure shows that the voltage–tension relation in the presence of AcS is altered such that higher tensions are generated at each membrane potential above the mechanical threshold. The voltage–tension relation increases monotonically well above +40 mV in this experiment. In this range of membrane potentials, all transient

inward membrane currents are suppressed or reversed despite marked potentiation of developed tension (Fig. 14, see also insets).

In tetrodotoxin-treated preparations, a slow transient inward current is often recorded on clamping the membrane between $-30$ to $+20$ mV. The time course of this current as well as its sensitivity to $[Ca]_o$ even in low

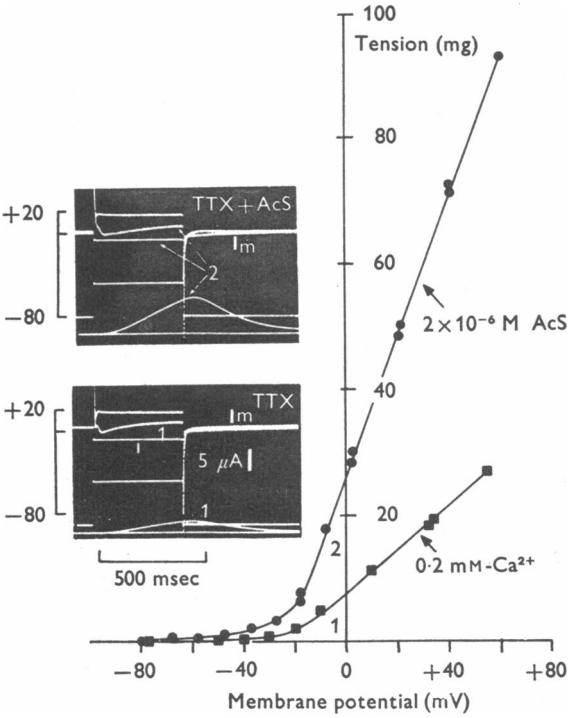


Fig. 14. Tension–voltage relation of ventricular strip in presence (circles) and absence of AcS (squares) in a preparation pretreated with tetrodotoxin (TTX, $10^{-6}$ M) to block the fast $I_{Na}$ (see inset traces marked $I_m$). Inset panels show selected clamp pulses and the accompanying membrane currents and tensions at points on the graph marked 1 and 2. Tension increases continuously at all potentials tested above the mechanical threshold in the presence of AcS. In each inset panel two clamp pulses are superimposed and the appropriate membrane currents indicate that at potentials around $-5$ to $-10$ mV, there is a transient inward current component. $[Ca]_o = 0{\cdot}2$ mM; strip diameter in test chamber 0·4 mm; length, 0·5 mm. $[AcS]_o = 2 \times 10^{-6}$ M. Temperature 24° C. Inward current plotted downward; clamp pulse duration = 400 msec.

$Na^+$ solutions has led a number of investigators to suggest that it may represent the contractile-activating calcium (Beeler & Reuter, 1970*b*; Reuter, 1973; Vassort & Rougier, 1972; New & Trautwein, 1972). Fig. 15 shows selected records of clamp pulses at various potentials in tetrodo-

toxin-Ringer before and after exposure of the muscle to AcS. A transient inward current is seen when the membrane is depolarized to −20 mV, simultaneous with the development of tension in tetrodotoxin-Ringer. The magnitude or duration of this slow inward current does not seem to increase in the presence of AcS despite a large potentiation of developed tension. Further, tension increases continuously with large depolarizations

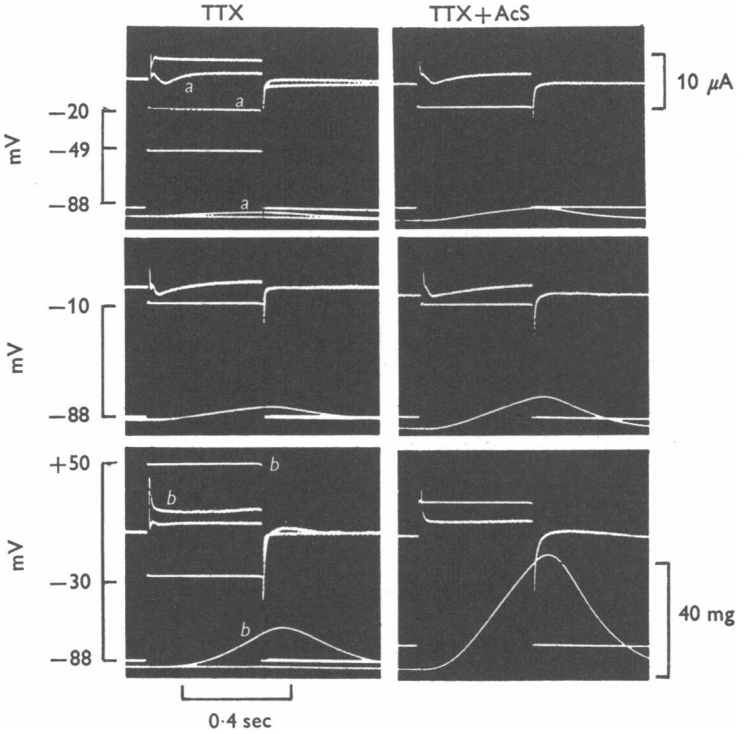


Fig. 15. Original records of square clamp pulses and membrane currents and developed tension in presence and absence of AcS ($2 \times 10^{-6}$ M) in TTX ($10^{-6}$ M) treated ventricular strips. In two of the panels, more than one clamp pulse has been illustrated. The current and tension accompanying the clamp step are appropriately marked. Note that the potentiation of tension in the presence of AcS is independent of amplitude or the direction of the slow transient inward current. Inward current downward. Holding potential = resting potential = −88 mV. Temperature 24° C. Diameter of ventricular strip in test chamber 0·4 mm, length 0·5 mm. $[Ca]_o = 0{\cdot}2$ mM.

while the transient inward current is abolished or reversed. In Fig. 16 the magnitude of the maximum inward current (which occurs 50 msec after the start of the test clamp) is plotted versus the absolute membrane potential. The transient inward current has a maximum at around −10 mV. The magnitude of this tetrodotoxin-insensitive inward current

varied from preparation to preparation but was never augmented by the addition of AcS. In fact, as seen in Fig. 16, the slow inward current is slightly reduced in the presence of the drug. The magnitude of slow inward current was measured either as the peak of net inward current or as the peak inward deflexion with respect to the initial outward current after the end of the capacitive surge (see also Beeler & Reuter, 1970*b*).

In later stages of drug action as the action potential shortens, the 'instantaneous' outward current is markedly increased. At this stage of the drug action, total membrane conductance at rest or during the plateau of the action potential is markedly enhanced.

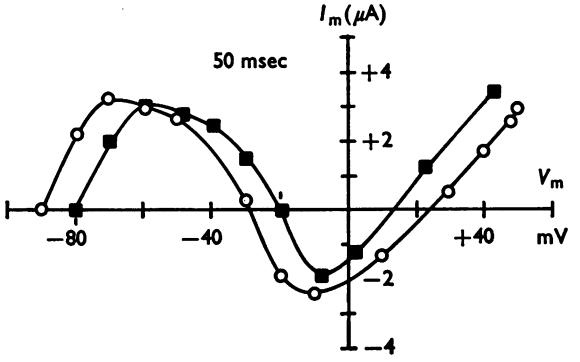


Fig. 16. Current–voltage relation of a ventricular strip pretreated with tetrodotoxin ($10^{-6}$ M) and exposed to AcS ($2 \times 10^{-6}$ M, squares). The currents were measured 50 msec after the start of the clamp step. This time generally corresponded with the largest transient inward deflexion in the current trace. In this experiment the magnitude of the transient inward current was measured with respect to the initial outward current after the end of the capacitive surge. In all of strips tested, generally there was either no change or small decrease in the magnitude of the secondary inward current. Temperature 24° C. Strip diameter 0·4 mm and length 0·5 mm. $[Ca]_o = 0{\cdot}2$ mM.

## DISCUSSION

Electromechanical observations reported in this investigation suggest that the presence of Ca in the extracellular space is required for AcS to potentiate twitch tension and to shorten the action potential of the frog ventricular muscle. In the absence of $Ca^{2+}$ digitalis not only fails to shorten the characteristically long 'zero'-$Ca^{2+}$ action potential, but often prolongs the plateau. Although the positive inotropic effect of AcS seems to be associated with an increased transmembrane influx of $Ca^{2+}$, no transient inward component of membrane current could be associated with the potentiation of tension on application of a clamp step. The AcS-induced potentiation of tension was unaffected by blocking the regenerative Na

system with tetrodotoxin and was independent of the direction or magnitude of the fast or slow components of $I_{Na}$. The positive inotropic effect of the drug persisted in tetrodotoxin-treated preparations where the membrane is clamped to potentials equal to or greater than $E_{Na}$ (see Figs. 14 and 15). These studies provide evidence that the various inotropic effects of digitalis and its analogues are mediated through independent molecular mechanisms and may be separated from each other by appropriate electrical or ionic interventions.

*Voltage-clamp studies and the positive inotropic effects of AcS.* The evidence from voltage-clamp studies indicates that the initial decline in the positive inotropic effect of AcS is due to the shortening of the action potential. Thus, if the action potential is controlled at a constant duration, the positive inotropic response of the drug is maintained for a longer period (Fig. 9). The shift in the voltage–tension relation caused by AcS without any marked alteration in the membrane currents at the initial stages of drug action suggests that the increase in $Ca^{2+}$ influx (Grossman & Furchgott, 1964) may be masked by an appropriate change in other ionic currents so that the net nembrane current remains unchanged.

The stimulation of a $K^+$ efflux mechanism by AcS to enhance $Ca^{2+}$ transport, similar to that postulated by Morad & Orkand (1971) to explain the positive slopes of voltage–tension relation in the vicinity of $E_{Ca^{2+}}$ could provide a unitary mechanism for the potentiation of tension and shortening of the action potential. The decrease of action potential duration normally observed after treatment of the ventricular strip with AcS did not occur in the absence of $Ca^{2+}$ in the bathing medium. This result suggests that the effect of AcS on the K permeability of the membrane requires the presence of $Ca^{2+}$ ions. Since Dutta & Marks (1969) have shown that decreased $[Ca]_o$ does not inhibit the binding or uptake of cardiac glycosides by the myocardium, AcS must influence a $Ca^{2+}$ dependent K efflux system to shorten the action potential. The critical question of whether such an ionic interaction is required in $Ca^{2+}$-containing solutions to potentiate tension is not supported by some of the recent radioflux experiments. In dog hearts treated with diphenylhydantoin (Scherlag, Helfont, Ricciutti & Damato, 1968) or in cases of respiratory acidosis induced in rabbit septum (G. A. Langer, personal communication), the digitalis-induced increase in $K^+$ efflux (Müller, 1965; Langer & Serena, 1970) was reduced without significant suppression of the positive inotropic response. However, the flux data in these studies were obtained in mammalian ventricular preparations where $Ca^{2+}$ is thought to be released in part from 'intracellular' pools and would not interact with the efflux of $K^+$ (for a comparison of frog and mammalian excitation–contraction coupling data, see Morad & Goldman, 1973; Beeler & Reuter, 1970*a*;

Wood, Heppner & Weidmann, 1969; Gibbons & Fozzard, 1971). Thus, specific radioflux data are not as yet available to determine if the increase in $Ca^{2+}$ influx in the presence of AcS is necessarily linked to an increase in $K^+$ to produce the inotropic effect of the drug in the frog heart. Since large outward currents, especially with higher drug doses, are often recorded on clamping the membrane during the positive inotropic effect of AcS, the increased efflux of $K^+$ must remain a viable possibility in mediating the inotropic effects of AcS.

*Is an electrogenic influx of $Ca^{2+}$ responsible for the positive inotropic effect of AcS?* A slow inward membrane current, highly sensitive to $[Ca]_o$, has often been observed in voltage-clamp experiments in a number of myocardial preparations (in dog, Beeler & Reuter, 1970; in sheep, Mascher & Peper, 1969; in cat, New & Trautwein, 1972; in frog atrium, Rougier, Vassort, Garnier, Gargouil & Caraboeuf, 1969; Vassort & Rougier, 1972). This current, which seems to be insensitive to high concentrations of tetrodotoxin, is activated at around $-30$ mV, shows a reversal potential around $+20$ mV, and has been identified by these investigators as the $Ca^{2+}$ current responsible for the activation of contraction. Although there is some controversy as to the existence or the ionic nature of this current (Johnson & Lieberman, 1971), such a current could often by recorded in our preparations (Fig. 14, inset; Fig. 15). Whether or not this slow and transient current represents a *true* $I_{Ca}$ or is an artifact reflecting the inhomogeneous distribution of potential in the preparation, its magnitude was unaltered by the exposure of the muscle to any concentration of AcS (Figs. 15, 16). Thus, no transient component of the inward membrane current could be identified to be appropriately changing so as to be responsible for the initiation or maintenance of the positive inotropic effect of AcS.

*Shortening of the action potential in the presence of AcS.* In all of the ventricular strips bathed in normal Ringer containing AcS, the duration of the action potential shortened markedly after an initial and transient period of prolongation of the plateau. Since digitalis enhances $K^+$ efflux (Müller, 1965; Langer & Serena, 1970), it could be argued that the decrease in action potential duration is due to the increased $K^+$ outward current. Consistent with these observations is the finding reported in this paper that the early (voltage-dependent) outward current markedly increases. Such an increase in outward current may reflect the enhanced $K^+$ efflux and the shortening of the action potential. However, the decrease in the action potential duration in the presence of AcS seems to be highly dependent on the $[Ca]_o$. In fact, in experiments where $Ca^{2+}$ was omitted from the bathing solution, AcS not only failed to shorten, but often prolonged the action potential (Figs. 4, 5). Such an increase in the duration of

the action potential may be in part due to the inhibition of an electrogenic $Na^+$ pump in the ventricular muscle (Haas, 1972). The contribution of such an electrogenic pump to the repolarization processes may be normally small and become measurable only when the processes which activate or enhance $K^+$ permeability are inhibited. Since the omission of $Ca^{2+}$ from the bathing solution seems to decrease the rate of repolarization and to prolong the plateau in the frog heart (Garnier *et al.* 1969; Harris & Morad, 1971; Harris & Morad, 1975), it is conceivable that under these conditions the activity of an electrogenic Na pump would be more apparent. Thus, the inhibition of Na pump by AcS in 'zero Ca' Ringer may prolong the action potential.

*Depolarization of the membrane and development of resting tension.* All ventricular strips treated with AcS eventually depolarize and develop a steady maintained tension. The rate of development of depolarization and the maintained tension, depended directly on the concentration of the drug. Membrane depolarization and the development of the steady tension could be prevented by bathing the preparation in solutions containing 5–10 mM-$Mg^{2+}$ or by clamping the membrane back to the resting potential.

Although there is no direct experimental evidence as to why membrane depolarizes in the presence of AcS, the loss of intracellular $K^+$ (Müller, 1965) and the suppression of depolarization by $Mg^{2+}$ may implicate the inhibition of $Na^+$-pump. It must then be argued that increasing the $[Mg]_o$ would reduce the inhibition of the Na/K ATPase by AcS or activate a quiescent moiety of the Na pump. There is no direct evidence for such an assertion in cardiac muscle. However, evidence from ox brain microsomal fractions points to a possible unmasking of new ATPase sites at higher $Mg^{2+}$ concentrations (Hansen & Skou, 1973).

The inhibition of $Na^+$-pump has been suggested as a possible unitary molecular mechanism to mediate all of the electromechanical effects of the drug (Langer & Serena, 1970; Langer, 1968). In such a hypothesis, the primary ionic requirement is an increase in $[Na]_i$ caused by the inhibition of the Na pump. The increase in $[Na]_i$ then is thought to increase the availability of $Ca^{2+}$ to the myofilaments either by increasing $Ca^{2+}$ influx via a $Ca^{2+}$–$Na^+$exchange mechanism (Baker, Blaustein, Hodgkin & Steinhardt, 1969) or by freeing more $Ca^{2+}$ from internal membrane binding sites (e.g. via $Ca^{2+}$–$Na^+$ competition mechanism, Lüttgau & Niedergerke, 1958). Thus, the continuous gain in $[Na]_i$ and loss of $K^+$ could provide a unitary mechanism for the potentiation of tension initially, and membrane depolarization and development of resting tension eventually. The evidence from voltage-clamp studies discussed in this report suggests that the inotropic effect of AcS is independent of the degree of activation or the direction of $Na^+$-current. In fact, in tetrodotoxin-treated ventricular strips

(with markedly reduced Na content, C.P. Bianchi, personal communication), reversal of the slow tetrodotoxin-insensitive $Na^+$ or $Ca^{+2}$ currents, by clamping the membrane above +40 to +60 mV, failed to alter the positive inotropic response of the drug (Figs. 14, 15). Under these experimental conditions it is difficult to postulate how the movement, or the internal concentration of $Na^+$, could be responsible for the (+) inotropic effect of AcS. On the other hand, our experiments are consistent with the possibility that the toxic manifestation of the drug action (i.e. depolarization of the membrane and development of resting tension) are mediated by inhibition of $Na^+$ pump.

*Uptake of $Ca^{2+}$ and the inotropic effect of AcS.* The shift in the voltage–tension relation or the mechanical threshold (Figs. 12, 14) can be brought about either by an increase in $Ca^{2+}$ influx or a suppression of the $Ca^{2+}$ sequestering system. Comparison of the rate of relaxation of contractions accompanying the clamp steps, in the presence and absence of AcS, showed no measurable change at the initial stages of drug action, suggesting that the impairment of the Ca uptake system was not primarily responsible for the positive inotropic action of the drug. However, with longer drug exposure times or at higher drug concentrations, the Ca sequestering system may be inhibited to such a degree as to contribute to the potentiated contractile state. In fact, when the ventricular strip was challenged with a continuous influx of Ca (via a KCl contracture) to test the capacity of the $Ca^{2+}$-removal system, the inhibition of the sequestering system could be more readily observed (see Fig. 8).

The effect of digitalis on the $Ca^{2+}$-pumping activity of fragmented sarcoplasmic reticulum is variable and depends on the source and method of preparation of the sarcoplasmic reticulum and on the experimental conditions (e.g. the age of the sarcoplasmic reticulum or the functional state of the myocardium which served as the source of the sarcoplasmic reticulum fragments). Although there are no data from sarcoplasmic reticulum studies to support our finding of minimal or no impairment of relaxation rate in the initial stages of AcS action (Chimoskey & Gergely, 1968; Pretorium, Pohl, Smithen & Inesi, 1969; Besch, Allen, Glick & Schwartz, 1970), there is also contradictory data which indicate that sarcoplasmic reticulum is strongly inhibited or stimulated by cardiac glycosides (Briggs, Gertz & Hess, 1966; Gertz, Hess, Lain & Briggs, 1967; Entman, Cook & Bressler, 1969; Carsten, 1967; Lee, Shin, Kang & Chan, 1969).

It should be remembered that the rate of transport of sarcoplasmic reticulum fragments is only one factor which determines the over-all function in relaxation processes. For instance, back-leak of $Ca^{2+}$ across the sarcoplasmic membrane or the intravesicular binding properties (Martonosi, 1972) and the total capacity of the sarcoplasmic reticulum may

also contribute to the relaxing effect. Our observations on the relaxing capability of ventricular strips suggest that (1) there is minimal or no inhibition of relaxation processes in the initial positive inotropic stage of the drug action; (2) with prolonged drug exposure the rate of relaxation is clearly suppressed; and (3) under the stress of a continuous $Ca^{2+}$ influx (i.e. KCl-induced contractures), impairment of relaxation is demonstrable even in the initial stages of drug action. These observations, coupled with the evidence from fragmented SR studies (Klaus & Lee, 1969; Lee & Klaus, 1971) suggest that AcS has a variable effect on the $Ca^{2+}$ pumping activity of the sarcoplasmic reticulum, but that it generally tends to inhibit the sequestering capacity by altering intravesicular binding properties. Thus, under conditions where the storage capacity of the sarcoplasmic reticulum is not compromised (e.g. the initial stage of drug action or at lower $[Ca^{2+}]_o$) the rate of relaxation is not demonstrably affected. On the other hand, with higher drug concentrations or longer exposure times, especially if the $Ca^{2+}$ load delivered has been increased, the $Ca^{2+}$ sequestering capacity is exceeded and the rate of relaxation is measurably impaired.

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