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DIFFERENTIAL EFFECTS OF OUABAIN AND 2,4-DINITROPHENOL ON CONTRACTILE TENSION OF AND ON SODIUM AND CALCIUM EFFLUX FROM FROG HEART VENTRICULAR STRIPS

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1 The efflux, from heart ventricular strips of *Rana pipiens*, of sodium (^{22}Na) and calcium (^{45}Ca) was measured simultaneously.

2 ^{22}Na efflux could be resolved into two first order kinetic components: $k_I = 0.105\ min^{-1}$, thought to represent efflux from the extracellular space, and $k_{II} = 0.0182\ min^{-1}$ representing efflux from the cells.

3 ^{45}Ca efflux was also resolved into an extracellular component, $k_I = 0.1216\ min^{-1}$; and an intracellular one, $k_{II} = 0.0102\ min^{-1}$. ^{45}Ca k_{II} was greatly increased by 2,4-dinitrophenol (DNP), but unchanged by caffeine. This suggests that it represents a mitochondrial calcium compartment.

4 ^{22}Na k_{II} was not changed by DNP. This indicates that, at the time of DNP addition, ^{22}Na was passively bound to undefined intracellular components.

5 Ouabain (10^{-6} M) decreased ^{45}Ca efflux (k_{II}) initially but at later periods slightly increased it. The former effect is thought to be due to an action at the plasma membrane level, while the latter probably represents an increased exchangeability of mitochondrial calcium. The same effects were always found when ouabain was applied at different times of strip superfusion.

6 Ouabain (0.25 to 4 μM) did not decrease the k_{II} of ^{22}Na efflux. Kinetic reasons are presented which indicate that, in this preparation, the activity of the sodium pump may be too fast to be measured by means of ^{22}Na efflux, therefore these findings do not necessarily mean that ouabain does not inhibit active sodium transport.

7 The time course of the inotropic effect of ouabain was also studied in ventricular strips of *Rana pipiens* heart that were stimulated at 0.2 Hz with biphasic, 2 ms pulses of supramaximal intensity, and incubated in Ringer solution containing 1.1 mM calcium, or in 'calcium-free' Ringer (residual calcium: 5.2 μM), or in 'calcium-free' Ringer with 0.1 mM of the calcium chelator ethyleneglycol bis (β-aminoethylether) N,N'-tetraacetic acid (EGTA).

8 In Ringer, the inotropic effect of ouabain was already observed at 5–10 s after steroid addition, even with the lowest concentration tested (0.25 μM), while signs of toxicity appeared only after 15 min in 4 μM ouabain, the highest concentration used.

9 When the strips were incubated in 'calcium-free' Ringer solution, force of contraction decayed to 1–2% of that in 1.1 mM calcium. Addition of 4 μM ouabain to these hypodynamic strips led to a progressive increase in contractile force of up to 300%, that started after a 50 s latency period. No signs of toxicity were observed.

10 Incubation of the strips in EGTA-Ringer also reduced contractile force to about 2% of that in Ringer, and 4 μM ouabain also increased force of contraction by approximately the same amount as seen in 'calcium-free' Ringer, but the effect began after a 10 min latency period. The concentration of calcium ion (Ca^{2+}) in the extracellular space of strips incubated in EGTA-Ringer, was approximately 800 fold lower than in Ringer, and 60 fold lower than in 'calcium-free' Ringer solution.

11 Caffeine (20 mM) induced, in strips previously incubated for 1 h in 4.4 mM calcium Ringer solution plus 10^{-6} M ouabain, a marked initial contracture, that relaxed spontaneously, and was followed by slow waves of contracture. This was not observed if the strips were incubated, prior to caffeine, in 4.4 mM calcium Ringer without ouabain, or in 1.1 mM calcium Ringer solution that contained 10^{-6} M ouabain.

12 Based on these findings, a hypothesis that can explain the inotropic effect of cardioactive steroids is presented.

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Introduction

Cardioactive steroids are potent inhibitors of active sodium and potassium ion transport in myocardial and other cell types (reviewed by Lee & Klaus, 1971; and by Akera & Brody, 1978). However, measurement of intracellular sodium concentration ($[Na]_i$), both chemically (Tuttle, Witt & Farah, 1961; Bentfeld, Lüllmann, Peters & Proppe, 1977; Ghysel-Burton & Godfraind, 1979) or with sodium-sensitive microelectrodes (Ellis, 1977; Deitmer & Ellis, 1978) has shown that low steroid concentrations, while increasing contractile force, do not increase $[Na]_i$ and may even decrease it. This does not refute the possibility that (as first suggested by Akera, Bennett, Olgaard & Brody, 1976) highly localized or transient increases in $[Na]_i$, that cannot be detected at present, do occur, but raises a reasonable doubt as to whether inhibition of the sodium pump is a prerequisite for the positive inotropic action of steroids (see also Okita, 1977). The mechanism by which inhibition of sodium and potassium ion transport leads to an increase in contractile force is not clear, although various hypotheses, based on different aspects of sodium-calcium exchange mechanisms (Crompton, Capano & Carafoli, 1976; Langer, 1977; Akera & Brody, 1978), have been put forward. A closely related problem refers to the unknown mechanism by which cardioactive steroids increase the amount of calcium available to the contractile system during each beat (the calcium 'transient'), as Allen & Blinks (1978) recently demonstrated in frog atrial trabeculae.

In the present experiments we have used frog heart ventricular strips, that were electrically stimulated and superfused *in vitro*, and in which the efflux of ^{22}Na, ^{45}Ca and contractile tension were measured simultaneously. This tissue was chosen because of its high sensitivity to cardioactive steroids, because of the simpler structure of frog myocardial cells, e.g. absence of transverse tubules and a less developed sarcoplasmic reticulum (SR) (Staley & Benson, 1968; Sommer & Johnson, 1969; Page & Niedergerke, 1972; Mazet, 1975), which could help in interpreting the results; and because of the much higher surface to volume ratio of these cells, relative to those of mammals, that may possibly magnify effects on plasma membrane ion fluxes. The points we first tried to clarify were: (a) Which are the kinetic components of ^{22}Na and ^{45}Ca efflux, and whether it is possible to deduce from them, which are the cell compartments from which the efflux of these ions originates; (b) Does ouabain at low, inotropic concentrations inhibit the sodium pump of these cells, and how do inotropism and sodium pump inhibition correlate; (c) Do cardiac glycosides, at non-toxic concentrations, change calcium efflux in some manner, and can this be correlated with inotropism, and with sodium pump inhibition.

During the course of these experiments, it was found that non-toxic concentrations of ouabain reduced ^{45}Ca efflux from the strips. This led us to investigate whether such an effect was relevant to the inotropic effect of the drug; the strips were washed for extended periods in 'calcium-free' Ringer, or this solution containing the calcium chelator, ethyleneglycol bis (β-aminoethyl ether) N,N'-tetraacetic acid (EGTA). As an inotropic effect of ouabain remained under these conditions where calcium influx is drastically reduced, it is suggested that at least part of the inotropic effect of steroids is due to this drug-induced reduction in calcium efflux.

Methods

Preparation of strips

Heart ventricular strips were obtained from *Rana pipiens* of either sex, weighing 49.5 ± 1.6 g, essentially by Dale's (1932) procedure, except that the strip was cut with two parallel safety razor blades cemented to a piece of acrylic plastic 1.5 mm thick. Both ends of the strip were tied with surgical silk (about 0.3 mm thick), and one of the silk threads was tied to a 30 × 0.8 mm steel rod which was rigidly attached to the cantilever of a vertically mounted Grass FT.0.3 force displacement transducer.

Incubation and superfusion

The strip, hanging from the transducer, was placed in a beaker containing 2 ml of ice cold frog Ringer solution of the following composition (mM): NaCl 110, KCl 2, $CaCl_2$ 1.1, $MgCl_2$ 0.6, KH_2PO_4 0.1, $NaHCO_3$ 2.4 and glucose 5.5 to which 25 μCi of ^{22}Na and 10 μCi of ^{45}Ca was added. This was continuously bubbled with 5% CO_2 in O_2; the pH was 6.8. After a 2 h period, during which the tissue became labelled with both isotopes, the strip still attached to the transducer was rinsed twice by immersing for about 5 s in non-radioactive Ringer solution and was then placed in the chamber used for superfusion and electrical stimulation. This chamber was a trough 6 cm long, by 2.5 cm wide, and 0.7 cm deep, in an acrylic block. The remaining free end of the silk thread was firmly tied to a silver wire ring fixed to the centre of one of the walls (2.5 cm wide). The trough was then filled, by means of an automatic syringe (Cornwall, B-D), with 8 ml of Ringer solution, kept at 20°C in a thermoregulated bath, and continuously bubbled with 5% CO_2 in O_2. The strip was stretched to a

resting tension of 1 g and electrical stimuli (0.2 Hz, 2 ms, supramaximal (40 V setting) biphasic pulses) generated by a Grass S4A stimulator were applied via two 3 cm long silver wires placed 2 cm apart from each other, and parallel on both sides of the strip. The contractions elicited by field stimulation were recorded on a Grass model 79 polygraph. The fluid bathing the strip was aspirated into an empty test tube and then replenished with fresh Ringer every 2 min. This process of fluid renewal lasted about 5 s. At the end of the experiment the strip was homogenized in 1 ml of water in an all glass (Kontes duall) homogenizer.

Other solutions used were: 'Calcium-free' Ringer solution which was the same as frog Ringer, but with no added calcium, and EGTA-Ringer which resembled 'calcium-free' Ringer, but also contained 0.1 mM EGTA. All solutions were kept in a thermoregulated water bath at 20°C, for at least 30 min before use.

Total calcium was measured with a Perkin Elmer 403 atomic absorption spectrophotometer. The concentration of ionized calcium (Ca^{2+}) was estimated from the measured total calcium concentration, pH and EGTA concentration, by means of a computer programme based on the Ca-EGTA association constant given by Portzehl, Caldwell & Rüegg (1964).

Three compartment model

For estimating (Ca^{2+}) in the tissue extracellular space, when (Ca^{2+}) was changed in the bulk fluid, a conventional model with three compartments in series was used. Compartment 1 represents the myocardial cells. Its initial concentration of total exchangeable calcium is taken as 0.49 mM and the fraction of tissue volume occupied by it is 0.815 (Niedergerke, 1963). Compartment 2 represents the extracellular fluid space, which occupies 0.185 of the tissue volume (Niedergerke, 1963). Its initial calcium concentration is assumed to be in equilibrium with that in the bulk fluid, i.e. 1.1 mM. Compartment 3 represents the bulk fluid that bathes the strip. As its volume (8 ml) is very much larger than the tissue volume (0.04 ml or less), and was renewed every 2 min, it was assumed as infinite. The rate constants for calcium transfer between the compartments were those measured below (Figure 1), and, for simplifying calculations, it was assumed that no calcium passed from compartment 2 to 1. This, although evidently untrue in normal calcium, results in an overestimate of (Ca^{2+}) in compartment 2. However, in very low (Ca^{2+}), where Ca^{2+} influx can be reduced over 800 fold, this 2 to 1 calcium transfer can be largely neglected.

The entry of substances, e.g. EGTA, from the bulk fluid into the extracellular space, was solved as a case of diffusion between two compartments, in which compartment 3 is very much larger than compartment 2, and substance concentration was initially zero in compartment 2, but remains constant in 3 (Simon, 1972). Thus, $(C_2)\ (t) = (C_3)\ (1-e^{-k_{3,2}t})$, where (C_2) and (C_3) represent substance concentration in compartments 2 and 3, respectively, t is time, and $k_{3,2}$ is the rate constant for 3 to 2 transfer.

The calcium concentration in the extracellular compartment at different times, $Ca_2\ (t)$, following omission of calcium in the bulk fluid ($Ca_3 = 5.2\ \mu M$), was estimated by the equation:

$$[Ca_2](t) = [Ca_2](O)e^{-k_{2,3}t} + \frac{V_1}{V_2}\ \frac{k_{1,2}}{k_{2,3}-k_{1,2}}$$

$$[Ca_1](O)[e^{-k_{1,2}t}-e^{-k_{2,3}t}] + [Ca_3](O)[1-e^{-k_{3,2}t}]$$

where $Ca_n(t)$ represents the total concentration of calcium in compartment n at time t; V_1 and V_2, the relative volumes of compartments 1 and 2, respectively; and $k_{i,j}$, the rate constant for calcium transfer from compartment i to compartment j. The first term on the right hand side of the equation thus represents the exponential decay of Ca_2 initially present in the extracellular fluid. The second term represents the change in Ca_2 produced by calcium transfer from compartment 1, also a first-order kinetic process, that is, in turn, followed by the exit of this calcium from compartment 2 to the bulk fluid (compartment 3).

The third term is a minor correction factor that was used, because in the first two terms analysed above it was implicit that $[Ca]_3$ was zero, and not 5.2 μM as actually measured. This simplification of the calculations led to a final $[Ca_2]$ also equal to zero. To correct for this, $[Ca_3] = 5.2\ \mu M$ was assumed to diffuse into compartment 2 under the same assumptions used for estimating the diffusion of EGTA between compartments 2 and 3, i.e. $[Ca_2]\ (t) = (Ca_3)\ (1-e^{-k_{3,2}t})$.

Radioactivity measurement

Radioactivity was measured with a Searle Analytical Mark II liquid scintillation counter in which two counting channels were used. One channel was set for counting the high energy particles of ^{22}Na exclusively, while the other lower energy channel measured both ^{45}Ca and ^{22}Na. The contribution of ^{22}Na to the counts in the low energy channel, known from appropriate ^{22}Na standards, was then subtracted, so that the net ^{45}Ca counts could be estimated. One ml of each collected fraction and 0.1 ml of the homogenate (made up to 1 ml with Ringer) was counted with 10 ml of an already described detergent-containing counting fluid (Orrego, Jankelevich, Ceruti & Ferrera, 1974). No quench corrections were necessary, except when 2,4-dinitrophenol (DNP) was present.

In this case, triplicate known amounts of both ^{45}Ca and ^{22}Na were counted separately in Ringer or in DNP-containing Ringer solution, so that appropriate correction factors could be calculated.

Expression of results

Mean values and s.e.mean are generally presented. When the different first-order kinetic components of the efflux of a given substance were studied, tissue desaturation curves (i.e. log% of substance remaining in tissue was plotted against time) were constructed, and the curves resolved into their different straight line components (Defares & Sneddon, 1960). However, when changes in efflux were studied following the addition of different drugs, the efflux was expressed as a fractional rate constant (f = amount of substance released during collection period (c.p.)/amount of substance present in the tissue at the beginning of c.p. × duration of c.p.), that is much more sensitive for this purpose, relative to a tissue desaturation curve (Vargas, Doria de Lorenzo & Orrego, 1977).

Statistical comparisons were made by Student's *t* test.

Materials

Carrier-free ^{22}Na and ^{45}Ca (25 mCi/mg) were from New England Nuclear Co. Ltd, and three times distilled water was used throughout.

Results

Efflux kinetics of ^{22}Na and ^{45}Ca

When ventricular strips, that had been previously incubated in Ringer containing both ^{22}Na and ^{45}Ca, were superfused with non-radioactive Ringer and tissue desaturation curves for both isotopes constructed (Figure 1), it was found that little ^{22}Na was retained by the tissue (i.e. 97% of the isotope present initially was lost in 60 min), and that its efflux could be resolved into three first order kinetic components (Figure 1a). The most rapid component ($T_{\frac{1}{2}}$:0.5 min) could also be seen in the absence of tissue, and thus represents the washout from the incubation chamber. The second kinetic component ($T_{\frac{1}{2}} = 6.6$ min; k_I:0.105 min^{-1}), is generally interpreted as washout from the extracellular space, a compartment that represents about 20% of the tissue fluid, and from which substances diffuse with comparable kinetics to those of this efflux component (Winegrad & Shanes, 1962; Van der Kloot & Dane, 1964; Lund-Andersen, 1974). A third, much slower kinetic component ($T_{\frac{1}{2}} = 38$ min; k_{II}:0.0182 min^{-1}), possibly

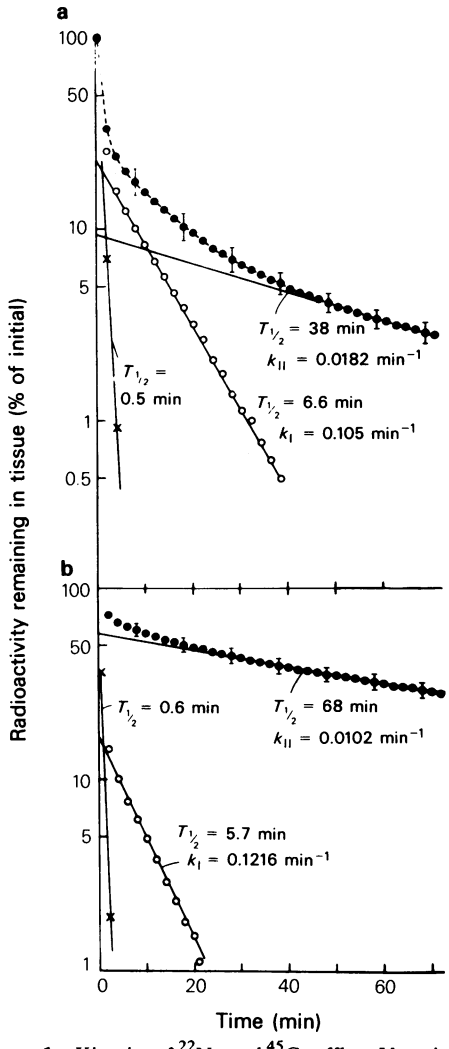

Figure 1 Kinetics of ^{22}Na and ^{45}Ca efflux. Ventricular strips were first incubated at 0°C for 2 h in 2 ml of frog Ringer solution containing 25 μCi of ^{22}Na and 10 μCi of ^{45}Ca, and then placed in a superfusion chamber at 20°C where they were stimulated at 0.2 Hz, and where the incubation fluid was renewed with non-radioactive Ringer every 2 min. Radioactivity remaining in the tissue, expressed as % of the initial (log scale), is plotted against time. (●) = Desaturation curve of ^{22}Na (in a), and of ^{45}Ca (in b), results of 9 experiments, in which the efflux of both isotopes was followed simultaneously. Each desaturation curve is resolved (see Methods) into its straight line (first order) components, next to which the $T_{\frac{1}{2}}$ values and, when appropriate, the rate constants (k), are shown.

representing ^{22}Na efflux from a cellular compartment was also present.

^{45}Ca was lost from the strips much less readily than

^{22}Na (Figure 1b). Its efflux could also be resolved into three first order kinetic components, of which two (k_I:0.1216 min^{-1} and k_{II}:0.0102 min^{-1}) represent efflux from the tissue. Probably, k_I also represents washout from the extracellular space, and k_{II} from a cellular compartment. These results with sodium and calcium efflux resemble those of Van der Kloot & Dane (1964), who used whole frog ventricles, except that calcium efflux was somewhat slower in the present experiments.

Effects of 2,4,-dinitrophenol and caffeine

When strips, previously incubated in ^{22}Na and ^{45}Ca, were superfused with non-radioactive Ringer for 26 min so that the extracellular kinetic components of ^{22}Na and ^{45}Ca efflux became negligible, i.e. less than 1% of the initial radioactivity, and then 0.1 mM DNP was added to the superfusion fluid, it was seen (Figure 2), that contractile force, after a brief initial increase, declined precipitously (half disappearance time: 4.5 min), and ceased altogether after 16 min. DNP induced, without measurable latency, an extremely large and progressive increase in ^{45}Ca efflux that showed no sign of saturation or of reaching a steady state until the end of the experiment. In contrast, the rate of efflux of ^{22}Na remained unchanged following DNP.

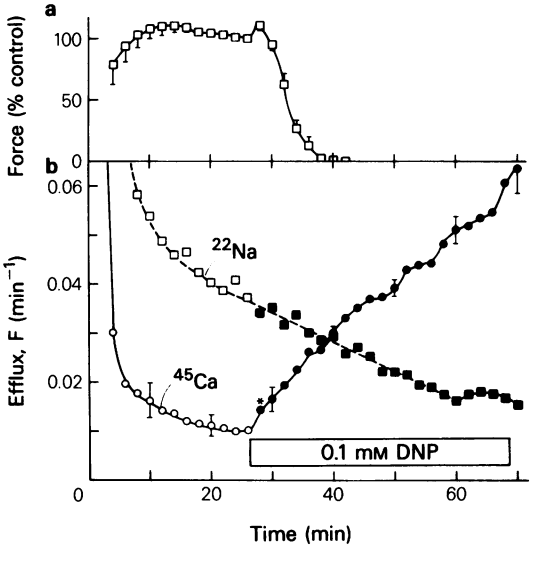

Figure 2 Effects of 2,4-dinitrophenol on contractile tension and on ^{45}Ca and ^{22}Na efflux in strips of frog ventricle. In (a), force of contraction expressed as % of that immediately before addition of DNP, is plotted against time. In (b), the efflux fractional rate constant for ^{22}Na and ^{45}Ca is shown as a function of time. The time during which 0.1 mM DNP was present is indicated. Filled symbols indicate efflux in the presence of DNP. Results of 3 experiments with ^{45}Ca, and of 2 independent ones with ^{22}Na. $*P<0.02$.

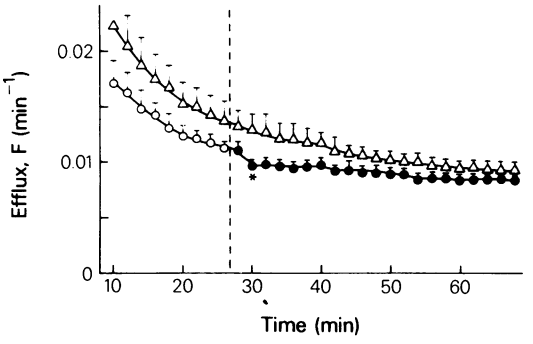

Figure 3 Effect of ouabain 10^{-6} M on ^{45}Ca efflux in frog ventricular strips. ^{45}Ca efflux is plotted against time. Ouabain (10^{-6} M) was added after 26 min of strip superfusion (lower curve, ●). Mean and s.e.mean of 6 control (△) and 5 ouabain-treated (○, ●) experiments. $*P<0.02$ for the slope of the ouabain curve between 28 and 30 min, compared (Student's paired *t* test) to the slope of the 26–28 min interval on the same curve.

Caffeine, at a concentration (20 mM) that produces a marked positive inotropism in this preparation (M.C. Ocampo & F. Orrego, unpublished observations), did not change the efflux rate of ^{45}Ca when added after 1 h of strip superfusion.

Effect of ouabain on ^{45}Ca efflux

When ouabain 10^{-6} M, a glycoside concentration that is non-toxic to this preparation, was added after 26 min of superfusion of strips with non-radioactive Ringer solution, it was seen (Figure 3) that the efflux rate of ^{45}Ca had decreased very significantly 4 min after addition of the drug. This was followed by a change in the slope of the efflux curve, which decreased less than in untreated controls. If 10^{-6} M ouabain was added after 8 min of superfusion (Figure 4), a decrease in ^{45}Ca efflux was again seen but now without latency, and this was also followed by a change of slope of the efflux curve, so that after 12 min in the presence of ouabain, ^{45}Ca efflux became slightly higher than in untreated controls. Qualitatively, the same effects were also observed when 0.25, 0.5, 1, 2 or 4 μM ouabain was present from the beginning of superfusion (7–8 experiments per concentration, results not shown).

Ouabain and sodium pump inhibition

Addition of 10^{-6} M ouabain after 26 min of superfusion, did not change the efflux of ^{22}Na that had been accumulated by the tissue, except for a small increase in efflux, seen after a latency of 8 min (Figure 5). The

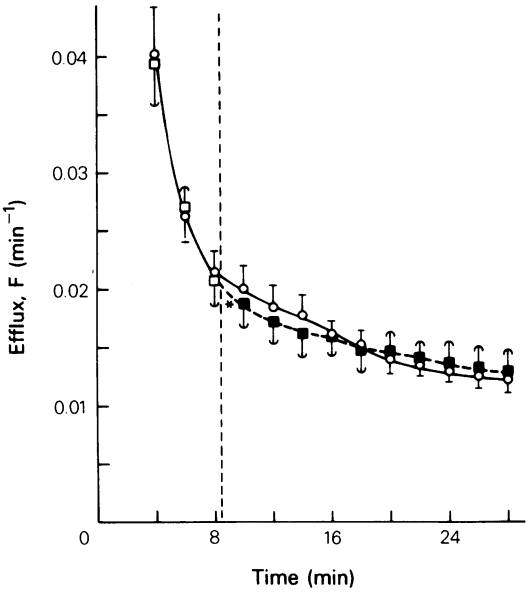

Figure 4 Effect of ouabain 10^{-6} M on early ^{45}Ca efflux in frog ventricular strips. Ouabain was added 8 min after the start of superfusion. *$P < 0.02$ for the comparison (Student's unpaired t test) between the slopes of ouabain-treated (□, ■ $n = 6$) and control (○, $n = 11$) curves in the 8 to 10 min interval.

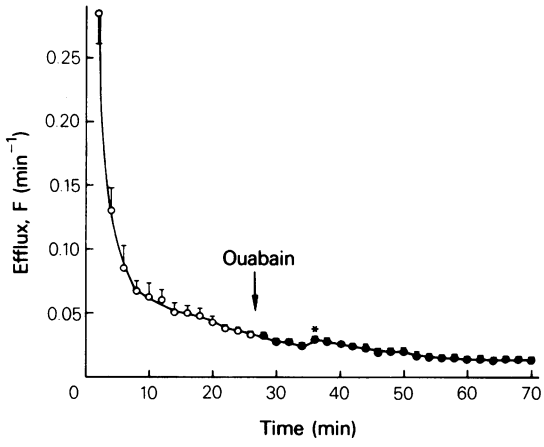

Figure 5 Effect of ouabain 10^{-6} M on ^{22}Na efflux in frog ventricular strips. Ouabain (10^{-6} M) was added after 26 min of superfusion (●). *$P < 0.05$ (paired t test) relative to the immediately preceding point. Results of 5 experiments.

presence of 0.25 to 4 μM ouabain from the beginning of superfusion was also ineffective in producing a significant reduction in ^{22}Na efflux.

Effects of ouabain in normal calcium

Ouabain, at concentrations that ranged from 2.5×10^{-7} M to 4×10^{-6} M, markedly increased the contractile force of strips incubated in frog Ringer solution containing 1.1 mM calcium. No signs of toxicity were observed, except with 4×10^{-6} M, where contractile force declined rapidly after about 15 min of drug action. The rate of onset of the inotropic effect, even at the lower ouabain concentrations, was extremely rapid, and significant increases in contractile force could be observed in the first or second beat following drug application (results not shown).

Inotropic action in low calcium

When strips were transferred from calcium-containing Ringer solution to one in which no calcium was added, but which still contained 5.2 μM Ca as a contaminant, it was observed (Figure 6) that contractile force declined exponentially at first ($T_{\frac{1}{2}} = 3.2$ min; $k = 0.217$ min^{-1}), but the rate of decline tapered off with time. If calcium-free Ringer containing 4×10^{-6} M ouabain, and in which the calcium concentration was also found to be 5.2 μM, was introduced after 40 min in low calcium, a marked, significant and progressive increase in contractile force of more than 300% could be observed (Figure 6). This positive inotropism started after a latency of about 50 s and did not decay even after 40 min in 4×10^{-6} M ouabain.

When the experiments shown in Figure 6 were repeated in 'calcium-free' Ringer containing EGTA (0.1 mM), very similar results were obtained (Figure 7), except that the ouabain-induced increase in contractile force started with a latency of 10 min, compared with 50 s in the absence of EGTA and that the effects of EGTA-Ringer and of ouabain showed greater variation (i.e. larger s.e.mean). In 2 out of 10 experiments, EGTA Ringer caused abolition of contractions, which was never observed in 'calcium-free' Ringer. The magnitude of the increase in force induced by ouabain in EGTA-Ringer was 5.3% of that found in the same strips in 1.1 mM calcium, and 3.1% when 'calcium-free' Ringer was used (Figure 6); these differences were not statistically significant.

Caffeine effects

When strips were first incubated for 1 h in Ringer that contained 4.4 mM calcium and 10^{-6} M ouabain (Figure 8), and then in a similar Ringer solution containing 20 mM caffeine, a marked initial contracture, that relaxed spontaneously, was always observed. This was followed, at irregular intervals, by other waves of contracture of variable intensity. This response to caffeine was never observed if the strips

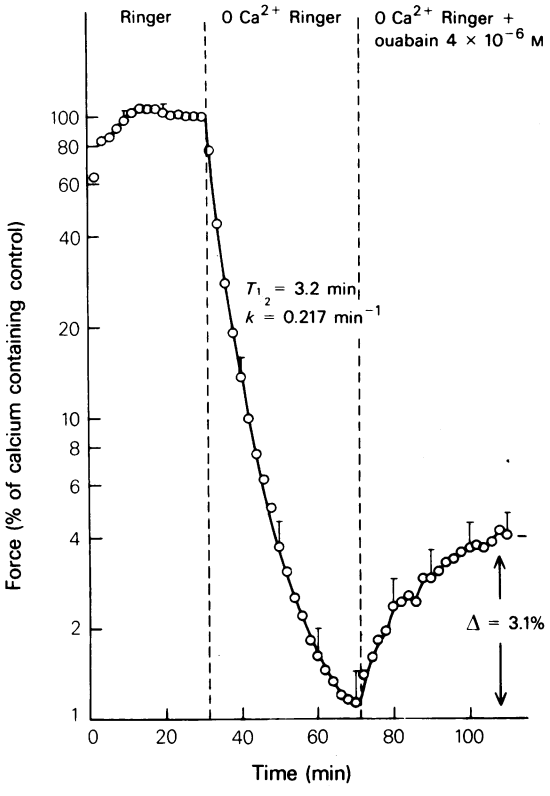

Figure 6 Inotropic effect of ouabain in calcium-free Ringer solution. Frog ventricular strips were stimulated at 0.2 Hz and incubation fluid was renewed every 2 min. Peak contractile force, expressed as % of that in the last period in Ringer, is plotted (log scale) against time. The composition of the superfusion fluid is shown at the top of the figure, and the time of solution change is shown by vertical interrupted lines. In $0\,Ca^{2+}$ Ringer, calcium concentration was 5.2 μM (see text). The $T_{\frac{1}{2}}$ and rate constant for the initial decay of force is shown. Results of 8 experiments; s.e.mean is shown at appropriate intervals. All the points with ouabain are significantly different ($P<0.01$ or better) relative to the last point before ouabain (Student's paired *t* test).

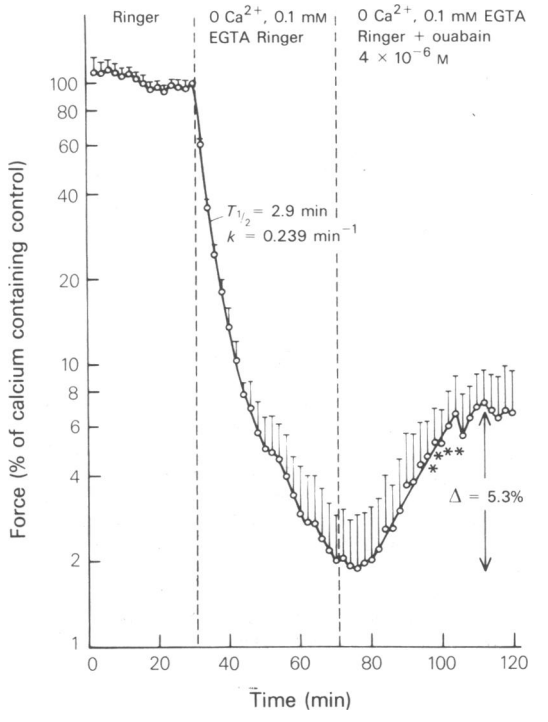

Figure 7 Effects of ouabain on frog ventricular strips incubated in EGTA-Ringer solution. The experimental set up was the same as in Figure 4, except that EGTA-Ringer was used instead of 'calcium-free' Ringer. Results of 8 experiments. $*P<0.01$; $**P<0.02$ relative to the last pre-ouabain point. The maximum increase in contractile force induced by ouabain (i.e. 5.3% of the tension generated by the strip in calcium-containing Ringer) is indicated.

were first incubated, also for 1 h, in 4.4 mM calcium Ringer without ouabain, or if the incubation prior to caffeine was in 1.1 mM calcium Ringer with 10^{-6} M ouabain.

Discussion

The finding that the H^+-ionophore DNP induced a large increase in the efflux rate of ^{45}Ca strongly indicates that the k_{II} for this ion largely, if not exclusively, represents efflux from mitochondria. This is consistent with the known capacity of these organelles to retain a high proportion of intracellular calcium in different tissues and species (Patriarca & Garafoli, 1968; Scarpa & Graziotti, 1973; Carafoli, Crompton, Malmström, Sigel, Salzmann, Chiesi & Affolter, 1977), and with the calcium-proton exchange mechanism of Ca^{2+} transport across the inner mitochondrial membrane (Carafoli *et al.*, 1977; Vercesi, Reynafarje & Lehninger, 1978). The fact that ^{45}Ca efflux rate following DNP can increase 5 fold or more, without showing signs of saturation, indicates that the capacity of the plasma membrane for extruding calcium is very high and, under normal conditions, not rate-limiting. The finding that the slope of ^{22}Na efflux remained unchanged following DNP, is also an indication that the small amount of this isotope still present in cells when DNP was added, was not actively retained by mitochondria. If the fall in contractile tension induced by DNP is interpreted as due to depletion of ATP stores, this lack of effect of DNP on ^{22}Na efflux indicates that, at the time of

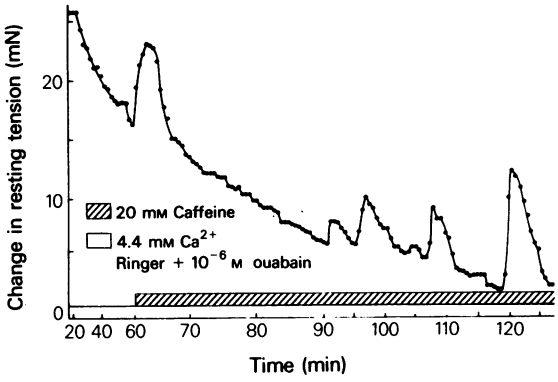

Figure 8 Effect of caffeine on resting tension of a frog ventricular strip. The resting tension of a strip is plotted against time. Superfusion, with solution changes every 5 min, was with Ringer that contained calcium 4.4 mM and ouabain 10^{-6} M. After 1 h of superfusion, caffeine 20 mM was also present. Note compressed time scale from 20 to 60 min. The strip, as in other experiments, was also stimulated at 0.2 Hz. Results of a single experiment. Similar results were obtained in three experiments, but the time at which contractures, other than the initial one, appeared, varied between experiments.

addition of DNP, ^{22}Na efflux does not represent active sodium extrusion, but only a passive mechanism (e.g. sodium-sodium exchange). In heart and other cell types, intracellular sodium is highly compartmentalized, and part of it is bound to nuclei by ion-exchange, passive mechanisms (Siebert, Langendorf, Hannover, Nitz-Litzow, Pressman & Moore, 1965; Pietrzyk & Heinz, 1974; Lee & Fozzard, 1975).

The lack of effect of caffeine on ^{45}Ca-k_{II}, indicates that this drug does not modify mitochondrial calcium efflux in intact cells, a result similar to that obtained with isolated mitochondria (Weber, 1968). This finding should not be taken as an indication that the SR, a caffeine-sensitive structure (Weber, 1968; Weber & Herz, 1968), is non-functional in these cells, since identical results would be expected if the ^{45}Ca turnover is very rapid in this compartment, i.e. only ^{40}Ca was present inside the SR by the time caffeine was added.

Ouabain, at all the concentrations tested, and at different times of application, induced a marked and consistent decrease in ^{45}Ca efflux. Such a decrease was also observed by Klaus & Kuschinsky (1962), who applied low concentrations of digitoxigenin to guinea-pig auricles, and more recently by Wood & Schwartz (1978), who used high (10^{-5} M) concentrations of ouabain and minced guinea-pig heart. As cardioactive steroids are known to exert their effects by acting on the external surface of plasma membranes (Lee & Klaus, 1971; Akera & Brody, 1978), we suggest this effect of ouabain also occurs at the plasma membrane level and not as a direct effect on mitochondria. The fact that ouabain, initially, while having a marked inotropic effect decreased the efflux of calcium that is predominantly stored in mitochondria, does not support the elegant hypothesis of Crompton *et al.* (1976), who thought that cardioactive glycosides might induce positive inotropism by releasing mitochondrial calcium by a sodium-calcium exchange mechanism, secondarily to an increase in $[Na^+]_i$ produced by inhibition of (Na^+, K^+)-ATPase. However, the small and delayed increase in calcium efflux seen following ouabain may be due to this type of mechanism, and may also be related to the increased calcium exchangeability induced by these drugs.

The lack of effect of ouabain on ^{22}Na efflux may be explained, alternatively, by one of the following two mechanisms: ^{22}Na is extruded from the cells by the sodium pump at a rate that is faster than that at which ^{22}Na clears the extracellular space (i.e. k_I), so that the kinetic component that represents active ^{22}Na efflux cannot be measured in this preparation. Such an interpretation explains why neither DNP nor ouabain inhibited ^{22}Na efflux. Perhaps the best indication that ouabain did inhibit the sodium pump, is that it *increased* ^{22}Na efflux after a relatively long latency (cf. Figure 5). This may be an indirect evidence for an increased $]Na]_i$ that displaced bound ^{22}Na from intracellular binding sites. A second possibility is that the steroid might not inhibit the sodium pump. This could not explain the lack of effect of DNP on ^{22}Na efflux, and seems unlikely, at least for the higher ouabain concentrations.

The finding that the inotropic effect of ouabain persisted in EGTA-Ringer and, especially, the fact that the absolute magnitude of the steroid-induced increase in contractile force was essentially the same in 'calcium-free' Ringer compared to EGTA-Ringer (although free extracellular Ca^{2+} was estimated as 60 fold lower in the latter medium) strongly suggests that the inotropic effect of ouabain is, at least in part, independent of a drug-induced increase in calcium influx. Electrophysiological, i.e. indirect, measurements of calcium influx into myocardial cells have always indicated that cardioactive steroids do not increase such influx (Sleator, Furchgott, De Gubareff & Krespi, 1964; Beeler, 1977; Kass, Lederer, Tsien & Weingart, 1978). In addition in brain slices, ouabain, while markedly reducing ^{45}Ca efflux, does not change its initial rate of influx (Cooke & Robinson, 1971); and at the neuromuscular junction, ouabain increases acetylcholine release (a process that has many features in common with muscle contraction), even in calcium-free physiological saline containing 1 mM EGTA (Baker & Crawford, 1975).

Our interpretation of the mechanism by which

ouabain increases contractile tension is as follows: Ca^{2+} is removed from the myoplasm by three competing transport systems located in the plasma membrane, in the SR, and in mitochondria, respectively, the two former being much faster than the mitochondrial one, which, in turn, has a greater capacity for calcium, relative to the SR (Scarpa & Graziotti, 1973; Kitazawa, 1976; Carafoli, 1977). When a cardioactive steroid is applied to the external face of the plasma membrane, the calcium extrusion mechanism of this structure becomes partly inhibited and the other competing systems, especially the SR, accumulate a greater fraction of myoplasmic Ca^{2+}. A progressive increase in (Ca^{2+}) thus occurs in the SR, part of which is released into the myoplasm during each beat. This leads to an increased Ca^{2+} transient, and to increased contractile force. The increased Ca^{2+} transient, in turn, also enhances the activation of the Ca^{2+}-activated potassium conductance present in these cells (Bassingthwaighte, Fry & McGuigan, 1976), so that the action potential shortens and Ca^{2+} influx becomes somewhat reduced (McDonald, Nawrath & Trautwein, 1975). This mechanism may be important in self-regulating steroid effects. When extracellular calcium is very low, mitochondria, because of their large calcium store, may keep supplying the myoplasm with calcium, for which the plasma membrane and the SR compete. A ouabain-induced reduction in calcium efflux might then also possibly lead to a greater calcium content of the SR (i.e. 'activator' calcium) and to an increase in contractile tension, which though lower in absolute magnitude relative to that seen in higher extracellular calcium, is, nevertheless, readily detected.

The mechanism by which ouabain decreases calcium efflux can be interpreted as due to an increase in $[Na^+]_i$ close to the plasma membrane, perhaps transient (Akera *et al.*, 1976). This, according to the sodium-calcium exchange mechanism (Jundt, Porzig, Reuter & Stucki, 1975), can readily lead to a decreased calcium efflux. As this, particularly at low steroid concentrations, has not been rigorously demonstrated, more direct steroid effects on the calcium extrusion mechanism cannot be excluded at present.

Although the SR occupies 0.5% of the myofibrillar volume of frog heart ventricular cells, with a relatively high proportion of it present close to the plasma membrane (Staley & Benson, 1968; Page & Niedergerke, 1972), and the isolated fragmented SR is able to accumulate calcium in the presence of ATP and to release it following caffeine (Fujino, Igarashi & Hoshi, 1979), its role in the contraction of the amphibian heart is disputed, mainly for kinetic reasons as Niedergerke (1956) found the time course of calcium action on frog ventricular strips was too slow to be accounted for by diffusion through the extracellular space but much too fast to involve an equilibration with intracellular calcium. This led him to postulate that the strength of contraction was controlled by the concentration of calcium in a 'superficially located region of the heart cell'. The presence of cyclic contractures similar to those induced by caffeine in the present work has been taken, in other species, as characteristic of the presence of a functioning SR (Endo, Tanaka & Ogawa, 1970; Fabiato & Fabiato, 1975). However, in frog ventricle cells 'skinned' mechanically, no such cyclic contractures can be observed, perhaps because of damage to the superficial SR during the skinning process (Fabiato & Fabiato, 1978). Possibly in some way related to the present findings are those of Nayler (1973), who found that ouabain increased the amount of calcium that could be displaced from myocardial cells by lanthanum, as well as those of Matsumura & Narita (1980), who showed that caffeine (30 mM) induced contracture in frog heart muscle only when calcium was 5.4 mM, or higher; and those of Otsuka & Nonomura (1963), who found that elevated extracellular potassium induced a much larger contracture in ouabain-pretreated frog ventricular strips, although the K^+-induced depolarizations were the same in control and ouabain-treated strips. These effects of K^+ can also be interpreted as due to a greater calcium content of the SR induced by ouabain. Although these latter findings, as well as those of Chapman & Miller (1974), support a functional role for the SR in the amphibian heart, and its possible participation in the inotropic effect of cardioactive steroids, a direct demonstration of both points is clearly needed before the hypothesis presented above is to be accepted.

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