Inotropic Effects and Changes in Sodium and Calcium Contents Associated with Inhibition of Monovalent Cation Active Transport by Ouabain in Cultured Myocardial Cells

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ABSTRACT Cultured monolayers of spontaneously contracting chick embryo ventricular cells were perfused with culture medium containing ouabain. Contractile state was monitored by an optical-video system recording amplitude and velocity of cell wall motion. Positive inotropic effects of 2.5×10^{-7} to 10^{-6} M ouabain were manifest within 1.5–2 min, and reached a stable plateau within 5–6 min. The inotropic effect was fully reversed within 5 min after washout of ouabain. Inhibition of uptake of $^{42}K^+$ (or the K^+ analog $^{86}Rb^+$) and efflux of $^{24}Na^+$ occurred 1.5–2 min after exposure to ouabin. The degree of inhibition of transport was closely related to the magnitude of the positive inotropic effect throughout the ouabain concentration range 10^{-7} to 10^{-6}M. After washout of ouabain from monolayers, the monovalent cation active transport rate returned to normal within 1 min. Thus, both the onset and offset of inotropic action of ouabain were closely related temporally to inhibition of the sodium pump. Exposure to ouabain caused significant increases in exchangeable Na and Ca contents that appeared to be developed within 5 min. These data support the hypothesis that inhibition of monovalent cation active transport by ouabain is causally related to the development of positive inotropy and are consistent with modulation of Ca content by intracellular Na^+ via the Na^+-Ca^{2+} exchange carrier mechanism.

INTRODUCTION

In 1938, Cattell and Gold demonstrated that digitalis exerts a direct positive inotropic effect on cardiac muscle. Since then, substantial evidence has accumulated indicating that the membrane-bound monovalent cation transport enzyme complex known as sodium, potassium-activated adenosine triphosphatase (NaK-ATPase) is the pharmacologic receptor for this effect of cardiac glycosides (Smith et al., 1972; Schwartz, 1976). Allen and Blinks (1978) have demonstrated that exposure of cardiac muscle to cardioactive

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steroid results in augmentation of the increase in intracellular free ionized calcium concentration, $[Ca^{2+}]_i$, that occurs during an action potential. However, the cellular mechanisms underlying this enhanced $[Ca^{2+}]_i$ transient, which presumably accounts for the positive inotropic effect observed, remain uncertain.

Several studies have supported the hypothesis that the positive inotropic effect of cardiac glycosides is related to inhibition of monovalent cation active transport (Ku et al., 1974; Langer, 1977; Hougen and Smith, 1978). Baker et al. (1969) demonstrated that resting Ca^{2+} influx in the perfused squid giant axon could be augmented by an increase in internal sodium concentration, $[Na^+]_i$, and presented evidence that movements of Na^+ and Ca^{2+} across the cell membrane are linked by a Na^+-Ca^{2+} exchange mechanism. These investigators proposed that an increase in Ca^{2+} influx associated with an increase in $[Na^+]_i$ could account for a positive inotropic effect in cardiac tissue after inhibition of monovalent cation active transport. The relationship between alterations in the transmembrane Na^+ concentration gradient and changes in Ca^{2+} fluxes has also been studied in myocardial tissue, providing evidence for the existence of a Na^+-Ca^{2+} exchange carrier mechanism (Reuter and Seitz, 1968; Reuter, 1974; DeMello, 1976; Ellis, 1977), which could function as an important component of the postulated link between inhibition of monovalent cation transport and enhanced contractility.

Further support of the sodium pump inhibition hypothesis has been sought by investigators attempting to show an increase in $[Na^+]_i$ in cardiac tissue induced by positively inotropic concentrations of cardiac glycosides. Langer and Serena (1970) obtained indirect evidence for an increase in tissue Na content during exposure to positively inotropic concentrations of acetylstrophanthidin in the arterially perfused rabbit interventricular septum preparation. However, careful studies by a number of other investigators have not demonstrated significant increases in $[Na^+]_i$ at positively inotropic but subtoxic concentrations of cardiac glycosides (Lee and Klaus, 1971; Bentfeld et al., 1977). This led Akera et al. (1976) to propose that inotropic but subtoxic cardiac glycoside concentrations increase only the $[Na^+]_i$ transient after depolarization, resulting in augmented Ca^{2+} entry even in the absence of a measureable increment in steady-state Na content.

In the experiments to be reported, we have used a cultured myocardial cell preparation to examine the temporal relationships between inhibition of monovalent cation active transport and contractile responses during exposure to and washout of ouabain, to define the ouabain concentration dependence of the degree of inhibition of monovalent cation active transport and the extent of positive inotropy, and to determine the effect of inotropic concentrations of ouabain on cellular contents of Na and Ca.

METHODS

Tissue Culture

Monolayer cultures of beating chick embryo ventricular cells were prepared by the method of DeHaan (1967) with modifications as previously described by Barry et al.

(1975). Briefly, 8–10-d-old chick embryo hearts were removed under sterile conditions, and the ventricles were cut into 0.5-mm^3 fragments and placed in Ca- and Mg-free Hanks' solution. The ventricular fragments were gently agitated in 10 ml of 0.025% (wt/vol) trypsin in Ca-and Mg-free Hanks' solution at 37°C for four cycles of 7 min each. The supernatant suspension, containing cells dissociated by each cycle, was placed in 20 ml of cold trypsin inhibitor medium (50% heat-inactivated fetal calf serum and 50% Ca-and Mg-free Hanks' solution). This suspension was centrifuged at 1,000 rpm for 10 min, the supernate was discarded, and the cells were resuspended in culture medium consisting of 6% heat-inactivated fetal calf serum, 40% Medium 199 (Grand Island Biological Company, Grand Island, N.Y.), 0.1% penicillin-streptomycin antibiotic solution, and 54% low-potassium balanced salt solution containing (in millimolar concentrations): NaCl, 116; NaH_2PO_4, 1.0; $MgSO_4$, 0.8; KCl, 1.18; $NaHCO_3$, 26.2; $CaCl_2$, 0.87; and glucose, 5.5. Major ion concentrations (millimolar) in the final culture medium were: Na, 145; K, 4.0; Ca, 1.2; HCO_3, 18; Mg, 0.8; and Cl, 131. The suspension of cells was diluted to 4×10^5 cells/milliliter and placed in plastic tissue culture dishes containing 25-mm circular coverglasses (VWR-Vanlab, thickness No. 2, VWR Scientific Inc., San Francisco, Calif.). Cultures were incubated in a humidified 5% CO_2, 95% air atmosphere at 37°C, and 30-40 coverslips were prepared at each culture. Confluent monolayers, in which at least 80% of cells were synchronously contracting, developed by 2–3 d, after which time contractility, ion flux, and ion content studies were performed.

Measurement of Contractility

A glass coverslip with attached heart cell monolayer was placed in a Sykes-Moore chamber (Bellco Glass, Inc., Vineland, N.J.) provided with inlet and exit ports for culture perfusion. The chamber was placed on the stage of an inverted phase contrast microscope (Diavert, E. Leitz, Inc., Rockleigh, N. J.), which was entirely enclosed in a lucite box in which was maintained a heated (37°C) room air atmosphere. The inlet to the Sykes-Moore chamber was connected by polyethylene tubing to four syringe pumps, so that each culture could be sequentially perfused with separate test solutions, each equilibrated with 95% air–5% CO_2. Flow characteristics in the chamber were estimated by indocyanine green dye washout. Medium bathing a cell in the center of the coverslip could be changed with a time constant (τ) of 15 s, at a flow rate of 0.96 ml/min. The optical apparatus was supported by an air table to damp building vibrations. The cells were magnified using a × 40 objective, and the image was monitored by a low light level silicon TV camera (DAGE 650 SSX, DAGE-MTI Inc., Michigan City, Ind.) attached to the microscope observation tube using a × 2 coupler. The TV camera video output was connected to a video motion detector (633, Colorado Video, Inc., Boulder, Col.) and displayed on a 9-in. TV monitor (Conrac Corp., Stamford, Conn.). The total magnification of the image on the monitor screen was × 2,000. The TV camera had an interlace defeat, so that the image was composed of 262 raster lines. The motion detector monitored a selected raster line segment and provided new position data every 16 ms for an image border of a single cardiac cell within the monolayer moving along the raster line. The analog voltage output from the motion detector was filtered at 15 Hz with a 48 dB/octave low-pass active filter, and calibrated to indicate the actual micrometers of motion. The first derivative was obtained electronically and was recorded as velocity of motion in micrometers per second. These analog traces were recorded using a Hewlett-Packard Co. (Palo Alto, Calif.) 4560 optical recorder. Cell monolayers contracted spontaneously at rates of 100–150 beats/min, and for a given monolayer the rate did not change significantly during perfusion. For contractility effect experiments, cells were perfused with media

containing 0.6 mM Ca^{2+} to facilitate the detection of positive inotropy (Barry et al., 1978). Under these conditions, control amplitude of motion ranged from 0.6 to 4 μm, averaging 0.9 μm.

Monovalent Cation Transport Measurement

$^{42}K^+$ and $^{86}Rb^+$ Uptake Uptake of K^+ by cardiac tissue provides a useful measure of the activity of the NaK-ATPase-mediated monovalent cation active transport system. $^{86}Rb^+$ is a convenient K^+ analog frequently used as an indicator of K^+ transport (Bernstein and Israel, 1970; Hougen and Smith, 1978). We used this approach to study ouabain-induced inhibition of active transport of monovalent cations in cultured monolayers of chick embryo ventricle cells.

Preliminary experiments were done to determine the amount of washing required to remove extracellular fluid in these cultures. After labeling cells to asymptote in media containing 2 μCi/ml ^{51}Cr EDTA, the "unwashed" extracellular fluid volume was 75 μl/mg protein. After three washes for 2 s each in physiologic salt solution at 22°C, this was reduced to 1.81 ± 0.03 μl/mg protein ($n = 35$). Three further washings did not significantly further lower the ^{51}Cr EDTA content of the monolayers. Thus, four washes were used in experiments utilizing radioactive isotopes. ^{51}Cr EDTA remaining on coverslips following four washes represents slowly exchanging extracellular space, and/or ^{51}Cr EDTA adherent to cell and glass surfaces.

Monolayers to be used for uptake experiments were exposed to L-[4,5-^{3}H, N]leucine (0.2 μCi/ml, total leucine concentration 28 mg/liter) for 24 h. Eight coverslips of cells labeled with [^{3}H]-leucine were placed in an uptake chamber containing fresh medium which was maintained at 37°C in a 95% air-5% CO_2 atmosphere and was gently agitated to diminish unstirred layer effects. At time zero, equilibration medium was removed and medium containing $^{86}Rb^+$ (5 μCi/ml, total [Rb^+] 0.1 mM) was added to the chamber. Coverslips were removed at 1-min intervals and rinsed four times as described above, and cells were dissolved in 0.5 N NaOH.

Aliquots of dissolved cells were then suspended in scintillation fluid and counted in a liquid scintillation spectrometer (model 3330-Packard Instrument Co., Downers Grove, Ill.). For each culture, the protein contents of aliquots from one-fourth of the separate coverslips were determined by the method of Lowry et al. (1951), and related to ^{3}H counts. The average relationship between ^{3}H counts and protein content for that culture was then calculated. Simultaneous counting of $^{86}Rb^+$ and ^{3}H thus permitted normalization of Rb^+ uptake per milligram of cell protein. This was necessary because of variability in the number of cells on each coverslip.

In this system Rb^+ uptakes were linear for 6 min, consistent with the reported τ of ~16 min for K^+ efflux from cultured chick ventricular cells (Horres and Lieberman, 1977). Uptake of K^+ was studied in a similar fashion, using 3.0 μCi/ml ^{42}K (total [K^+] 4.0 mM). In all experiments, Rb^+ or K^+ uptake was measured in some cultures in the presence of 10^{-3}M ouabain to define passive uptake in nanomoles per milligram protein/minute. This value was subtracted from uptake rates in the absence of ouabain or in the presence of stated ouabain concentrations, to determine the effect of ouabain on active transport of Rb^+ or K^+.

$^{24}Na^+$ Efflux Alterations in monovalent cation active transport were also assessed by measurement of $^{24}Na^+$ efflux rates. Cells grown on coverslips were labeled with [^{3}H]leucine as described above. They were labeled to steady state by 30 min of exposure to 5 μCi/ml $^{24}Na^+$-containing culture medium (total [Na^+] 145 mM). Individual coverslips were then transferred to a perfusion chamber with a total volume of 0.45 ml. The cells were superfused with unlabeled washout medium at 1.5

ml/min at 37°C. Initial experiments demonstrated that at this flow rate $^{24}Na^+$ efflux was not perfusion rate-limited. The effluent was collected at 30-s intervals and counted as previously described for determinations of $^{24}Na^+$ efflux/30 s time. From these data the rate constant for Na^+ efflux was calculated. Cells from the same culture were superfused with media containing ouabain to determine the effects of ouabain on Na^+ transport as reflected by changes in the rate constant of $^{24}Na^+$ efflux.

Effects of Ouabain on Na and Ca Content

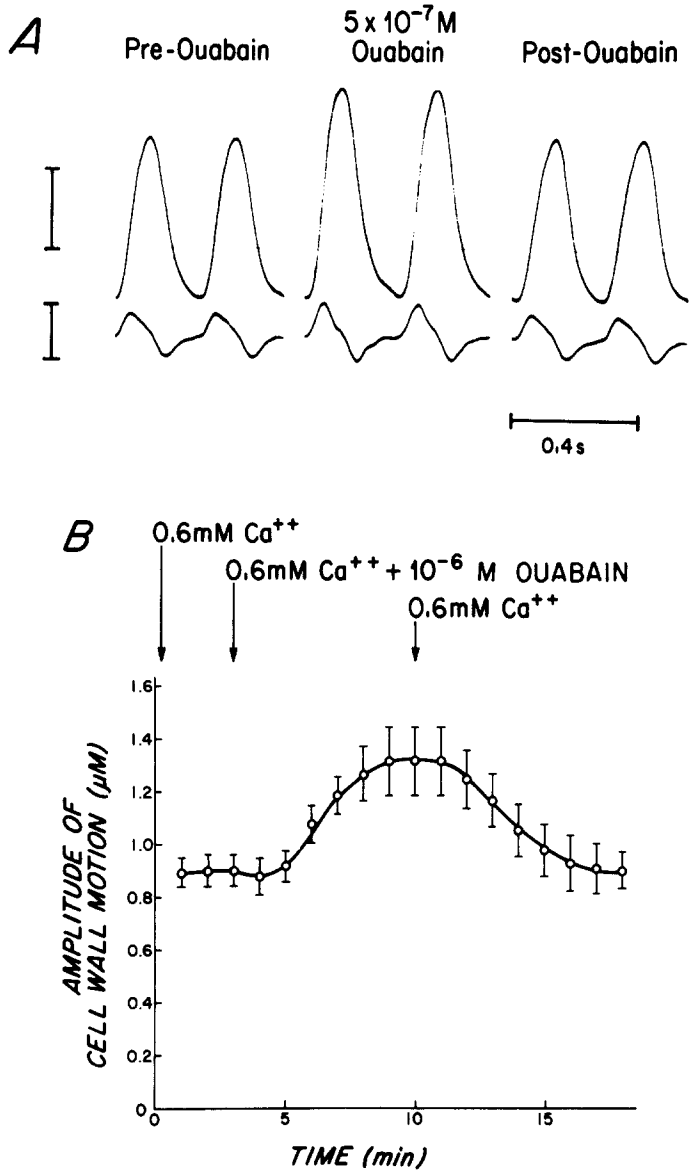
Isotopic methods were used to assess changes in Na and Ca content in cultured heart cells after exposure to ouabain. For estimation of Na content, cells labeled with [^{3}H] leucine were incubated in culture medium containing $^{24}Na^+$ (5 μCi/ml at total [Na^+] 145 mM) for 5, 10, and 30 min in the presence of various concentrations of ouabain, washed for 2 s each in four vessels containing 25 ml of physiologic buffer at 22°C, and dissolved in 1 ml of 0.5 M NaOH. Aliquots were then suspended in scintillation fluid and counted. Results from each coverslip were normalized for cell density variation as described above and data were calculated as Na content in nanomoles per milligram protein.

For determination of Ca content, [^{3}H]leucine-labeled cells were incubated in the presence of $^{45}Ca^{2+}$ (5 μCi/ml, total [Ca^{2+}]0.725 mM) for 5, 10, and 120 min and 24 h in the presence of stated ouabain concentrations. These specific times were chosen because preliminary $^{45}Ca^{2+}$ efflux studies demonstrated two kinetically distinguishable cellular compartments, one with a τ for efflux of 45 s and one with a τ of 26 min. After labeling, coverslips were washed four times as described above and cells were then dissolved and counted. Ca content of cells on each coverslip was normalized for protein content using double isotope counting of $^{45}Ca^{2+}$ and ^{3}H as previously described, and the data were calculated as Ca content in nanomoles per milligram protein.

RESULTS

Effects of Ouabain on Contractility

Cultured monolayers of chick embryo ventricular cells exhibited distinct positive inotropic responses to ouabain. Fig. 1 *A* shows representative changes in contraction amplitude and velocity of a single cell of a monolayer culture after initiation of perfusion with culture medium containing 5×10^{-7} M ouabain. Increases in contraction amplitude and velocity had a rapid onset, reached plateau values within 5–6 min, and were rapidly reversed on washing out ouabain from the culture chamber. Fig 1 *B* summarizes results obtained in 12 cultures demonstrating the time-course of the onset of inotropic effects after initiation of perfusion of the cultures with 10^{-6}M ouabain. Also illustrated is the time-course of the disappearance of the positive inotropic effect during washout with ouabain-free culture medium. Allowing for a 30–40 s lag between institution of perfusion of the culture with ouabain-containing solution and the attainment of greater than 90% completion of the concentration change in the chamber, ouabain at this concentration induced a readily demonstrable positive inotropic effect within 1.5–2 min. After washout of ouabain, control levels of contraction amplitude and velocity were restored



within 6–7 min. Average beating rate did not change significantly during exposure to ouabain over the concentration range 10^{-7} to 10^{-6} M.

Effects of Ouabain on Monovalent Cation Transport

To validate the use of Rb^+ as a K^+ analog, we performed studies of the simultaneous uptake by cultured heart cells of $^{42}K^+$ (total [K^+] 4.0 mM) and $^{86}Rb^+$ (total [Rb^+] 0.1 mM). As shown in Table I, the experimentally determined ratios of both active and passive uptake of K^+: Rb^+ approximated

40:1, consistent with our previous experience with other myocardial cells (Smith et al., 1972; Hougen and Smith, 1978) and indicating that Rb^+ at 0.1mM concentration is a suitable K^+ analog for the study of active and passive transport. In other studies, K^+ uptake by cultures in media containing $^{42}K^+$ as the only radioisotope was compared to K^+ uptake by cultures in

TABLE I

Rb^+ AND K^+ UPTAKE BY CULTURED HEART CELLS

	Control	10^{-6} M Ouabain	10^{-3} M Ouabain
Rb^+ uptake, *nmol/mg protein · min*	3.45 ±0.33	1.89 ±0.38	1.40 ±0.30
K^+ uptake, *nmol/mg protein · min*	138.65 ±13.61	76.02 ±4.81	56.24 ±8.03
K^+ uptake/Rb^+ uptake ratio	40.2	40.2	40.2

Coverslips with attached heart cell monolayers labeled with [^{3}H]leucine were exposed to medium containing $^{86}Rb^+$ and $^{42}K^+$ (5 μCi/ml $^{86}Rb^+$, 0.1 mM Rb; 3 μCi/ml $^{42}K^+$, 4 mM K^+) in the presence of zero, 10^{-6} M, and 10^{-3} M ouabain concentrations. Each value represents the slope (± SEM) of a regression line obtained from two experiments wih six time points each at 1-min intervals. Correlation coefficients were > 0.95 for all regression lines.

media containing both $^{42}K^+$ and $^{86}Rb^+$ at a total Rb^+ concentration of 0.1 mM. Results were essentially identical, further indicating that at these low concentrations, Rb^+ does not measureably alter K^+ transport.

The time-course of the effect of 10^{-6}M ouabain on active transport of Rb^+ in monolayer cultures is shown in Fig. 2 *A*. It is apparent that inhibition of active transport of Rb^+ is demonstrable within 2 min after exposure to 10^{-6}M

FIGURE 1. (*Opposite*) Effect of ouabain on contractility of cultured heart cells. (*A*) Recording of amplitude of contraction in micrometers (upper trace) and velocity of cell wall motion in micrometers per second (lower trace). Pre-ouabain control amplitude and velocity (left panel) were obtained during perfusion of the culture with medium containing 0.6 mM Ca at 37°C. The middle panel shows the change in amplitude and velocity of cell wall motion 7 min after initiation of perfusion with medium containing 0.6 mM Ca and 5×10^{-7}M ouabain. The post-ouabain recordings (right panel) were obtained after 7 min of perfusion of the culture with ouabain-free medium. The upper vertical bar indicates 1 μm of motion amplitude and the lower vertical bar 24 μm/s of motion velocity. (*B*) Mean (± SEM) values obtained in 12 cultures during exposure to and washout of 10^{-6}M ouabain. Amplitude of cell wall motion in micrometers is plotted against time in minutes. After 10 min of equilibration, cultures were perfused with 10^{-6}M ouabain for 7 min. The contraction amplitude began to increase after 2 min of exposure to ouabain and reached a plateau (47% mean increase) within 6 min. During washout of ouabain, the effect was rapidly reversed and control level was restored after 6 min.

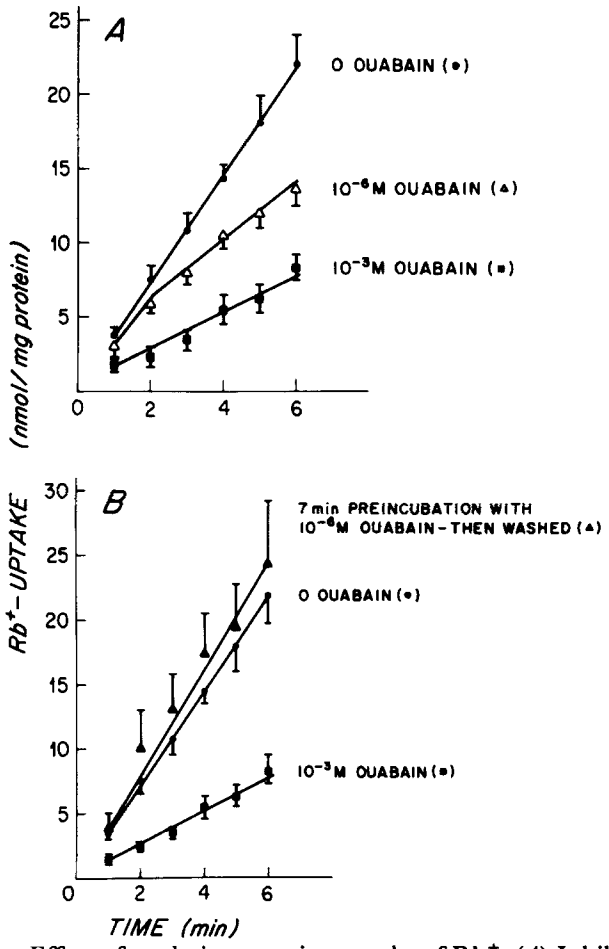


FIGURE 2. Effect of ouabain on active uptake of Rb^+. (*A*) Inhibition of uptake during exposure to ouabain. The upper line shows Rb^+ uptake (n = 10, means ± SEM) in nanomoles per milligram protein vs. time in the absence of ouabain. The uptake in the presence of 10^{-3}M ouabain added at time zero is shown in the lower line and represents nonspecific (passive)Rb^+ uptake. The difference in slope of the control and 10^{-3}M ouabain lines was defined as active uptake. The curve in presence of 10^{-6} ouabain demonstrates an uptake rate similar to control for the first 2 min after exposure to ouabain, but then declines significantly ($P < 0.001$). The reduction in rate of Rb^+ uptake appeared stable after 2 min of exposure to ouabain, before full development of the inotropic effect. (*B*) Recovery of monovalent cation active transport after washout of ouabain. The lower (10^{-3} M ouabain) and middle (control) curves were obtained as described previously. To obtain the upper curve, monolayers were exposed to 10^{-6}M ouabain for 7 min, a dose and time sufficient to cause a 47% increase in contractility and 75% inhibition of Rb^+ uptake. Monolayers were then washed for 1 min to remove ouabain and exposed at time zero to $^{86}Rb^+$-containing medium. Active transport of Rb^+ recovered to control values within 2 min of ouabain removal. Mean uptake was slightly greater than control, but the difference was not statistically significant.

ouabain, as shown by deviation from control values of the Rb^+ uptake curve for cultures exposed to ouabain. The passive uptake of Rb^+ by cultures is defined by the lower curve showing Rb^+ uptake in the presence of 10^{-3}M ouabain. It should be noted that this concentration of ouabain inhibited contraction of the cells within a few seconds. Thus this curve demonstrates the passive uptake of $^{86}Rb^+$ by quiescent cells.

Fig. 2*B* summarizes the results of experiments designed to define the time-course of changes in Rb^+ transport after washout of ouabain. The washout procedure required 1 min to restore ouabain-free medium, and the uptake of Rb^+ by cultures was completely recovered to control values at the earliest time transport rates could be determined. These findings indicate that inhibition of active transport of monovalent cations is closely related temporally to the onset of inotropic effects, although the inotropic response requires several minutes to develop fully after the development of a stable degree of active transport inhibition. During washout, recovery of monovalent cation active transport is very rapid in this system and precedes the disappearance of the positive inotropic effect.

In order to define further the effects of ouabain on monovalent cation active transport, we examined the effect of 10^{-6}M ouabain on the rate of $^{24}Na^+$ efflux in cells preloaded with this tracer. There was a significant decrease in the rate of $^{24}Na^+$ efflux from cultured cells perfused with ouabain-containing culture medium. After 2 min of perfusion the rate constant for $^{24}Na^+$ loss was 0.590 ± 0.048 min^{-1} in control cultures (mean $\pm$ SEM, $n = 12$) as compared with 0.385 ± 0.035 in cells perfused with 10^{-6}M ouabain ($n = 12$, $P < 0.001$). Thus, both Na^+ efflux and Rb^+ uptake data demonstrate that inhibition of active transport of monovalent cations after exposure to ouabain is quite rapid in onset and corresponds closely to the time of onset of the positive inotropic response.

We next examined the relationships among ouabain concentration, the degree of inhibition of monovalent cation active transport, and changes in contraction amplitude observed in matched cultures. These results are summarized in Fig. 3. The threshold concentrations for effects of ouabain both on active transport and on contractile amplitude were between 1 and 2.5×10^{-7}M ouabain. Between 10^{-7} and 10^{-6}M ouabain there was a correlation between the degree of NaK-ATPase inhibition as judged by reduced monovalent cation active transport and the degree of inotropic response. We observed no significant inotropic response without detectable inhibition of monovalent cation active transport. No evidence of toxicity manifested by irregularity of beating or marked changes in spontaneous beating rate occurred, even after exposure to ouabain for several hours within the range 10^{-7} to 10^{-6}M. Higher concentrations of ouabain ($\geq 10^{-5}$M) led to cessation of beating, as mentioned previously.

Effects of Ouabain on Na and Ca Content

As shown above, inotropically effective concentrations of ouabain clearly inhibited active transport of monovalent cations in these cultured cells. In order to determine if changes in intracellular Na concentration occurred as a

consequence of this inhibition of cation transport, and in view of evidence suggesting an important role of Na^+-Ca^{2+} exchange in modulating Ca content, we studied the effects of ouabain on cellular Na and Ca contents. In Fig. 4 are shown the effects of 5×10^{-7}M ouabain on calculated Na content after 5, 10, and 30 min of exposure. In this and the following experiments, Na (or Ca) content was calculated assuming complete exchange of tracer with the intra-

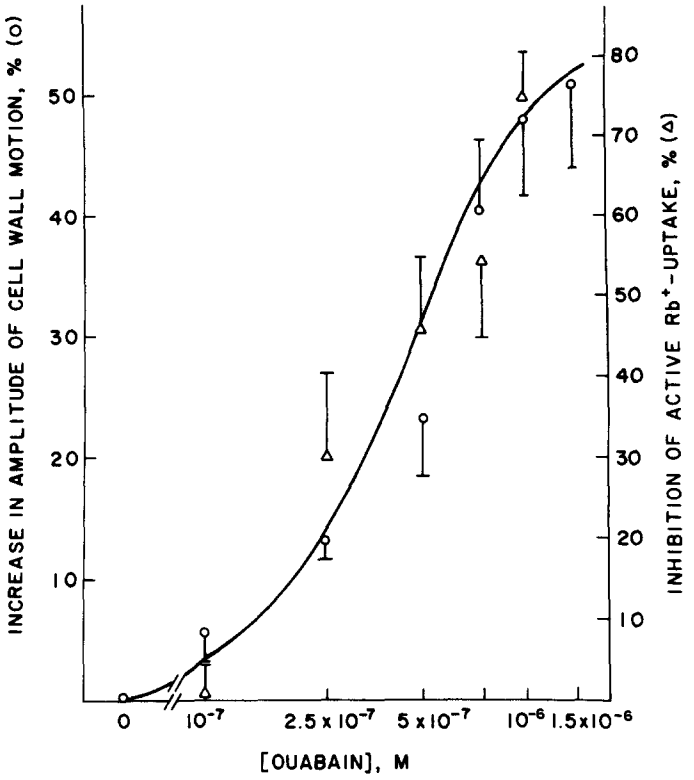


FIGURE 3. Concentration-effect curve showing responses to ouabain of amplitude of cell wall motion (left scale) and inhibition of Rb^+ active transport (right scale). Points are means ± SEM of 4–14 experiments. The increase in contraction amplitude in percent shows the maximal effect at each ouabain concentration. Thresholds for significant inhibition of Rb^+ active transport and increase of contractility were between 10^{-7} and 2.5×10^{-7} M ouabain. The similar dependence of percent increase in amplitude of wall motion and percent inhibition of Rb^+ transport on ouabain concentration is evident.

cellular pool, from the counts per minute observed (corrected for cell density) and the specific activity of $^{24}Na^+$ in the uptake medium. Thus, calculated Na content is less than the true exchangeable Na content until equilibrium is reached. Calculated Na content of the cells reached a plateau after 30 min of exposure to $^{24}Na^+$. There was a significant increase in Na content after

exposure to ouabain that appeared to be fully developed within 5 min, corresponding to the time when the inotropic effect was fully developed.

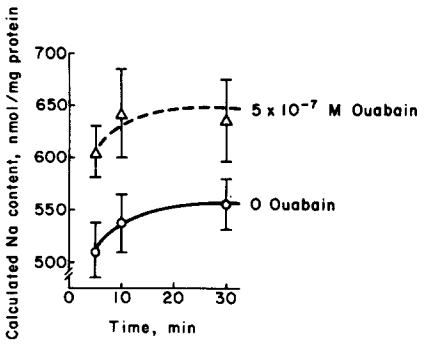


FIGURE 4. Calculated sodium content of cultured heart cells. Monolayer cultures labeled with [^{3}H]leucine were exposed to $^{24}Na^+$-containing medium (5 μCi/ml, total [Na^+] 145 mM) for the indicated times in the absence or presence of 5 × 10^{-7} M ouabain. Data were corrected for milligrams of protein, and sodium content was calculated using the known specific activity of the incubation medium. Points are means ± SEM of 25–35 experiments. There was a significant ($P < 0.01$) increase in sodium content after 5 min of exposure to 5 × 10^{-7} M ouabain, which was stable over the remaining uptake period.

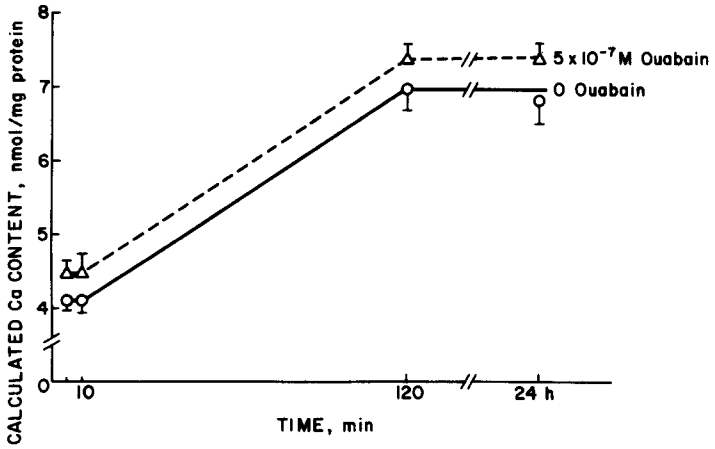


FIGURE 5. Calculated calcium content of cultured heart cells. Monolayers were exposed to $^{45}Ca^{2+}$-containing medium (5 μCi/ml, total [Ca^{2+}] 0.725 mM) for the indicated times in the absence or presence of 5 × 10^{-7}M ouabain. Points are means ± SEM of 28–35 experiments. At 5 min a small but significant ($P < 0.01$) increase in calcium content induced by 5 × 10^{-7}M ouabain was found. Complete equilibrium of Ca^{2+} exchange was reached within 120 min, but no further increase in Ca content occurred after the initial 5 min of uptake.

The effects of ouabain on calculated Ca content are shown in Fig. 5. Exposure to 5 × 10^{-7}M ouabain significantly increased Ca content by 5 min, at a time when the increase in Na content and the positive inotropic effect

were fully manifest. The increment above control Ca content was stable over the remainder of the incubation period.

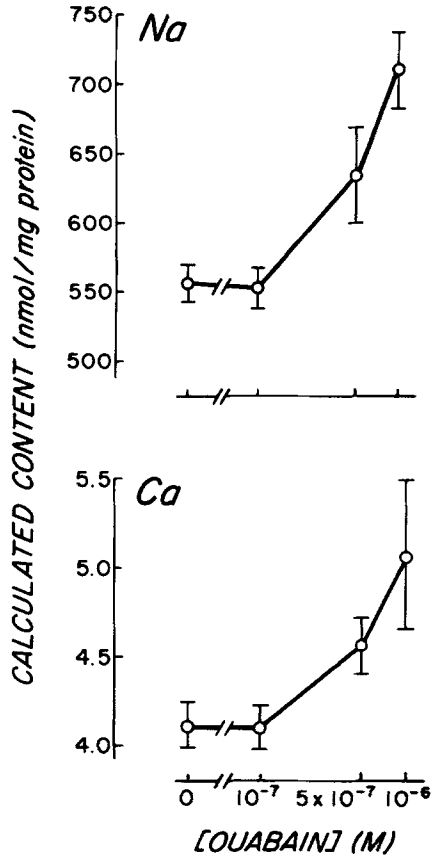


FIGURE 6. Effects of ouabain on Na and Ca content. Upper panel: dependence of cellular sodium content on ouabain concentration (log scale). Monolayers were exposed to $^{24}Na^{+}$ for 30 min as described in Fig. 4. At this time equilibrium with exchangeable intracellular Na^{+} was reached. Points are means ± SEM of 25–38 experiments. At 10^{-7} M ouabain, there was no change in sodium content whereas there was a significant increase at 5×10^{-7} M ($P < 0.01$) and at 10^{-6} M ouabain ($P < 0.001$). Lower panel: dependence of cellular calcium content on ouabain concentration. Monolayers were exposed to $^{45}Ca^{2+}$ for 10 min as described in Fig. 5. Points are means ± SEM of 28–35 experiments. At 10^{-7} M ouabain, the change in Ca content was not statistically significant, but an increase could be demonstrated at 5×10^{-7} ($P < 0.01$) and at 10^{-6} M ouabain ($P < 0.001$).

The relationships between ouabain concentration and cellular Na and Ca contents were next examined. Calculated Na contents after 30 min of exposure were determined in the presence of 10^{-7}, 5×10^{-7}, and 10^{-6}M ouabain. The results are summarized in Fig. 6. A direct relationship between ouabain concentration and cell Na content was noted (Fig. 6, upper curve), consistent

with the inhibition of Na^+ efflux previously observed. Ca content after 10 min of incubation in media containing various concentrations of ouabain is also shown in Fig. 6. There was a direct relationship between ouabain concentration and the magnitude of the increase in calculated Ca content. No significant increase in calculated Ca content was observed at an ouabain concentration (10^{-7}M) that failed to produce either an appreciable increase in Na content or a positive inotropic effect.

DISCUSSION

For more than a decade, controversy has existed regarding the mechanism of the positive inotropic effects of digitalis on cardiac muscle. The extensive literature has been well summarized in reviews by Lee and Klaus (1971), Schwartz et al. (1975), and Akera and Brody (1978). Specific areas of controversy have included the role of inhibition of NaK-ATPase in the development of positive inotropy, and the importance of an increase in steady-state $[Na^+]_i$ in mediating this effect.

The data reported here support the hypothesis that inhibition of monovalent cation active transport by cardiac glycosides is causally related to the development of their positive inotropic effect on heart muscle. During exposure to ouabain, a decrease in active transport both of the K^+ analog Rb^+ and of Na^+ in cultured heart cells preceded development of the full inotropic effect. In addition, during washout of ouabain, recovery of active transport to normal preceded loss of the positive inotropic effect.

In our experiments, the degree of inhibition of active transport and the degree of positive inotropic response showed a similar dependence on ouabain concentration. Data have been reported suggesting that cardiac glycosides stimulate the NaK-ATPase pump. (For review, see Lee and Klaus, 1971.) More recently, Cohen et al. (1976) interpreted their electrophysiologic observations as evidence consistent with stimulation of monovalent cation action active transport in sheep Purkinje fibers by ouabain at low concentrations (5×10^{-8}M) that have been reported to have positive inotropic effects in this tissue (Blood and Noble, 1976). Our data show no evidence for stimulation of monovalent cation transport by inotropically effective concentrations of ouabain and are consistent with the findings of Hougen and Smith (1978), who also observed inhibition of myocardial monovalent cation active transport by inotropically effective, but subtoxic, plasma concentrations of ouabain (10^{-9} to 10^{-8}M) in intact dogs.

The Na content of cultured heart cells was increased at positively inotropic concentrations of ouabain. This finding, taken together with the observation that a stable degree of inhibition of active transport preceded full development of the positive inotropic effect, is consistent with a causal role of increased $[Na^+]_i$ in the mediation of the positive inotropic effects of cardiac glycosides. This hypothesis is also supported by studies demonstrating a positive inotropic effect of agents that increase $[Na^+]_i$ by enhanced opening of the fast Na channel (Horackova and Vassort, 1974), by non-glycoside inhibition of NaK-ATPase (Ku et al., 1975; Temma et al., 1978; Yamamoto et al., 1978), or by

more direct enhancement of Na^+ entry by Na^+ ionophores (Sutko et al., 1977; Shlafer et. al., 1978). It is not obvious why other careful studies in intact tissue have not shown an increase in $[Na^+]_i$ after exposure to positively inotropic, but subtoxic, concentrations of cardiac glycosides (Lee and Klaus, 1971; Bentfeld et al., 1977). The properties of previously studied intact muscle preparations may be different from those of the cultured cells used in our experiments.

The kinetics of $^{45}Ca^{2+}$ uptake in our experiments (Fig. 5) are consistent with the presence of two kinetically separable Ca compartments: one rapidly equilibrating (within 5 min) and containing the incremental Ca content resulting from exposure to ouabain; and a more slowly exchanging compartment, fully labeled by 120 min and showing no further change up to 24 h. These two compartments may correspond to the rapidly exchanging compartment (τ for efflux 1.66 min) and the more slowly exchanging compartment (τ for efflux ~27 min) described by Langer and Frank (1972) in their study of Ca exchange in monolayers of cultured neonatal rat ventricular cells.

Other investigators have observed an increase in exchangeable myocardial Ca content after exposure to cardioactive steroids (Langer and Serena, 1970; Bailey and Sures, 1971; Wood and Schwartz, 1978). Some authors have proposed that this may result from an increase in the affinity of the NaK-ATPase complex, or some other membrane component, for Ca^{2+}. A positive inotropic effect might then ensue due to enhanced availability of coupling Ca^{2+} (Nayler, 1973; Schwartz, 1976; Gervais et al., 1977). It has also been proposed that elevation in $[Na^+]_i$ produces an increase in slow inward current in Purkinje fibers (Weingart et al., 1978) and in frog atrial muscle (Goto et al., 1978), following pump inhibition. Although the studies reported here do not exclude these mechanisms, enhanced contractility, increased Na content, and increased exchangeable Ca content in our experiments all appear to develop with a similar time-course after exposure to ouabain. Thus, our data support the hypothesis of Baker et al. (1969) and of Langer (1977) that the inotropic effects of cardiac glycosides develop as a consequence of Na^+-Ca^{2+} exchange. Since Na^+-Ca^{2+} exchange is bidirectional, a net increase in Ca content following elevation of $[Na^+]_i$ could develop because of diminished Ca^{2+} efflux (Vassort et al., 1978) and/or enhanced Ca^{2+} influx (Blaustein, 1977; Fabiato and Fabiato, 1979; Horakova and Vassort, 1979).

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