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EVIDENCE THAT CONCENTRATIONS OF OUABAIN WHICH INDUCE POSITIVE INOTROPIC EFFECTS ON THE PERFUSED GUINEA-PIG HEART INCREASE THE AMOUNT OF CALCIUM IN A RAPIDLY-EXCHANGEABLE CELLULAR COMPARTMENT

PAMELA C. MAITLAND, SUZANNE V. LAMONT and GREGORY J. BARRITT*
Department of Clinical Biochemistry, Flinders University School of Medicine, Flinders Medical Centre, Bedford Park, South Australia 5042

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Abstract—The effects of ouabain and adrenaline on kinetically distinct compartments of exchangeable calcium associated with myocardial muscle cells were investigated using a Langendorff perfused guinea-pig heart preparation, a $^{45}Ca^{2+}$ outflow exchange technique, and ^{51}Cr-EDTA to monitor the rate of loss of freely-diffusable $^{45}Ca^{2+}$ from the vascular and interstitial spaces. A non-linear least squares curve-fitting procedure was used to analyse the data. The minimum number of compartments of exchangeable calcium required to adequately describe each set of data for the loss of ^{45}Ca from the heart was five. These consisted of freely-diffusible exchangeable calcium in the vascular and interstitial spaces (monitored using ^{51}Cr-EDTA) and three kinetically distinct compartments of exchangeable calcium associated with the muscle cells. A concentration of ouabain (0.15 μM), which enhanced the force of contraction of the heart by 60% without evidence of toxicity, increased the flux and quantity of exchangeable calcium in the vascular space and in the cellular compartment with the highest fractional transfer rate. Both ouabain and adrenaline decreased the flux and fractional transfer rate of exchangeable calcium in the two cellular compartments with the lowest fractional transfer rates. The results are consistent with the conclusion that one of the actions of ouabain on myocardial muscle cells is to increase the quantity of rapidly-exchangeable calcium bound to extracellular sites on the sarcolemma and/or present in the myoplasm.

The actions of cardiac glycosides on the heart have been the subject of extensive studies. However, the mechanisms by which these drugs induce an increase in contraction of myocardial muscle fibres appear to be complex and are still not well understood (reviewed in [1]). A common feature of at least three of the proposed mechanisms [1] is the prediction that the drugs increase the quantity and rate of exchange of Ca^{2+} in a rapidly exchangeable cellular compartment which consists of Ca^{2+} invlcved in the contraction–relaxation cycle of myocardial muscle cells.

While the results of a number of earlier studies indicated that cardiac glycosides modify the transport and distribution of Ca^{2+} in cardiac muscle [2–5], the nature of the compartments of exchangeable Ca^{2+} affected by the drugs was not well defined. More recent experiments have provided evidence that cardiac glycosides alter the kinetic parameters of a compartment of exchangeable Ca^{2+} with a high fractional transfer rate [6–8]. However, these investigations were performed with aged tissue [6] or minced cardiac muscle [7], or with cardiac muscle which had previously been perfused with a Ca^{2+}-free medium [8]. Thus the question of the nature of the compartments of exchangeable Ca^{2+} affected by cardiac glycosides under more physiological conditions has not been clearly resolved.

In a previous study conducted in this laboratory, it was shown that glucagon alters the rates of exchange of Ca^{2+} in two kinetically distinct compartments of cellular exchangeable Ca^{2+} in the perfused rat heart [9]. In these experiments $^{45}Ca^{2+}$ outflow exchange curves obtained under steady-state conditions were analysed using an iterative, non-linear least squares curve-fitting procedure and [^{14}C]sucrose or [^{3}H]inulin was employed to monitor the loss of freely diffusible Ca^{2+} from the vascular and interstitial spaces [9]. The aim of the present experiments was to investigate the effects of the cardiac glycoside, ouabain, on the kinetic properties of cellular compartments of exchangeable Ca^{2+} in the guinea-pig heart using the $^{45}Ca^{2+}$ outflow exchange techniques developed previously [9]. The effects of ouabain were compared with those of adrenaline since this β-adrenergic agonist, which has been shown to stimulate Ca^{2+} inflow across the sarcolemma and the rate of Ca^{2+} transport by the sarcoplasmic reticulum (reviewed in [10]) is also expected to increase the amount and/or rate of exchange of rapidly exchangeable cellular Ca^{2+}.

In the present series of experiments, ^{51}Cr-EDTA was used to monitor the loss of freely diffusible $^{45}Ca^{2+}$ from the vascular and interstitial spaces, and hence to distinguish between Ca^{2+} present in these spaces and cellular Ca^{2+} (which includes Ca^{2+} bound to external sites on the sarcolemma). A number of previous studies by others have provided evidence

* Author to whom correspondence should be sent.

which indicates that ^{51}Cr-EDTA is a reasonable marker for the loss of freely diffusible molecules from the vascular and interstitial spaces [11–14]. The results reported here provide evidence which indicates that concentrations of ouabain which induce a positive inotropic effect on the heart without evidence of toxicity increase the quantity of Ca^{2+} in a rapidly exchangeable compartment of cellular Ca^{2+}.

MATERIALS AND METHODS

Materials. Ouabain octahydrate, the sodium salt of adrenaline, and bovine serum albumin fraction V were obtained from the Sigma Chemical Co. (St. Louis, MO); $^{45}CaCl_2$ and [^{3}H]inulin from Amersham Australia Pty. Ltd. (Sydney, New South Wales); and ^{51}Cr-EDTA from the Australian Atomic Energy Commission (Sutherland, New South Wales). All other reagents were of the highest grade available.

Heart perfusions. The hearts of male guinea-pigs (Institute of Medical and Veterinary Science, Adelaide) of 200–300 g body wt were perfused by the Langendorff method through a cannula inserted in the aorta. The perfusion medium [15] was composed of 118 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM potassium phosphate, 25 mM $NaHCO_3$, 0.002% (w/v) phenol red, 11 mM glucose, 0.9 mM $CaCl_2$ and other additions as indicated. The pH was adjusted to 7.4 (NaOH) and the medium equilibrated with O_2:CO_2 (95:5). Preparation of the isolated perfused heart and its attachment to the perfusion apparatus [16] were conducted as described by Clark *et al.* [15]. The heart, maintained at a temperature of 31° [17], was suspended in air in a water-jacketed chamber from which the perfusate was collected or recirculated as indicated. The perfusion medium was continuously equilibrated with O_2:CO_2 (95:5) [18], the perfusion pressure was 100 cm H_2O, and the flow rate was 10 ml/min per g wet wt tissue.

The hearts were electrically paced with square wave pulses of 5.4 V and 4.2 msec duration at a frequency of 2.6 per sec using a Grass S5 stimulator (Grass Instrument Company, Quincy, MA). Platinum electrodes were attached to the metal cannula and apex of the heart. The force of contraction of the heart was estimated by measuring isometric shortening of a portion of ventrical muscle using a Grass FTO3C force-displacement transducer connected to a Grass polygraph recorder, model 7C.

Measurement of rates of $^{45}Ca^{2+}$, ^{51}Cr-EDTA and albumin outflow. Hearts were initially perfused for 5 min with a non-recirculated medium, then for 40 min with a recirculated medium. $^{45}CaCl_2$ (1.3 MBq) and ^{51}Cr-EDTA (3 MBq) were then added to the perfusion medium (approx. 150 ml total volume). In some experiments (indicated in the text), [^{3}H]inulin (2 MBq) was used in place of ^{51}Cr-EDTA. Perfusion of the hearts with this recirculated medium was continued for a further 35 min. At this time, the washout of $^{45}Ca^{2+}$ and ^{51}Cr-EDTA was initiated by changing the perfusion medium (using a three-way tap) to one of identical composition (including the presence of 0.9 mM $CaCl_2$), except that no $^{45}Ca^{2+}$ and ^{51}Cr-EDTA were present. The perfusate was collected using a fraction collector at intervals of 0.2 min (0–2 min), 0.5 min (2–20 min), and 1 min (20–30 min after initiation of the washout period).

When present, 1.5 ml of 100 μM adrenaline or 0.1 ml of 240 μM ouabain (dissolved in water) was added at the same time as the $^{45}Ca^{2+}$ and ^{51}Cr-EDTA to give the final concentrations indicated in the figures. For experiments conducted in the presence of adrenaline it was found necessary to include 0.2% (w/v) albumin in the perfusion medium in order to prevent inactivation of the adrenaline. Control experiments showed that the presence of this concentration of albumin alone had no effect on the kinetic parameters of $^{45}Ca^{2+}$ and ^{51}Cr-EDTA outflow.

The weight of perfusate collected in each fraction eluted from the heart was measured. A sample (100 μl) of each fraction was mixed with 4 ml of Aqueous Counting Scintillator (ACS II, Amersham Corporation, Arlington Heights, IL) and the amount of ^{45}Ca present measured by liquid scintillation using an Isocap 300 liquid scintillation spectrometer (Searle Analytic Inc., Des Plaines, IL). The amount of ^{51}Cr-EDTA present in the samples (100 μl) of each fraction was measured using a Searle γ counter (model 1185). Control measurements showed that under the detection conditions employed, radiation emitted by ^{51}Cr did not interfere with the measurement of ^{45}Ca by liquid scintillation.

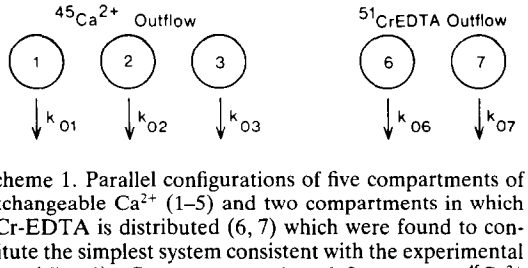
The concentration of ^{45}Ca and ^{51}Cr-EDTA in the recirculated perfusion medium at the end of the 35 min period of exposure of the heart to the isotopes was estimated by measuring the radioactivity present in samples (100 μl) of the recirculated perfusate. At the end of the washout period (i.e. after perfusion in the absence of $^{45}Ca^{2+}$ and ^{51}Cr-EDTA for 30 min), the heart was removed from the cannula, weighed, and homogenised in 5 ml of 250 mM sucrose/5 mM HEPES–KOH (pH 7.4) using a Polytron homogeniser (Kinematica GmbH, Lucerne, Switzerland). Samples of the homogenate (50 μl) were dissolved in 200 μl of NCS Tissue Solubilizer (Amersham Australia Pty. Ltd.), acidified with 10 μl of glacial acetic acid, and the amount of ^{45}Ca present estimated by liquid scintillation. Samples (100 μl) of the homogenate were also removed for estimation of ^{51}Cr-EDTA.

In order to determine the kinetic parameters of the albumin space, bovine serum albumin [1 ml of a 20% (w/v) solution per 150 ml perfusion medium, final concentration of 0.13% (w/v)] was added to the perfusion medium 85 min after perfusion of the heart with recirculated medium was first begun. After a further 15 min, the perfusion medium was changed to an identical one which contained no albumin. The amount of albumin present in fractions eluted from the heart, and in the equilibration medium, was determined by the method of Lowry *et al.* [19].

Calculation of rates of $^{45}Ca^{2+}$ outflow. The total amount of $^{45}Ca^{2+}$ associated with the heart at the beginning of the washout period, the initial dose of $^{45}Ca^{2+}$ in the heart (I.D.Ca_T becquerels), was calculated by summing the quantities of isotope present in each fraction eluted from the heart and the quantity of isotope present in the heart at the end of the washout period. (About 2% of the initial dose of $^{45}Ca^{2+}$ and less than 1% of the initial dose of ^{51}Cr-

EDTA remained in the heart at the end of the 30 min washout period.) For each fraction of perfusate collected, the rate of loss of $^{45}Ca^{2+}$ (dq_{Ca}/dt becquerels per min) was expressed as a fraction of the initial dose of $^{45}Ca^{2+}$ in the heart, $(dq_{Ca}/dt)/(I.D.Ca_T) \times 100\%$ per min.

Calculation of rates of ^{51}Cr-EDTA outflow. In order to determine which kinetically distinct compartments of exchangeable Ca^{2+} associated with the heart represent $^{45}Ca^{2+}$ in the ^{51}Cr-EDTA-accessible space (vascular and interstitial $^{45}Ca^{2+}$), it was necessary to express the rate of loss of ^{51}Cr-EDTA in the same units as those employed for the rate of loss of $^{45}Ca^{2+}$. This was done by expressing the rate of loss of ^{51}Cr-EDTA in terms of the fraction of the initial dose of $^{45}Ca^{2+}$ associated with the whole heart lost per min. The calculation was performed in the following steps. (1) The total amount of ^{51}Cr-EDTA associated with the heart at the beginning of the washout period, the initial dose of ^{51}Cr-EDTA (I.D.Cr becquerels), was calculated by summing the quantities of isotope present in each fraction eluted from the heart. (2) For each fraction of perfusate collected from the heart, the rate of loss of ^{51}Cr-EDTA (dq_{Cr}/dt becquerels per min) was expressed as a fraction of the initial dose of ^{51}Cr-EDTA, $(dq_{Cr}/dt)/(I.D.Cr)$ per min. (3) The volume of the ^{51}Cr-EDTA-accessible space (V_{Cr}) was calculated by dividing the initial dose of ^{51}Cr-EDTA associated with the heart (I.D.Cr) by the concentration of ^{51}Cr-EDTA present in the perfusion medium at the end of the period of equilibration with the isotopes. (4) The initial dose of $^{45}Ca^{2+}$ present in the ^{51}Cr-EDTA-accessible space ($I.D.Ca_{Cr}$ becquerels) was calculated by multiplying the volume of the ^{51}Cr-EDTA-accessible space (V_{Cr}) by the concentration of $^{45}Ca^{2+}$ in the perfusion medium at the end of the period of equilibration with isotopes. (5) The rate of loss of ^{51}Cr-EDTA was expressed in units of the rate of loss of $^{45}Ca^{2+}$ from the ^{51}Cr-EDTA-accessible space, $(dq_{Cr}/dt)/(I.D.Cr) \times (I.D.Ca_{Cr})$ becquerels per min. (6) Finally, the rate of loss of ^{51}Cr-EDTA was expressed in the same units as those employed for the rate of loss of $^{45}Ca^{2+}$, that is as $(dq_{Cr}/dt)/(I.D.Cr) \times (I.D.Ca_{Cr})/(I.D.Ca_T) \times 100\%$ initial dose of $^{45}Ca^{2+}$ in the heart lost per min.



Scheme 1. Parallel configurations of five compartments of exchangeable Ca^{2+} (1–5) and two compartments in which ^{51}Cr-EDTA is distributed (6, 7) which were found to constitute the simplest system consistent with the experimental data (Fig. 1). Compartments 1 and 2 represent $^{45}Ca^{2+}$ distributed in the vascular and interstitial spaces, respectively, and compartments 3, 4 and 5 cellular exchangeable Ca^{2+} (including Ca^{2+} bound to extracellular sites on the sarcolemma). The kinetic properties of compartments 1 and 2 are assumed to be identical to those of compartments 6 and 7, respectively, in which ^{51}Cr-EDTA is distributed. That is $k_{01} = k_{06}$, $R_{01} = R_{06}$, and $k_{02} = k_{07}$ and $R_{02} = R_{07}$. The evidence upon which this conclusion is based is described in the Appendix. The constants k_{0j} (min^{-1}) represent the fractional transfer rates (rate constants) for the loss of $^{45}Ca^{2+}$ or ^{51}Cr-EDTA from compartment j to the medium (compartment 0). The flux of Ca^{2+} from compartment j and the quantity of exchangeable Ca^{2+} in compartment j are represented by the constants R_{0j} (nmoles/min per mg wet wt) and Q_j (nmole per mg wet wt), respectively.

Analysis of $^{45}Ca^{2+}$ and ^{51}Cr-EDTA outflow curves and estimation of the values of the kinetic constants. The results from individual experiments performed under a given condition (the absence of a drug or in the presence of ouabain or adrenaline) were combined and plotted as a function of time. Parallel compartment configurations which describe the loss of $^{45}Ca^{2+}$ and ^{51}Cr-EDTA from the heart, e.g. those shown in Scheme 1, were fitted simultaneously to the experimental data for $^{45}Ca^{2+}$ and ^{51}Cr-EDTA outflow using an iterative, non-linear, least squares curve-fitting procedure, as described previously [9]. Calculation of statistical weights and assessment of the goodness of fit of a given compartment configuration to the experimental data were conducted as described previously [9]. When configurations with one more or one less compartment of exchangeable Ca^{2+} or in which certain pairs of rate constants were constrained to be equal were tested, the significance of the difference between the residual weighted sums of squares was assessed using the F-ratio test [28].

Numerical values for the fractional transfer rate, k_{0j}* (min^{-1}) and flux, R_{0j} (nmole Ca^{2+}/min per mg wet wt) for the outflow of Ca^{2+} from compartment j to the medium, for the compartment configuration under consideration, were obtained from each analysis [9]. The quantity of exchangeable Ca^{2+} present in a given compartment, Q_j nmole per mg wet wt, was calculated using the expression $Q_j = R_{0j}/k_{0j}$. For these calculations, the initial dose of $^{45}Ca^{2+}$ associated with the whole heart was expressed as nmole per mg wet wt.

The various compartment configurations were also fitted to $^{45}Ca^{2+}$ and ^{51}Cr-EDTA outflow curves obtained from individual experiments; and to outflow curves describing the rate of loss of albumin and [^{3}H]inulin. In these cases, the standard deviation of each value for the rate of loss of isotope, dq/dt, was estimated using the expression S.D. $= \sqrt{dq/dt}$.

RESULTS

Curves for the rate of loss of $^{45}Ca^{2+}$ and ^{51}Cr-EDTA from guinea-pig hearts perfused at 31° in the presence of 0.9 mM Ca^{2+} under control conditions, and in the presence of 0.15 μM ouabain or 1 μM adrenaline are shown in Fig. 1. The parallel compartment configurations shown in Scheme 1 were found to constitute the simplest system which gave the best fit to each of the three sets of $^{45}Ca^{2+}$ and ^{51}Cr-EDTA outflow data (Fig. 1 and Table 1). The basis on which this conclusion was reached is described in the Appendix.

* Constants k_{0j}, R_{0j} and Q_j are defined in the legend of Scheme 1.

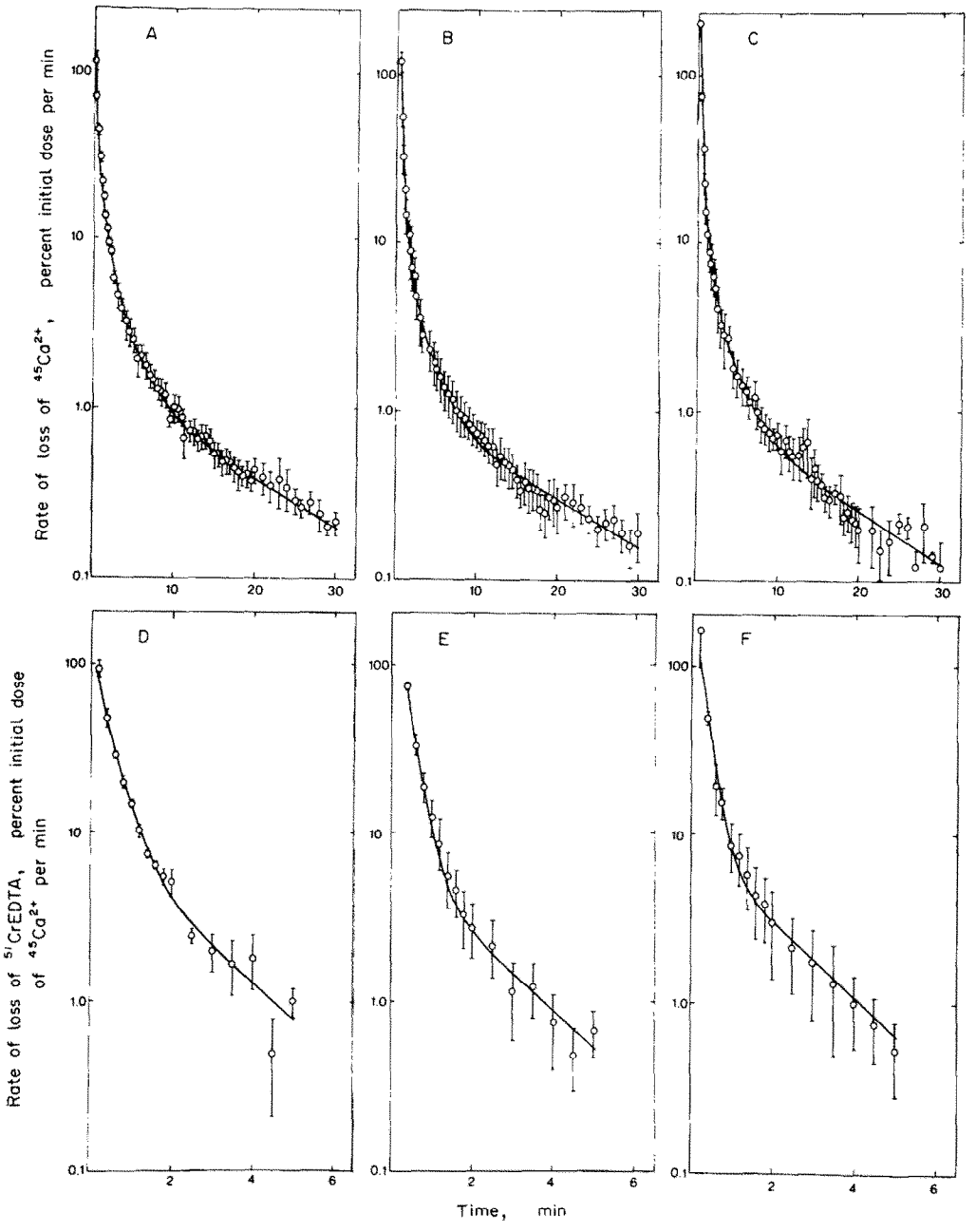


Fig. 1. Semilogarithmic plots of the rate of loss of $^{45}Ca^{2+}$ (A–C) or ^{51}Cr-EDTA (D–F) from isolated perfused guinea-pig hearts as a function of time under control conditions (A, D) and in the presence of 0.15 μM ouabain (B, E) or 1 μM adrenaline (C, F). Heart perfusions and measurement of the rate of loss of isotope, expressed as the percentage of the initial dose of $^{45}Ca^{2+}$ in the heart per min, were conducted as described in Materials and Methods. Each data point represents the mean ± S.E.M. of 4 (A, D) or 3 (B, C, E, F) separate experiments. The solid lines were drawn using the numerical values of the constants (Table 1) obtained from a fit of Scheme 1 to the data.

The volumes occupied by fluid in ^{51}Cr-EDTA-accessible compartments 6 and 7 (Scheme 1) in hearts perfused under control conditions were found to be 0.96 ± 0.2 and 0.68 ± 0.14 ml per g wet wt (mean ± S.E.M., $n = 4$), respectively. Analysis of data for the rate of loss of albumin, a marker for the vascular space [22], from hearts previously equilibrated with albumin under control conditions gave a value of 1.17 ± 0.09 ml for the volume of the albumin space and 2.7 ± 0.4 min^{-1} ($n = 3$) for the fractional transfer rate for the loss of material from the albumin-accessible compartment [compare with the value of the fractional transfer rate k_{01}, for the loss of Ca^{2+} from compartment 1 (Table 1)].

When ^{51}Cr-EDTA was replaced by [^{3}H]inulin as a marker for the extracellular space, two

Table 1. Values and S.D. of the kinetic constants obtained for a fit of the compartment configurations shown in Scheme 1 to the $^{45}Ca^{2+}$ and ^{51}Cr-EDTA outflow data of Fig. 1*

Kinetic parameter	Treatment of hearts		
	Control	Ouabain (0.15 μM)	Adrenaline (1 μM)
Flux of Ca^{2+} across compartment boundary (nmole/min per mg wet wt)			
R_{01} (Vascular)	2.05 ± 0.48	7.04 ± 1.11‖	6.75 ± 1.41§
R_{02} (Interstitial)	0.19 ± 0.06	0.22 ± 0.04	0.22 ± 0.04
R_{03}	1.22 ± 0.48	1.88 ± 0.40	0.97 ± 0.26
R_{04}	0.13 ± 0.04	0.052 ± 0.009†	0.034 ± 0.006‡
R_{05}	0.030 ± 0.007	0.006 ± 0.003§	0.002 ± 0.004‖
Quantity of exchangeable Ca^{2+} (nmole/mg wet wt)			
Q_1 (Vascular)	0.83 ± 0.18	1.59 ± 0.22§	1.10 ± 0.16
Q_2 (Interstitial)	0.37 ± 0.09	0.32 ± 0.05	0.32 ± 0.04
Q_3	0.35 ± 0.09	0.70 ± 0.10§	0.37 ± 0.05
Q_4	0.29 ± 0.07	0.28 ± 0.05	0.24 ± 0.06
Q_5	0.45 ± 0.10	0.29 ± 0.43	0.33 ± 0.76
Fractional transfer rate for Ca^{2+} outflow (min^{-1})			
k_{01} (Vascular)	2.47 ± 0.17	4.42 ± 0.22‖	6.16 ± 0.49‖
k_{02} (Interstitial)	0.52 ± 0.06	0.68 ± 0.04†	0.69 ± 0.06
k_{03}	3.51 ± 0.75	2.71 ± 0.28	2.66 ± 0.45
k_{04}	0.45 ± 0.08	0.19 ± 0.03§	0.14 ± 0.04§
k_{05}	0.068 ± 0.004	0.019 ± 0.022†	0.006 ± 0.05

* Analysis of the data and estimation of the values of the kinetic constants were performed as described in Materials and Methods. The values and S.E.M. of the initial doses of $^{45}Ca^{2+}$ associated with the heart were 1.98 ± 0.22 (4), 1.82 ± 0.14 (3) and 1.48 ± 0.10 (3) nmole per mg wet wt for hearts perfused under control conditions and in the presence of ouabain and adrenaline, respectively. The results analysed (Fig. 1) are those obtained for 4 (control conditions) and 3 (0.15 μM ouabain or 1 μM adrenaline) separate experiments.

The degrees of significance, P, determined using the *t*-test [28], are †$P < 0.05$, ‡$P < 0.02$, §$P < 0.01$ and ‖$P < 0.001$.

kinetically distinct [^{3}H]inulin-accessible compartments with fractional transfer rates similar to those obtained using ^{51}Cr-EDTA were obtained (results not shown). The results obtained using ^{51}Cr-EDTA, albumin and [^{3}H]inulin, together with previous work of others [12, 14] are consistent with the conclusion that compartments 1 and 2 of exchangeable Ca^{2+} (Scheme 1) represent Ca^{2+} present in the vascular and interstitial spaces, respectively, while compartments 3, 4 and 5 represent exchangeable Ca^{2+} associated with the ^{51}Cr-EDTA-non-accessible space (cellular Ca^{2+}). Ca^{2+} which is bound to external sites on the sarcolemma [14] may be included in the latter compartments.

Low concentrations of ouabain (0.15 μM) increased the force of contraction of the heart by 60% (Figs. 2A and 2B) without evidence of toxic effects (irregularity or a reduction of beat amplitude). In hearts perfused in the presence of ouabain, the positive inotropic effect of the drug was reversed after perfusion for a further 25 min in the absence of ouabain (results not shown). The main effects of ouabain on exchangeable Ca^{2+} associated with the heart were to increase the flux, quantity and fractional transfer rate of Ca^{2+} in the vascular space (compartment 1), and the quantity of exchangeable Ca^{2+} in the cellular compartment with the highest fractional transfer rate (compartment 3), and to decrease the flux and fractional transfer rate of Ca^{2+} in each of the other two compartments of cellular exchangeable Ca^{2+} (compartments 4 and 5) (Table 1).

Adrenaline (1 μM) increased the force of contraction of the heart by 50% (Fig. 2A and 2C). This change was associated with an increase in the flux, quantity and fractional transfer rate of exchangeable Ca^{2+} in the vascular space (compartment 1) and a decrease in the flux and fractional transfer rate of exchangeable Ca^{2+} in the two cellular compartments with the lowest fractional transfer rates (compartments 4 and 5) (Table 1).

DISCUSSION

The application of standard procedures for determination of the minimum number of compartments

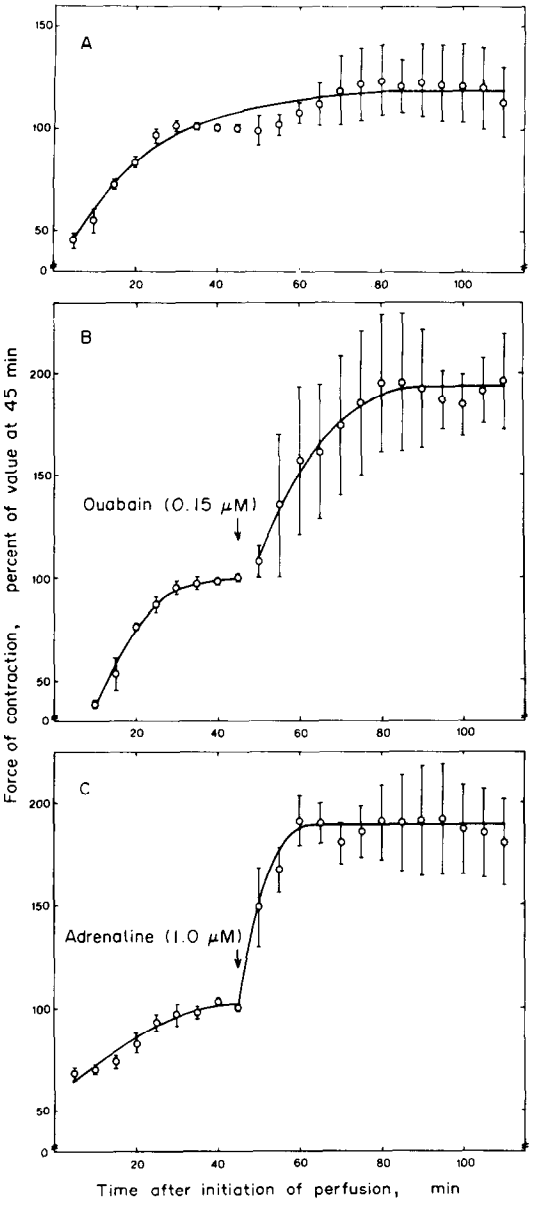


Fig. 2. The force of contraction plotted as a function of time for guinea-pig hearts perfused under control conditions (A) and in the presence of 0.15 μM ouabain (B) and 1 μM adrenaline (C). Heart perfusions and the measurement of the isometric shortening of a portion of ventricle muscle were conducted as described in Materials and Methods. For each experiment, contractile force was expressed as a percentage of the value attained at 45 min after initiation of the perfusion. Ouabain (B) and adrenaline (C) were added at the times indicated by the arrow. Each data point is the mean ± S.E.M. of the results of 4 (A) and 3 (B and C) separate experiments. The solid lines were drawn from a fit of the data points by eye.

of exchangeable Ca^{2+} associated with the heart has shown that a relatively complex system of five compartments of exchangeable Ca^{2+} is the simplest compartmental model which is consistent with the $^{45}Ca^{2+}$ and ^{51}Cr-EDTA outflow curves obtained under each of the three sets of conditions investigated. Since the values of ten kinetic parameters were estimated, the fractional standard deviation of some of the parameters, including, R_{03}, Q_5 and k_{05}, is reasonably large.

It is likely that Ca^{2+} in the interstitial and cellular compartments does not exchange directly with Ca^{2+} in the medium. Hence these compartments are probably arranged in a configuration which is more complex than the parallel model shown in Scheme 1. At present insufficient information is available to determine a unique compartment configuration. Since the numerical values of the fractional transfer rates k_{03}, k_{04} and k_{05} differ by 5- to 10-fold and the quantity of exchangeable Ca^{2+} in the perfusion medium is much larger than that present in the heart, the numerical values obtained for the constants Q_j, k_{0j} and R_{0j} for the parallel configuration (Scheme 1) are also approximate estimates [23] of the values of these parameters for the compartments of exchangeable Ca^{2+} if they are arranged in a more complex (series) configuration.

The values obtained for the fractional transfer rates of the first two compartments of cellular exchangeable Ca^{2+} in control hearts, k_{03} and k_{04}, (3.5 and 0.5 min^{-1}) are similar to those of 2.8 and 0.39 min^{-1} reported for rapidly exchangeable cellular Ca^{2+} in perfused hamster hearts [24]. Since Ca^{2+} involved in the contraction–relaxation cycle of myocardial muscle cells is expected to exchange very rapidly [6, 14, 24], it is likely that the cellular compartment of exchangeable Ca^{2+} with the highest fractional transfer rate represents Ca^{2+} which is directly involved in the contraction–relaxation cycle. This may include Ca^{2+} present in the myoplasm [25], sarcoplasmic reticulum [26] and/or Ca^{2+} bound to external sites on the sarcolemma [14]. The fractional transfer rate of exchangeable Ca^{2+} in the major compartment detected in isolated guinea-pig heart mitochondria incubated at concentrations of free Ca^{2+}, Mg^{2+} and inorganic phosphate similar to those estimated to be present in the myoplasm, has been found to be 0.05 min^{-1}*. This value is similar to that for the slowest compartment of cellular exchangeable Ca^{2+} in the perfused guinea-pig heart, indicating that this compartment of exchangeable Ca^{2+} includes mitochondrial Ca^{2+} (compare with [9, 27]).

The observation that ouabain increases the quantity of Ca^{2+} in the compartment of cellular exchangeable Ca^{2+} with the highest fractional transfer rate is consistent with other recent observations [6–8] on the actions of this drug, and may reflect an increase in the amount of Ca^{2+} bound to sites on the sarcolemma [29–31]. The failure to detect a significant effect of adrenaline on rapidly exchangeable cellular Ca^{2+} suggests that effects of adrenaline on this compartment of Ca^{2+} are not large enough to be detected using the techniques employed in the present study.

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* S. V. Lamont and G. J. Barritt, unpublished observation.

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APPENDIX

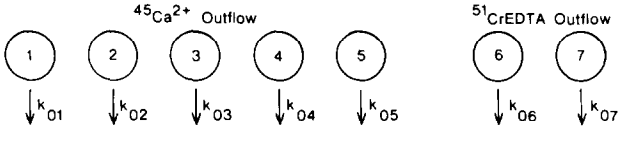
Determination of the minimum number of compartments of exchangeable Ca^{2+} required to fit the $^{45}Ca^{2+}$ outflow data

Curves of the type shown in Fig. 1 can be adequately described by equations which are the sums of exponential terms [6, 9, 20]. These are, in turn, expressions for the rate of loss of isotope from kinetically distinct compartments of isotope associated with the heart [9, 21]. The first step in analysis of the data consisted of fitting the compartment configurations for the distribution of $^{45}Ca^{2+}$ and ^{51}Cr-EDTA shown in Scheme 2 to each set of experimental data (Fig. 1) using the SAAM computer programme [32] as described in Materials and Methods. The programme is constructed so that the 3-compartment parallel configuration ($^{45}Ca^{2+}$ outflow) is fitted to the data for $^{45}Ca^{2+}$ outflow and the 2-compartment parallel configuration (^{51}Cr-EDTA outflow) fitted to the data for ^{51}Cr-EDTA outflow. Values of the fractional transfer rates (k_{0i}) and fluxes (R_{0i}) are adjusted by an iterative procedure so that the sum of the residual weighted sums of squares for the fits of the compartment configurations to the $^{45}Ca^{2+}$ and ^{51}Cr-EDTA outflow data reaches a minimum value.

The compartment configurations shown in Scheme 2 were found to constitute the simplest system which was consistent with the data obtained under each of the three experimental conditions tested. A system consisting of only two compartments of exchangeable Ca^{2+} gave a poor fit to the data, and the addition of a fourth compartment of exchangeable Ca^{2+} did not significantly decrease the residual weighted sums of squares.

In the fit of the configurations shown in Scheme 2 to a given set of experimental data, the numerical values of the fractional transfer rates k_{01} and k_{02} ($^{45}Ca^{2+}$ outflow) were found to be similar to the values of k_{06} and k_{07} (^{51}Cr-EDTA outflow), respectively. The compartment configurations shown in Scheme 2 were then fitted to the data for $^{45}Ca^{2+}$ and ^{51}Cr-EDTA outflow with the values of the fractional transfer rates k_{01} and k_{02} constrained to be equal to those of k_{06} and k_{07}, respectively. For a given set of experimental conditions the residual weighted sums of squares was not significantly greater than that obtained in the absence of these constraints (Table 2).

On the basis of this observation and the evidence that ^{51}Cr-EDTA represents freely diffusible $^{45}Ca^{2+}$ in the vascular and interstitial spaces (summarised in the Results section) further analysis of the data was conducted using compartment configurations in which the values of members of the pairs of kinetic parameters k_{01}, k_{06}; R_{01}, R_{06};



Scheme 2. Parallel configurations of three compartments of exchangeable Ca^{2+} (1–3) and two compartments in which ^{51}Cr-EDTA is distributed (6, 7) which were found to constitute the simplest system consistent with the experimental data (Fig. 1) when the analysis was performed without constraining any kinetic parameters for $^{45}Ca^{2+}$ outflow to be equal to those for ^{51}Cr-EDTA outflow. The constants k_{0j} (min^{-1}) represent the fractional transfer rates (rate constants) for the loss of isotope from compartment j to the medium (compartment 0).

Table 2. Values of the residual weighted sums of squares obtained for fits of compartment configurations consisting of 3–5 compartments of exchangeable Ca^{2+}, with and without constraints on the values of some of the kinetic constants, to the $^{45}Ca^{2+}$ and ^{51}Cr-EDTA outflow data of Fig. 1*

Number of compartments of exchangeable Ca^{2+}	Kinetic constants constrained to be equal	Residual weighted sums of squares (per cent initial dose of $^{45}Ca^{2+})^2$		
		Control	Ouabain	Adrenaline
3 (Scheme 2)	None	0.191	0.122	0.088
3 (Scheme 2)	$k_{01} = k_{06}$ $k_{02} = k_{07}$	0.203†	0.142†	0.098†
3 (Scheme 2)	$k_{01} = k_{06}$; $R_{01} = R_{06}$ $k_{02} = k_{07}$; $R_{02} = R_{07}$	1.095‡	0.563‡	0.225‡
4	$k_{01} = k_{06}$; $R_{01} = R_{06}$ $k_{02} = k_{07}$; $R_{02} = R_{07}$	0.249‡	0.196‡	0.098‡
5 (Scheme 1)	$k_{01} = k_{06}$; $R_{01} = R_{06}$ $k_{02} = k_{07}$; $R_{02} = R_{07}$	0.189	0.092	0.068

* Compartment configurations were fitted to the experimental data by a non-linear, iterative, least squares curve fitting procedure as described in Materials and Methods. The significance of differences between values of the residual weighted sums of squares obtained for fits of different compartment configurations to the same set of experimental data were determined using the F-ratio test [28].

† Not significantly different from the value obtained for the 3-compartment configuration with no constraints; ‡ significantly different (at the 5% level) from the value obtained for the 5-compartment configuration.

k_{02}, k_{07}; and R_{02}, R_{07} were constrained to be equal. That is, the values of the kinetic parameters for compartments 1 and 2 ($^{45}Ca^{2+}$ outflow) were constrained to be equal to those for compartments 6 (proposed vascular space) and 7 (proposed interstitial space), respectively. (As described in Materials and Methods, the rate of loss of ^{51}Cr-EDTA is expressed in the same units as the rate of loss of $^{45}Ca^{2+}$).

When these constraints were applied, the 3-compartment configuration for $^{45}Ca^{2+}$ outflow (Scheme 2) was found not to be consistent with the data (Table 2). The minimum residual weighted sums of squares (Table 2) was only achieved when two more compartments of exchangeable Ca^{2+} (compartments 4 and 5) were added as in Scheme 1. When only four compartments of exchangeable Ca^{2+} were present, the residual weighted sums of squares was significantly higher than that obtained for the fit of the five-compartment configuration (Table 2), and a systematic deivation of the line of best fit from the experimental data for $^{45}Ca^{2+}$ outflow was observed (results not shown). Thus it is concluded that the compartment configurations shown in Scheme 1 represent the simplest system which is consistent with the data for both $^{45}Ca^{2+}$ and ^{51}Cr-EDTA outflow when the kinetic parameters (k_{0j} and R_{0j}) of compartments 1 and 2 ($^{45}Ca^{2+}$ outflow) are constrained to be equal to those of compartments 6 and 7 (^{51}Cr-EDTA outflow), respectively.