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A kinetic investigation of the effects of adrenaline on $^{45}Ca^{2+}$ exchange in isolated hepatocytes at different Ca^{2+} concentrations, at 20° C and in the presence of inhibitors of mitochondrial Ca^{2+} transport

Janice C. PARKER,* Gregory J. BARRITT*‡ and John C. WADSWORTH†
**Department of Clinical Biochemistry, Flinders University School of Medicine, Flinders Medical Centre, Bedford Park, South Australia 5042, and †C.S.I.R.O. Division of Animal Production, P.O. Box 239, Blacktown, N.S.W. 2148, Australia*

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1. The effects of adrenaline on $^{45}Ca^{2+}$-exchange curves for isolated hepatocytes incubated under various steady-state conditions were investigated. Kinetic analysis showed that the simplest compartment configuration consistent with each set of data was a series configuration of a three-compartment closed system comprising compartment 1 (C_1), the extracellular medium, and two kinetically distinct compartments of cellular exchangeable Ca^{2+}, C_2 and C_3 ($C_1 \rightleftharpoons C_2 \rightleftharpoons C_3$). 2. Subcellular fractionation of hepatocytes labelled with $^{45}Ca^{2+}$ at 0.1 mM-Ca^{2+} indicated that C_3 includes exchangeable Ca^{2+} in the mitochondria and endoplasmic reticulum. 3. The following results were obtained from experiments conducted at 37°C at five different extracellular Ca^{2+} concentrations. For both untreated and adrenaline-treated cells, plots of the flux from C_1 to C_2 as a function of the extracellular Ca^{2+} concentration were best described by straight lines consistent with Ca^{2+} influx across the plasma membrane being a diffusion process. Adrenaline increased the value of the permeability constant for Ca^{2+} influx by 40%. For untreated cells, plots of the flux between C_2 and C_3 as a function of the concentrations of Ca^{2+} in these compartments approached a plateau at high Ca^{2+} concentrations. Adrenaline caused a 3-fold increase in the concentration of Ca^{2+} that gives half-maximal rate of Ca^{2+} transport from C_2 to C_3. 4. At 1.3 mM extracellular Ca^{2+}, a decrease in incubation temperature from 37°C to 20°C decreased the quantity of Ca^{2+} in C_3 and the flux and fractional transfer rates for the transport of Ca^{2+} between C_2 and C_3. At 20°C adrenaline increased the quantity of Ca^{2+} in C_3 and the fractional transfer rates for the transfer of Ca^{2+} from C_1 to C_2, and from C_2 to C_3. 5. At 37°C and 2.4 mM extracellular Ca^{2+}, antimycin A plus oligomycin decreased the quantity of Ca^{2+} in C_3 and increased the fractional transfer rate for the transport of Ca^{2+} from C_3 to C_2. In the presence of antimycin A and oligomycin, adrenaline did not increase the quantity of Ca^{2+} in C_2 or the flux and fractional transfer rate for the transport of Ca^{2+} from C_1 to C_2, whereas these parameters were increased in the absence of the inhibitors.

Previous kinetic studies of $^{45}Ca^{2+}$ exchange in isolated hepatocytes have revealed two intracellular compartments of exchangeable Ca^{2+}, with turnover times of about 1 and 10 min respectively. These have been tentatively identified as exchangeable Ca^{2+} in the cytoplasm and intracellular organelles, respectively (Barritt *et al.*, 1981*b*; Parker & Barritt, 1981). Evidence was obtained that adrenaline increases the quantity of Ca^{2+} in the compartment with the shorter turnover time by inducing (*a*) the outflow of Ca^{2+} from the compartment with the longer turnover time and (*b*) the inflow of Ca^{2+} from the medium (Barritt *et al.*, 1981*b*). The conclusion that one of the actions of adrenaline is to induce the outflow of Ca^{2+} from intracellular organelles is consistent with that reached by using other techniques (Chen *et al.*, 1978; Babcock *et al.*, 1979; Blackmore *et al.*, 1979; Murphy *et al.*, 1980;

‡ To whom reprint requests should be addressed.

Berthon *et al.*, 1981; Reinhart *et al.*, 1982*b*; Kimura *et al.*, 1982; but see also Althaus-Salzmann *et al.*, 1980; Poggioli *et al.*, 1980). However, the possible role of enhanced Ca^{2+} inflow across the plasma membrane in the actions of α-adrenergic agonists (Keppens *et al.*, 1977; Assimacopoulos-Jeannet *et al.*, 1977; Foden & Randle, 1978) has not been fully resolved (Blackmore *et al.*, 1982; Reinhart *et al.*, 1982*b*). Furthermore, many studies of the effects of α-adrenergic agonists on Ca^{2+} distribution in liver cells have been conducted at low concentrations of extracellular Ca^{2+} (Chen *et al.*, 1978; Babcock *et al.*, 1979; Blackmore *et al.*, 1979; Murphy *et al.*, 1980; Blackmore *et al.*, 1982), and few attempts have been made to study the effects of agonists over a range of extracellular Ca^{2+} concentrations.

The aims of the present experiments were to investigate further the nature of the kinetically distinct compartments of exchangeable Ca^{2+} in isolated hepatocytes, the kinetic properties of the processes for the transport of Ca^{2+} between these compartments, and to define more clearly the effects of adrenaline. $^{45}Ca^{2+}$-exchange experiments have been conducted over a range of extracellular Ca^{2+} concentrations, at a lower temperature, and in the presence of inhibitors of mitochondrial Ca^{2+} transport, and the data were analysed by the technique developed previously (Barritt *et al.*, 1981*b*). The effects of adrenaline on $^{45}Ca^{2+}$ exchange by isolated hepatocytes incubated at 0.1mM extracellular Ca^{2+} and the distribution of $^{45}Ca^{2+}$ in subcellular fractions isolated from these cells were investigated in a previous study (Barritt *et al.*, 1981*b*). However, the exchange data were not sufficiently extensive to permit a kinetic analysis to be performed, and the method employed for subcellular fractionation resulted in a significant loss of $^{45}Ca^{2+}$ before homogenization of the cells. Therefore an additional aim of the present experiments was to obtain more extensive $^{45}Ca^{2+}$-exchange data at 0.1mM-Ca^{2+}, to subject this to kinetic analysis, and to determine the amount of $^{45}Ca^{2+}$ present in isolated subcellular fractions by using a modified cell-fractionation procedure.

The results are consistent with the conclusion that the entry of Ca^{2+} to hepatocytes across the plasma membrane occurs by a process of diffusion, and indicate that adrenaline increases the permeability constant for Ca^{2+} inflow to the cell. This effect of adrenaline appears to require the normal function of mitochondria.

Experimental

Chemicals

Antimycin A, oligomycin, ATP, digitonin and desiccated firefly lanterns were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. All other reagents were purchased from the sources described previously (Barritt *et al.*, 1981*b*).

$^{45}Ca^{2+}$ exchange

The isolation of parenchymal cells from the livers of fed rats, assessment of cell integrity and the measurement of $^{45}Ca^{2+}$ exchange under steady-state conditions were performed as described previously (Parker & Barritt, 1981). The integrity of isolated hepatocytes was routinely assessed before and after $^{45}Ca^{2+}$-exchange incubations by examining the ability of the cells to exclude Trypan Blue (final concn. 0.4%, w/v). The dye was excluded from 90–95% of freshly prepared cells. After incubation for 45min this value fell by an average of 4%. The amounts of ATP present in neutralized $HClO_4$ extracts of samples of cell incubation mixtures were determined by the method of Stanley & Williams (1969).

Analysis of $^{45}Ca^{2+}$-exchange curves obtained under steady-state conditions

Fits of the series (Scheme 1) and parallel (not shown) configurations of a three-compartment closed system to the data were performed by using a non-linear iterative curve-fitting procedure as described previously (Barritt *et al.*, 1981*b*). For each set of data subjected to kinetic analysis (Figs. 1, 3 and 4), both the series and parallel configurations were found to be the simplest systems consistent with the data. Although the parallel configuration cannot be excluded (Barritt *et al.*, 1981*b*), only results for the series configuration are reported. However, the major conclusions in the present paper are also valid for kinetic constants obtained for fits of the parallel configuration to the data. Degrees of significance between the values of a given kinetic constant obtained for untreated and treated cells were assessed by the *t* test (Boxenbaum *et al.*, 1974).

Values for the amount of exchangeable Ca^{2+} associated with the cells and the quantities of exchangeable Ca^{2+} in compartments 2 and 3 are expressed as nmol of exchangeable Ca^{2+}/mg wet wt. Either the quantities of exchangeable Ca^{2+} in compartments 2 and 3 have also been expressed in units of mM and nmol/mg of organelle protein, respectively, or the factors for the conversion from units of nmol/mg wet wt. into these units are given. The calculations were performed as follows. From the values obtained for the protein and marker-enzyme contents of subcellular fractions enriched in the mitochondria and endoplasmic reticulum (see the Results section), the total amounts of protein in the mitochondria and endoplasmic reticulum were calculated to be 45mg of mitochondrial protein and 50mg of endoplasmic-reticulum protein per g wet wt. [cf. values of 50mg (Carafoli, 1967; van Rossum, 1970; Claret-Berthon *et al.*, 1977) and 82mg (Blackmore *et al.*, 1979) per g wet wt. for

mitochondrial protein, and 30mg (Claret-Berthon *et al.*, 1977), 75mg (calculated from the data of Krack *et al.*, 1980) and 24mg (Joseph *et al.*, 1983) per g wet wt. for endoplasmic-reticulum protein]. It was assumed that, for exchangeable Ca^{2+} in compartment 3, 1nmol of Ca^{2+}/mg wet wt. is equal to $1/(0.045+0.05)=10.5$nmol of Ca^{2+}/mg of organelle protein. For exchangeable Ca^{2+} in compartment 2 (the cytoplasm, excluding the mitochondria and endoplasmic reticulum) units of nmol/mg wet wt. were converted into mM by using the relationships: intracellular fluid volume (ml) = 0.5 × wet wt. of cells (g) (Krebs *et al.*, 1974) and the volume occupied by freely diffusible Ca^{2+} in the cell cytoplasm (excluding intracellular organelles) = 0.63 × intracellular fluid volume (Baker & Knight, 1978). Thus, for exchangeable Ca^{2+} in compartment 2, 1nmol of Ca^{2+}/mg wet wt. of cells is equal to 3.3mM total Ca^{2+}.

Calculation of the permeability constant of the liver cell plasma membrane for Ca^{2+}

On the basis of the theory proposed by Hodgkin & Katz (1949), the influx of Ca^{2+} across the plasma membrane by diffusion can be described by the equation

$$R_{21}=P\exp(-2FE/RT)\,Ca_o^{2+F}-P\,Ca_i^{2+F} \quad (1)$$

where R_{21} is the Ca^{2+} flux expressed as $mol\cdot cm^{-2}\cdot s^{-1}$, Ca_o^{2+F} and Ca_i^{2+F} represent the concentrations (M) of free Ca^{2+} in the extracellular medium and cell cytoplasm respectively, P ($cm\cdot s^{-1}$) is the permeability constant, F ($9.652\times10^4\cdot mol^{-1}$) is the Faraday constant, E (V) is the membrane potential, R ($8.31\ J\cdot mol^{-1}\cdot K^{-1}$) is the gas constant, and T (K) is the absolute temperature (Ferreira & Lew, 1976). For a membrane potential of −34mV (Claret & Mazet, 1972; Jenkinson & Koller, 1977), the value of $\exp(-2FE/RT)$ is 12.8. [Although α-adrenergic agonists have been shown to decrease the membrane potential of hepatocytes isolated from some mammalian species (Haylett & Jenkinson, 1972; Jenkinson & Koller, 1977), this effect is transient in comparison with the much longer time of exposure of hepatocytes to adrenaline used in the present steady-state experiments.]

Since Ca_i^{2+F} is very much lower than Ca_o^{2+F}, the term PCa_i^{2+F} in eqn. (1) will be small compared with $P\exp(-2FE/RT)Ca_o^{2+F}$. Moreover, for a given experimental condition (untreated or adrenaline-treated cells) homoeostatic mechanisms will act to decrease the variation in Ca_i^{2+F} at different values of Ca_o^{2+F} (Brinley *et al.*, 1977; Tiffert & Brinley, 1981). Therefore plots of R_{21} as a function of Ca_o^{2+F} can be approximated by a straight line with a slope equal to $P\exp(-2FE/RT)$ ($cm\cdot s^{-1}$) and passing near the origin (cf. Fig. 2*a*).

Values of P were calculated from the slope of plots of R_{21} as a function of the concentration of total extracellular Ca^{2+} (Ca_o^{2+T}) (assuming that $Ca_o^{2+T}=Ca_o^{2+F}$) by using a value of 12.8 for $\exp(-2FE/RT)$. Fluxes were converted from units of nmol of Ca^{2+}/min per mg wet wt. of cells into $mol\cdot cm^{-2}\cdot s^{-1}$ by using a value of $2.8\,cm^2\cdot mg$ wet $wt.^{-1}$ for the relationship between surface area and wet wt. of the liver cell (Claret & Mazet, 1972).

Preparation of subcellular fractions from isolated hepatocytes

Isolated hepatocytes were incubated as described previously (Barritt *et al.*, 1981*b*) in the presence of 0.1mM extracellular Ca^{2+} with or without adrenaline (0.1μM) for 15min before the addition of $^{45}CaCl_2$ (0.3MBq). After a further 20min, the cells were precipitated by centrifugation and homogenized as described previously (Barritt *et al.*, 1981*b*), except that the step in which the cells were washed in sucrose/Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]/EGTA before homogenization was omitted.

Subcellular fractions were isolated by centrifugation of: the homogenate at 150*g* for 6min, the supernatant from this step at 4100*g* for 5min [this precipitate is fraction 2 (enriched in mitochondria)], the supernatant from the precipitate of fraction 2 at 14000*g* for 10min (this precipitate is fraction 3, enriched in 'microsomes' derived from the endoplasmic reticulum), and the supernatant from this step at 70000*g* for 90min. Precipitates of fractions 2 and 3 were washed once and resuspended in sucrose/Hepes/EGTA, and the amounts of $^{45}Ca^{2+}$, glutamate dehydrogenase and glucose 6-phosphatase present were determined as described previously (Barritt *et al.*, 1981*b*).

Results

Effects of adrenaline on $^{45}Ca^{2+}$ exchange at 0.1mM, 0.5mM and 5.0mM extracellular Ca^{2+}

$^{45}Ca^{2+}$-exchange curves obtained under steady-state conditions for untreated cells and cells treated with adrenaline at 0.1mM, 0.5mM and 5.0mM extracellular Ca^{2+} are shown in Fig. 1. Other experiments have shown that at 0.1mM- and 2.4mM-Ca^{2+} (*a*) the effects of adrenaline on $^{45}Ca^{2+}$ exchange are completely inhibited by the α-adrenergic antagonist phenoxybenzamine (results not shown) and (*b*) the effects of phenylephrine, a more selective α-adrenergic agonist, on $^{45}Ca^{2+}$-exchange curves are similar to those of adrenaline (Barritt *et al.*, 1981*b*). At 0.1mM- and 0.5mM-Ca^{2+}, adrenaline decreased the plateau of the exchange curve (Figs. 1*a* and 1*b*) and caused no change (Fig. 1*a*) or a small stimulation (Fig. 1*b*) in the amount of $^{45}Ca^{2+}$

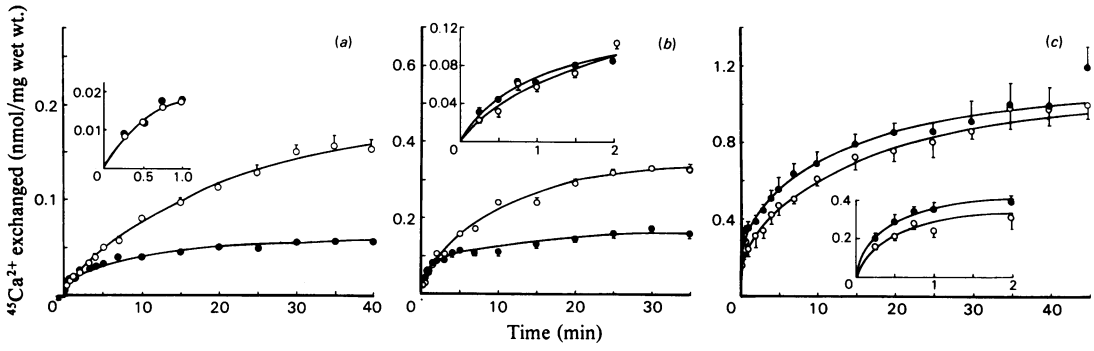
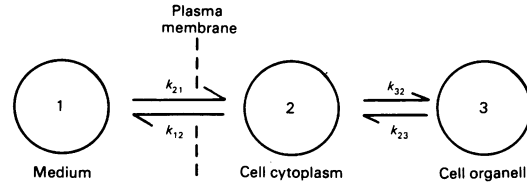


Fig. 1. *Effects of adrenaline on $^{45}Ca^{2+}$ exchange at 0.1 mM, 0.5 mM and 5.0 mM extracellular Ca^{2+}*
Hepatocytes were incubated at 37°C in the presence of (*a*) 0.1 mM, (*b*) 0.5 mM or (*c*) 5.0 mM extracellular Ca^{2+} in the absence (○) or in the presence (●) of 0.1 μM-adrenaline for 15 min before the addition of tracer amounts of $^{45}Ca^{2+}$. A second addition of adrenaline (0.1 μM) was made to the adrenaline-stimulated cells at 1 min before the addition of $^{45}CaCl_2$. The composition of the incubation medium and measurement of the amount of $^{45}Ca^{2+}$ associated with the cells were as described in the Experimental section. Each point is the mean ± S.E.M. for 8–18 (0.1 mM-Ca^{2+}), 10–20 (0.5 mM-Ca^{2+}) or 4–8 (5.0 mM-Ca^{2+}) determinations. The insets show the quantities of $^{45}Ca^{2+}$ exchanged during the first 1 or 2 min. The lines were drawn by using the values of the constants (Table 1) and appropriate equations for a fit of the series configuration of three compartments of exchangeable Ca^{2+} (see the Experimental section) to the data.

exchanged within the first 1 min. Increases in both the initial rate of $^{45}Ca^{2+}$ exchange and the plateau of the exchange curve were observed in the presence of adrenaline at 5.0 mM-Ca^{2+} (Fig. 1*c*).

A series configuration of a three-compartment closed system (Scheme 1) was found to be the simplest system consistent with each set of data shown in Fig. 1. The values of the kinetic constants are shown in Table 1. At 0.1 mM-Ca^{2+} adrenaline caused large decreases in the quantity of exchangeable Ca^{2+} in compartment 3 (Q_3) and in the flux (R_{32}) and fractional transfer rate (k_{32}) for the transport of Ca^{2+} from compartment 2 to compartment 3. An increase in the quantity of exchangeable Ca^{2+} in compartment 2 (Q_2) was also observed, but there was no change in the fractional transfer rate for the transport of Ca^{2+} from the medium to compartment 2 (R_{21}).

In order to determine the intracellular distribution of exchangeable Ca^{2+} and the sites from which it is lost after treatment of cells with adrenaline, hepatocytes incubated in the presence of 0.1 mM-$^{45}Ca^{2+}$ as described in the Experimental section were subjected to subcellular fractionation. In contrast with previous experiments (Barritt *et al.*, 1981*b*), the cells were not washed before homogenization. This decreased the loss of cellular $^{45}Ca^{2+}$. For control cells, fraction 2 (enriched in mitochondria) contained 44 ± 8% of the total cellular glutamate dehydrogenase activity, 7 ± 0.3% of the glucose 6-phosphatase activity, 0.020 ± 0.002 mg of protein/mg wet wt. of cells, and 0.027 ± 0.003 nmol of $^{45}Ca^{2+}$/mg wet wt. of cells (1.4 nmol of $^{45}Ca^{2+}$/mg of protein),



Scheme 1. *Schematic representation of a series configuration of a three-compartment closed system proposed to describe the distribution of exchangeable Ca^{2+} between the medium and intracellular locations in isolated hepatocytes*
The three kinetically distinct compartments of exchangeable Ca^{2+} are the medium (compartment 1) and two compartments of cellular exchangeable Ca^{2+} (compartments 2 and 3). Previous studies have provided evidence indicating that kinetically distinct cellular compartment 2 represents exchangeable Ca^{2+} in the cytoplasm, whereas exchangeable Ca^{2+} in the mitochondria and endoplasmic reticulum contributes to kinetically distinct cellular compartment 3 (Barritt *et al.*, 1981*b*; Parker & Barritt, 1981). The quantity of exchangeable Ca^{2+} in each compartment is represented by Q_1, Q_2 and Q_3 (nmol/mg wet wt.). The fractional transfer rate (rate constant) and flux for the transfer of Ca^{2+} from compartment j ($j = 1$, 2 or 3) to compartment i ($i = 1$, 2 or 3) are represented by k_{ij} (min^{-1}) and R_{ij} (nmol/min per mg wet wt.) respectively.

whereas fraction 3 (enriched in 'microsomes' derived from the endoplasmic reticulum) contained 3 ± 0.8% of the glutamate dehydrogenase activity, 15 ± 1.2% of

Table 1. *Effect of adrenaline on the kinetic parameters obtained for a fit of the series configuration of three compartments of exchangeable Ca^{2+} to $^{45}Ca^{2+}$-exchange data obtained at 0.1 mM, 0.5 mM and 5.0 mM extracellular Ca^{2+}*

The series configuration (Scheme 1) was fitted to the data of Fig. 1 as described in the Experimental section. The values ± S.D. of each parameter (defined in the legend of Scheme 1) are shown. The quantity of exchangeable Ca^{2+} in compartment 1 (Q_1), the extracellular medium, was 3 (0.1 mM-Ca^{2+}), 16.3 (0.5 mM-Ca^{2+}) and 163 (5.0 mM-Ca^{2+}) nmol/mg wet wt. Q_2 and Q_3 can be expressed in units of mM and nmol/mg of organelle protein, respectively, by using the relationships: 1 nmol of Ca^{2+}/mg wet wt. = 3.3 mM (compartment 2) or 10.5 nmol/mg of organelle protein (compartment 3), as described in the Experimental section. The degrees of significance for comparison of the value obtained in the presence of adrenaline with that for control cells were: $*P<0.05$; $**P<0.01$; $***P<0.001$.

	0.1 mM-Ca^{2+}		0.5 mM-Ca^{2+}		5.0 mM-Ca^{2+}	
	Control	Adrenaline present	Control	Adrenaline present	Control	Adrenaline present
Fluxes (nmol/min per mg wet wt.)						
R_{21}	0.046 ± 0.011	0.038 ± 0.003	0.11 ± 0.02	0.13 ± 0.01	0.97 ± 0.14	1.28 ± 0.19
R_{32}	0.010 ± 0.001	0.003 ± 0.001***	0.034 ± 0.007	0.0062 ± 0.0029**	0.050 ± 0.006	0.052 ± 0.007
Fractional transfer rates (min^{-1})						
k_{21}	1.42 ± 0.36 ($\times 10^{-2}$)	1.17 ± 0.10 ($\times 10^{-2}$)	0.6 ± 0.1 ($\times 10^{-2}$)	0.78 ± 0.08 ($\times 10^{-2}$)	0.60 ± 0.08 ($\times 10^{-2}$)	0.78 ± 0.12 ($\times 10^{-2}$)
k_{12}	3.38 ± 1.56	1.53 ± 0.24	1.38 ± 0.72	1.45 ± 0.26	3.74 ± 0.83	3.74 ± 0.74
k_{32}	0.74 ± 0.19	0.082 ± 0.025**	0.44 ± 0.21	0.070 ± 0.038	0.19 ± 0.04	0.153 ± 0.029
k_{23}	0.0536 ± 0.0092	0.105 ± 0.034	0.12 ± 0.03	0.073 ± 0.044	0.066 ± 0.010	0.076 ± 0.013
Compartment sizes (nmol/mg wet wt.)						
Q_2	0.014 ± 0.003	0.025 ± 0.002**	0.076 ± 0.024	0.088 ± 0.008	0.26 ± 0.03	0.34 ± 0.02*
Q_3	0.187 ± 0.019	0.032 ± 0.003***	0.28 ± 0.03	0.085 ± 0.017***	0.75 ± 0.04	0.69 ± 0.04

the glucose 6-phosphatase activity, 0.0075 ± 0.001 mg of protein/mg wet wt. of cells and 0.016 ± 0.001 nmol of $^{45}Ca^{2+}$/mg wet wt. of cells (2.1 nmol of $^{45}Ca^{2+}$/mg of protein). Fractions 2 and 3 isolated by similar procedures from cells treated with adrenaline contained 0.011 ± 0.002 ($P<0.01$) and 0.010 ± 0.005 ($P<0.005$) nmol of $^{45}Ca^{2+}$/mg wet wt. of cells (0.6 and 1.3 nmol of $^{45}Ca^{2+}$/mg of organelle protein), respectively (means ± S.E.M., $n = 3$–14 and 6 for enzyme and $^{45}Ca^{2+}$ determinations respectively).

Assuming that the net loss or gain of $^{45}Ca^{2+}$ by the mitochondria and endoplasmic reticulum during homogenization and subcellular fractionation is small, the total amounts of exchangeable Ca^{2+} in these organelles are estimated to be 0.06 and 0.11 nmol/mg wet wt. respectively (1.3 and 2.2 nmol/mg of organelle protein), by using values for the amounts of marker enzymes, $^{45}Ca^{2+}$ and protein present in each fraction. The sum of the values of exchangeable Ca^{2+} in the mitochondria and endoplasmic reticulum (0.17 nmol/mg wet wt. of cells) is similar to the quantity of exchangeable Ca^{2+} in kinetically distinct compartment 3 at 0.1 mM-Ca^{2+} (Table 1).

The effects of adrenaline on the values of the kinetic parameters obtained at 0.5 mM-Ca^{2+} were similar to those observed at 0.1 mM-Ca^{2+}, although less pronounced (Table 1). At 5.0 mM-Ca^{2+} adrenaline increased the quantity of exchangeable Ca^{2+} in compartment 2 (Q_2), and the flux (R_{21}) and fractional transfer rate (k_{21}) for the transport of Ca^{2+} from the medium to this compartment.

Effect of changes in extracellular Ca^{2+} concentration on kinetic parameters of cellular exchangeable Ca^{2+}

Plots of flux, R_{ij}, as a function of the concentration or amount of Ca^{2+} in comparment j were made by using the data of Figs. 1 and 4(*a*) and data from previous experiments (Barritt *et al.*, 1981*a*,*b*; Parker & Barritt, 1981). For both control and adrenaline-treated cells, plots of R_{21} as a function of the extracellular Ca^{2+} concentration were best described by a straight line (Fig. 2*a*), indicating (see the Discussion section) that the transfer of Ca^{2+} from compartment 1 (the medium) to compartment 2 (the cytoplasm) represents the transport of Ca^{2+} across the plasma membrane by diffusion. Values of the permeability constant, P, were calculated as described in the Experimental section and found to be 7.9×10^{-11} and 11.2×10^{-11} cm·s^{-1} in the absence and presence of adrenaline respectively.

Other plots of R_{ij} as a function of Q_j approached a plateau at high Ca^{2+} concentrations (Figs. 2*b*–2*d*), although for plots of R_{12} as a function of Q_2 the plateau was not well defined (Fig. 2*b*). Except for the plot of R_{32} as a function of Q_2 for untreated cells

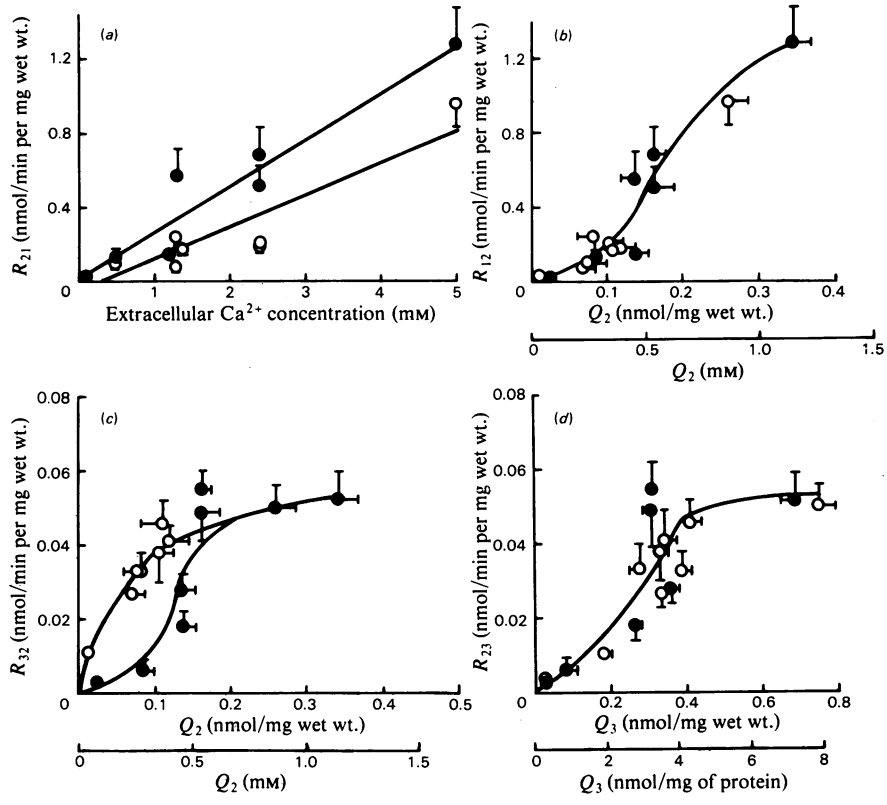


Fig. 2. *Plots of flux (R_{ij}) for the transport of Ca^{2+} from compartment j to compartment i as a function of the quantity of Ca^{2+} in compartment j*

Values and standard deviations (represented by the error bars) for the Ca^{2+} fluxes R_{21} (=R_{12}) and R_{32} (=R_{23}) and quantities of exchangeable Ca^{2+}, Q_2 and Q_3, were obtained from fits of the series configuration of three compartments of exchangeable Ca^{2+} to $^{45}Ca^{2+}$-exchange data obtained at 37°C at 0.1mM, 0.5mM and 5.0mM extracellular Ca^{2+} (Table 1), 1.3mM-Ca^{2+} [Table 2, and Table 4 of Barritt *et al.* (1981*b*), Table 2 of Parker & Barritt (1981) and Table 1 of Barritt *et al.* (1981*a*)] and 2.4mM-Ca^{2+} [Table 3, and Table 5 of Barritt *et al.* (1981*b*)] in the presence (●) or in the absence (○) of 0.1 μM-adrenaline. The units of Q_2 and Q_3 were converted from nmol of Ca^{2+}/mg wet wt. into mM total exchangeable Ca^{2+} and nmol/mg of organelle protein, respectively, as described in the Experimental section. The lines in (*a*) were drawn from a fit of the equation $R_{12} = P\exp(-2FE/RT)Ca_o^{2+T}$ (see the Experimental section) to the data by linear regression. The values of the slopes, $P\exp(-2FE/RT)$, were 0.17 and 0.25nmol·min^{-1}·mg wet $wt.^{-1}$·mM^{-1} for control and adrenaline-treated cells respectively. The line for data from control cells in (*c*) was drawn by using the equation $R_{32} = 0.064Q_2/(0.07 + Q_2)$. The values of the constants 0.064nmol/min per mg, the maximum flux, and 0.07nmol/mg wet wt., the quantity of Ca^{2+} in compartment 2 that gives half-maximal flux, were determined from linear-regression analysis of plots of $1/R_{32}$ as a function of $1/Q_2$. The lines in (*b*), (*c*) (adrenaline-treated cells) and (*d*) were drawn by eye.

(Fig. 2*c*), these plots are sigmoidal (Figs. 2*b*–2*d*) and non-linear in double-reciprocal form (results not shown). Adrenaline increased the concentration of Ca^{2+} that gives half-maximal stimulation of flux R_{32} from 0.05 to 0.15nmol/mg wet wt. of cells (0.17 to 0.5mM total cytoplasmic Ca^{2+}) (Fig. 2*c*), but did not markedly affect the shape of the other plots (Figs. 2*b* and 2*d*).

Effect of adrenaline at 20°C

A series configuration of a three-compartment closed system was also the simplest system found to be consistent with $^{45}Ca^{2+}$-exchange data obtained at 20°C in the presence and absence of adrenaline (Fig. 3 and Table 2). When the values of the kinetic constants obtained for untreated cells incubated at 20°C were compared with those obtained at 37°C, the decrease in temperature was found to decrease the quantity of Ca^{2+} in compartment 3 (Q_3), the flux between compartments 2 and 3 (R_{32}) and the fractional transfer rates (k_{32}, k_{23}) for the transport of Ca^{2+} between these compartments (Table 2).

At 20°C, adrenaline increased the amount of $^{45}Ca^{2+}$ exchanged at short times and the plateau of

the exchange curve (Fig. 3). Inspection of the kinetic constants showed that the main effect of the hormone was to increase the quantity of exchangeable Ca^{2+} in compartment 3 (Q_3) and the fluxes and fractional transfer rates for the transfer of Ca^{2+} from the medium to compartment 2 and from compartment 2 to compartment 3.

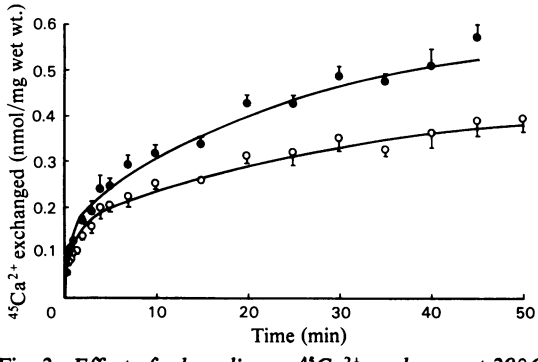


Fig. 3. *Effect of adrenaline on $^{45}Ca^{2+}$ exchange at 20°C in the presence of 1.3 mM extracellular Ca^{2+}*

Isolated hepatocytes were incubated at 20°C for 15 min in the presence of 1.3 mM-$CaCl_2$ and in the presence (●) or absence (○) of 0.1 μM-adrenaline before the initiation of $^{45}Ca^{2+}$ exchange. The composition of the incubation medium and measurement of the amounts of $^{45}Ca^{2+}$ associated with the cells were as described in the Experimental section. Each point is the mean ± S.E.M. for 4–15 separate determinations. The lines were drawn by using values of the constants (Table 2) and appropriate equations for a fit of the series configuration of three compartments of exchangeable Ca^{2+} to the data.

Effects of antimycin A plus oligomycin, phosphate ions and digitonin

Incubation of hepatocytes in the presence of antimycin A plus oligomycin decreased the plateau of the $^{45}Ca^{2+}$-exchange curves obtained at 2.4 or 0.1 mM extracellular Ca^{2+}, with little effect on the amount of $^{45}Ca^{2+}$ exchanged within the first 1 min (Fig. 4). At 2.4 mM-Ca^{2+}, the main effect of the inhibitors was to decrease the quantity of exchangeable Ca^{2+} in compartment 3 (Q_3) and increase the fractional transfer rate (k_{23}), for the transport of Ca^{2+} from compartment 3 to compartment 2 (Table 3). Exposure of hepatocytes to antimycin A plus oligomycin for 35 min did not alter the viability of the cells, as judged by measurement of the proportion of cells that excluded Trypan Blue, or the amount of lactate dehydrogenase in the extracellular medium. At 5, 20 and 35 min after the addition of the inhibitors the amount of cellular ATP was 0.6 ± 0.05, 0.4 ± 0.03 and 0.25 ± 0.04 nmol/mg wet wt. (means ± S.E.M., n=4–6), compared with values of 0.88 ± 0.08, 0.65 ± 0.15 and 0.71 ± 0.06 nmol/mg wet wt. respectively, for control cells incubated for these time periods.

In the presence of oligomycin and antimycin A, adrenaline did not alter the amount of $^{45}Ca^{2+}$ exchanged between 0 and 10 min and decreased the plateau of the exchange curve slightly (Fig. 4). Analysis of the data obtained at 2.4 mM-Ca^{2+} showed that, in contrast with the effects of adrenaline in the absence of inhibitors, the hormone did not markedly increase the quantity of exchangeable Ca^{2+} in compartment 2 (Q_2), or increase the flux (R_{21}) and fractional transfer rate (k_{21}) for the

Table 2. *Effects of decreasing the incubation temperature from 37°C to 20°C, and of adrenaline at 20°C, on the kinetic parameters obtained for a fit of the series configuration of three compartments of exchangeable Ca^{2+} to $^{45}Ca^{2+}$-exchange data obtained at 1.3 mM extracellular Ca^{2+}*

The series configuration (Scheme 1) was fitted to the data of Fig. 3 (20°C) and a $^{45}Ca^{2+}$-exchange curve obtained at 37°C in the presence of 1.3 mM-Ca^{2+} (four to six separate determinations at each of 17 time points; not shown) as described in the Experimental section. The values ± S.D. of each parameter (defined in the legend of Scheme 1) are shown. The quantity of exchangeable Ca^{2+} in compartment 1 (Q_1), the medium, was 42 nmol/mg wet wt. Q_2 and Q_3 can be expressed in units of mM and nmol/mg of organelle protein, respectively, by using the relationships: 1 nmol of Ca^{2+}/mg wet wt. = 3.3 mM (compartment 2) or 10.5 nmol/mg of organelle protein (compartment 3), as described in the Experimental section. The degrees of significance for comparison of the value obtained for control cells at 20°C with that for control cells at 37°C were: $^{*}P<0.05$; $^{***}P<0.001$.

	Control, 37°C	Control, 20°C	Adrenaline present, 20°C
Fluxes (nmol/min per mg wet wt.)			
R_{21}	0.18 ± 0.04	0.19 ± 0.03	0.25 ± 0.04
R_{32}	0.046 ± 0.006	0.009 ± 0.002***	0.016 ± 0.004
Fractional transfer rates (min^{-1})			
k_{21}	0.0043 ± 0.0009	0.0044 ± 0.0007	0.0058 ± 0.0009
k_{12}	1.6 ± 0.7	1.1 ± 0.3	1.26 ± 0.32
k_{32}	0.40 ± 0.15	0.048 ± 0.019*	0.082 ± 0.029
k_{23}	0.11 ± 0.011	0.030 ± 0.013***	0.038 ± 0.015
Compartment sizes (nmol/mg wet wt.)			
Q_2	0.11 ± 0.03	0.18 ± 0.02	0.20 ± 0.02
Q_3	0.41 ± 0.03	0.28 ± 0.05*	0.42 ± 0.07

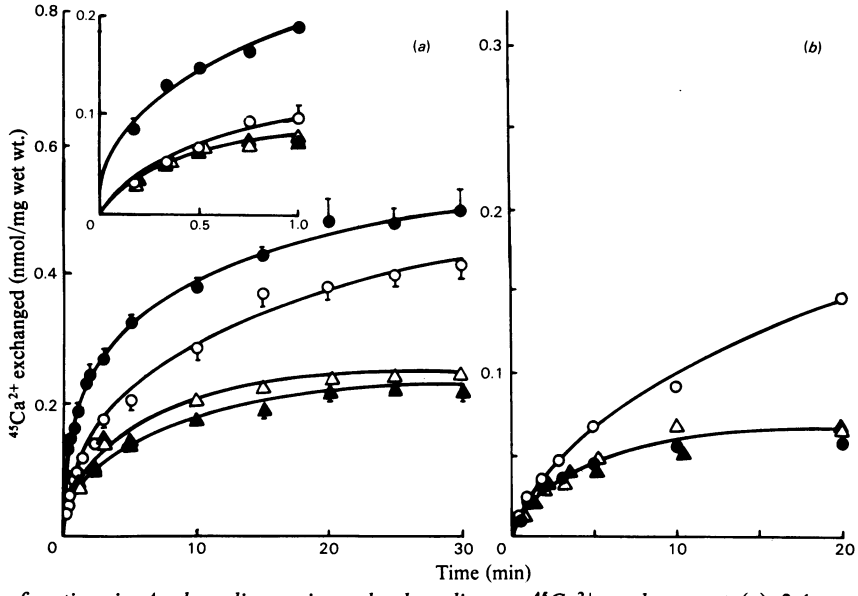


Fig. 4. *Effects of antimycin A plus oligomycin and adrenaline on $^{45}Ca^{2+}$ exchange at (a) 2.4 mM and (b) 0.1 mM extracellular Ca^{2+}*

Isolated hepatocytes were incubated at 37°C for 15 min in the presence of (*a*) 2.4 mM- or (*b*) 0.1 mM-$CaCl_2$, in the presence (●, ▲) or absence (○, △) of 0.1 μM-adrenaline and in the presence (△, ▲) or absence (○, ●) of antimycin A (5 μg/ml) and oligomycin (10 μg/ml). The composition of the incubation medium and measurement of the amounts of $^{45}Ca^{2+}$ associated with the cells were as described in the Experimental section. Each point is the mean ± S.E.M. for five to eight separate determinations (*a*) or the means of two to four determinations (*b*). The inset in (*a*) shows the quantities of $^{45}Ca^{2+}$ exchanged in 1 min. The lines were drawn by using the values of the constants (Table 3) and appropriate equations for a fit of the series configuration of three compartments of exchangeable Ca^{2+} to the data (*a*), or from a fit of the data by eye (*b*).

transport of Ca^{2+} from the medium to compartment 2 (Table 3). A small decrease in the quantity of exchangeable Ca^{2+} in compartment 3 (Q_3) and decreases in the flux (R_{32}) and fractional transfer rates (k_{32}, k_{23}) for the transport of Ca^{2+} between compartments 2 and 3 were observed in the presence of adrenaline (Table 3).

The incubation of hepatocytes in media containing 0, 1 mM-, 5 mM- or 10 mM-phosphate ions (adjusted with NaH_2PO_4) did not significantly affect the amount of $^{45}Ca^{2+}$ exchanged between 2 and 30 min at 1.3 mM-Ca^{2+} (results not shown). When the experiments conducted at 0, 1 mM-. 5 mM- and 10 mM-phosphate ions were repeated at an extracellular Ca^{2+} concentration of 0.4 mM, similar results were obtained.

The possibility that exchangeable Ca^{2+} in compartment 3 represents mitochondrial Ca^{2+} in a small population of damaged hepatocytes was tested by comparing $^{45}Ca^{2+}$-exchange curves (conducted at 1.3 mM-Ca^{2+} as described in the legend of Fig. 1) for cells treated for 15 min with 0.005% (w/v) digitonin before the addition of $^{45}Ca^{2+}$ (3% of the cells excluded Trypan Blue) with exchange curves for untreated cells (90% of the cells excluded Trypan Blue). Over the period 1–30 min after the addition of $^{45}Ca^{2+}$, the shapes of the exchange curves for treated and untreated cells were similar. The amount of $^{45}Ca^{2+}$ exchanged by cells treated with digitonin was 20% greater than that exchanged by untreated cells (results not shown).

Discussion

Cellular compartments of exchangeable Ca^{2+}

The simplest kinetic system that adequately describes the $^{45}Ca^{2+}$-exchange curves obtained under each of the experimental conditions investigated is that shown in Scheme 1 (Barritt *et al.*, 1981*b*; Parker & Barritt, 1981). This scheme is consistent with both the results of the kinetic analysis of $^{45}Ca^{2+}$-exchange curves obtained in the presence of antimycin A and oligomycin and a comparison of the results of the kinetic analysis and subcellular-fractionation experiments conducted in the absence of inhibitors at 0.1 mM-Ca^{2+}.

It is considered unlikely that compartment 3 (mitochondria and endoplasmic reticulum) represents a large quantity of exchangeable Ca^{2+} in the

Table 3. *Effects of antimycin A plus oligomycin and of adrenaline on the kinetic parameters obtained for a fit of the series configuration of three compartments of exchangeable Ca^{2+} to $^{45}Ca^{2+}$-exchange data obtained at 37°C in the presence of 2.4 mM extracellular Ca^{2+}*

The series configuration (Scheme 1) was fitted to the data of Fig. 4(*a*) as described in the Experimental section. The values ± S.D. of each parameter (defined in the legend of Scheme 1) are shown. The quantity of exchangeable Ca^{2+} in compartment 1 (Q_1), the medium, was 78 nmol/mg wet wt. Q_2 and Q_3 can be expressed in units of mM and nmol/mg of organelle protein, respectively, by using the relationships: 1 nmol of Ca^{2+}/mg wet wt. = 3.3 mM (compartment 2) or 10.5 nmol/mg of organelle protein (compartment 3), as described in the Experimental section. The degrees of significance for comparison of the value under test with the appropriate control (for columns 3 and 4, the control is no inhibitors and no adrenaline; for column 5 the control is inhibitors present and no adrenaline) were: $*P<0.05$; $**P<0.02$; $***P<0.01$; $****P<0.001$.

	No adrenaline		Adrenaline present	
	No inhibitors	Antimycin A + oligomycin	No inhibitors	Antimycin A + oligomycin
Fluxes (nmol/min per mg wet wt.)				
R_{21}	0.214 ± 0.043	0.217 ± 0.054	0.691 ± 0.130***	0.156 ± 0.023
R_{32}	0.038 ± 0.008	0.026 ± 0.006	0.055 ± 0.007	0.012 ± 0.005**
Fractional transfer rates (min^{-1})				
k_{21}	2.75 ± 0.56 ($\times 10^{-3}$)	2.78 ± 0.69 ($\times 10^{-3}$)	8.86 ± 1.67 ($\times 10^{-3}$)***	2.00 ± 0.29 ($\times 10^{-3}$)
k_{12}	2.03 ± 0.75	2.84 ± 1.16	4.29 ± 1.14	1.45 ± 0.38
k_{32}	0.360 ± 0.129	0.345 ± 0.135	0.343 ± 0.064	0.112 ± 0.061
k_{23}	0.115 ± 0.026	0.155 ± 0.036	0.177 ± 0.022	0.091 ± 0.045
Compartment sizes (nmol/mg wet wt.)				
Q_2	0.105 ± 0.019	0.076 ± 0.014	0.161 ± 0.012**	0.107 ± 0.014
Q_3	0.330 ± 0.022	0.170 ± 0.014****	0.311 ± 0.016	0.133 ± 0.019****

mitochondria and endoplasmic reticulum of a small number of damaged hepatocytes in which the organelles have been directly exposed to high concentrations of extracellular Ca^{2+}. Thus pretreatment of hepatocytes with digitonin did not markedly increase the quantity of exchangeable Ca^{2+} present. Moreover, the values obtained for the quantity of exchangeable Ca^{2+} in compartment 3 in studies with isolated hepatocytes (Barritt *et al.*, 1981*b*; Parker & Barritt, 1981; the present work) are similar to those obtained in studies with the perfused liver (Claret-Berthon *et al.*, 1977).

The present results are consistent with the conclusion that kinetically distinct compartment 2 represents rapidly exchangeable Ca^{2+} in the cytoplasm (Scheme 1) although further experiments are required before a conclusive assignment of this compartment can be made. At an extracellular Ca^{2+} concentration of 1.3 mM, the concentration of total exchangeable Ca^{2+} in compartment 2 is estimated to be 0.3 mM [cf. a value of 0.25 mM for the concentration of Ca^{2+} in a similar compartment of the liver cell calculated from the data of Joseph *et al.* (1983)]. Most of the exchangeable Ca^{2+} in compartment 2 is likely to be bound to metabolites, proteins and phospholipids, and possibly sequestered in organelles. If 16% were free (ionized) Ca^{2+}, as observed for exchangeable Ca^{2+} in the erythrocyte cytoplasm (Simonsen *et al.*, 1982), the concentration of free Ca^{2+} in the liver cell cytoplasm would be 50 μM. This is about 150 times the measured concentration, 0.2–0.3 μM free Ca^{2+} (Murphy *et al.*, 1980; Charest *et al.*, 1983). Therefore it may be concluded that a significant quantity of Ca^{2+} in compartment 2 is bound or sequestered in organelles (excluding slowly exchangeable Ca^{2+} in the mitochondria and endoplasmic reticulum) by processes that are not present in the cytoplasm of the erythrocyte.

Mechanism of Ca^{2+} inflow

Comparison of eqn. (1) (see the Experimental section) with plots of R_{21} as a function of Ca_o^{2+T}, the total extracellular Ca^{2+} concentration, indicates that the transport of Ca^{2+} from compartment 1 (extracellular medium) to compartment 2 (cytoplasm) occurs by a process of simple or facilitated diffusion. This conclusion is supported by the observation that plots of Na^+-independent Ca^{2+} influx as a function of the extracellular Ca^{2+} concentration are linear for the squid giant axon (Hodgkin & Keynes, 1957; Baker *et al.*, 1971; Baker & McNaughton, 1976). It has been proposed that in this system Ca^{2+} influx is by diffusion (DiPolo & Beaugé,

1980). Plots of Ca^{2+} flux as a function of extracellular Ca^{2+} concentration have also been reported to be linear for the inflow of Ca^{2+} to exocrine cells of the pancreas (Kondo & Schultz, 1976), and approximately linear for the inflow of Ca^{2+} to Ehrlich ascites-tumour cells (Hinnen *et al.*, 1979). Taken together, these results are consistent with the conclusion that the transfer of Ca^{2+} between compartments 1 and 2 represents transport across the liver cell plasma membrane (Scheme 1).

The value for the rate of Ca^{2+} influx to the liver cell at 1.3mM extracellular Ca^{2+} calculated from the present data is 1.4×10^{-12} mol·cm^{-2}·s^{-1}. This is in reasonable agreement with estimates of about 5×10^{-11} mol·cm^{-2}·s^{-1} for this parameter, which can be calculated from the primary data of Krell *et al.* (1979) and Reinhart *et al.* (1982*b*) for the uptake of Ca^{2+} by perfused livers under non-steady-state conditions. These values are considerably greater than that of 4×10^{-14} mol·cm^{-2}·s^{-1} obtained for the Na^+-independent pathway of Ca^{2+} inflow across the plasma membrane of the squid giant axon (DiPolo, 1979; DiPolo & Beaugé, 1980).

Kinetics of intracellular Ca^{2+}-transport processes

The results indicate that the process for the transport of Ca^{2+} from compartment 2 (cytoplasm) to compartment 1 (extracellular medium) is only about 20% saturated with Ca^{2+} at physiological concentrations of extracellular Ca^{2+} (1.3mM) [cf. ATP-dependent Ca^{2+} efflux in the giant axon of the squid (DiPolo & Beaugé, 1979)]. In contrast, the processes for the transport of Ca^{2+} between compartments 2 and 3 appear to be closer to saturation with Ca^{2+}. This observation suggests that under physiological conditions the uptake of Ca^{2+} by the mitochondria and endoplasmic reticulum is close to saturation.

These results are consistent with those of Joseph *et al.* (1983), who have shown that in hepatocytes treated with digitonin the amounts of Ca^{2+} in the mitochondria and endoplasmic reticulum are near maximal at physiological concentrations of cytoplasmic Ca^{2+}. Measurement of the apparent K_m value for Ca^{2+} of microsomal fractions incubated under physiological conditions also indicates that this process is likely to be near saturation at physiological cytoplasmic Ca^{2+} concentrations (Brattin *et al.*, 1982). However, the apparent K_m value for Ca^{2+} of isolated mitochondria incubated in the presence of KCl and Mg^{2+} is about 10μM (Affolter & Carafoli, 1981), suggesting that under physiological conditions Ca^{2+} uptake by these organelles is far from saturated. The apparent discrepancy between this prediction and the present results may be due to differences between the incubation conditions used for isolated mitochondria *in vitro* and those present in the cell cytoplasm, and the fact that kinetically distinct compartment 3 is composed of exchangeable Ca^{2+} in at least two types of organelles. Although the plots of flux R_{32} as a function of the quantity of Ca^{2+} in compartment 2 appear to reach a plateau, the possibility that the processes for the transport of Ca^{2+} from compartment 2 to compartment 3 are only partially saturated with Ca^{2+} cannot be competely excluded.

The sigmoidal nature of plots of R_{12} as a function of Q_2, and of R_{32} as a function of Q_2 or Q_3, may reflect complex kinetics (Vinogradov & Scarpa, 1973; Reed & Bygrave, 1975; Ferreira & Lew, 1976; Black *et al.*, 1981; Famulski & Carafoli, 1982; Muallem & Karlish, 1982) for the interaction of Ca^{2+} with Ca^{2+} transporters, including the postulated plasma-membrane $(Ca^{2+} + Mg^{2+})$-dependent ATPase (Garnett & Kemp, 1975; Hope-Gill & Nanda, 1979; Lotersztajn *et al.*, 1981; Iwasa *et al.*, 1982; Kraus-Friedmann *et al.*, 1982), the fact that Q_2 and Q_3 represent total rather than free Ca^{2+}, the presence of more than one type of transporter for the transfer of Ca^{2+} between two given compartments, and/or the possibility that changes in the structure and properties of the plasma membrane are induced at very low (Kolb & Adam, 1976; Loewenstein, 1981) or very high concentrations of extracellular Ca^{2+}.

Effects of low temperature and inhibitors of mitochondrial Ca^{2+} transport

The main effect of lowering the temperature from 37°C to 20°C was to decrease the flux of Ca^{2+} between compartments 2 and 3 (cf. Krell *et al.*, 1979). No accumulation of cellular Ca^{2+} or decrease in the rate of Ca^{2+} outflow across the plasma membrane was observed at 20°C. These results contrast with the net accumulation of Ca^{2+} observed at much lower temperatures (van Rossum *et al.*, 1976).

The predominant effect of adrenaline at 20°C is a stimulation of Ca^{2+} inflow to intracellular Ca^{2+} stores. No evidence was obtained for a stimulation of Ca^{2+} outflow from intracellular stores or the cell. Moreover, at 20°C adrenaline does not induce a loss of Ca^{2+} from isolated hepatocytes incubated in the presence of 0.1mM-Ca^{2+} (G. J. Barritt, unpublished work). These results differ from those obtained at 37°C, where the predominant effect of adrenaline is a stimulation of Ca^{2+} outflow from intracellular stores (Chen *et al.*, 1978; Babcock *et al.*, 1979; Blackmore *et al.*, 1979; Murphy *et al.*, 1980; Berthon *et al.*, 1981; Barritt *et al.*, 1981*b*; Reinhart *et al.*, 1982*b*).

The results of the steady-state kinetic analysis indicate that incubation of hepatocytes with antimycin A plus oligomycin does not significantly affect Ca^{2+} transport across the plasma membrane

under the experimental conditions used. Although a decrease in the cellular ATP concentrations was observed in the presence of the inhibitors, other studies (Whiting & Barritt, 1982) have shown that Ca^{2+} outflow induced by ionophore A23187 at 0.1 mM extracellular Ca^{2+} is not impeded by treatment of cells with dinitrophenol, an agent which also decreases the intracellular concentration of ATP (Berry *et al.*, 1980; Blackmore *et al.*, 1982). The absence of an effect of adrenaline on flux R_{21} and fractional transfer rate k_{21} for the transport of Ca^{2+} from the medium to the cell in cells incubated with antimycin A plus oligomycin indicates that normal mitochondrial function is required for this action of adrenaline on the liver cell [compare with the requirement for mitochondrial function in other actions of α-adrenergic agonists on the liver (Reinhart *et al.*, 1982*a*) and the inhibition by antimycin A of agonist-induced $^{45}Ca^{2+}$ influx in the parotid gland (Poggioli *et al.*, 1983)].

If it is assumed that antimycin A plus oligomycin deplete mitochondrial exchangeable Ca^{2+}, but do not significantly alter that present in the endoplasmic reticulum and other intracellular organelles, the data of Table 3 indicate that at 2.4 mM extracellular Ca^{2+} the quantities of exchangeable Ca^{2+} are 0.16 nmol/mg wet wt. (3.5 nmol/mg of mitochondrial protein) and 0.17 nmol/mg wet wt. (3.4 nmol/mg of endoplasmic-reticulum protein) in the mitochondria and endoplasmic reticulum respectively. These values and those obtained from cell-fractionation studies conducted at 0.1 mM-Ca^{2+} can be compared with estimates of 2.7 and 6.8 nmol/mg of mitochondrial protein for total mitochondrial Ca^{2+} at 0.01 mM- and 1.3 mM-Ca^{2+} (Reinhart *et al.*, 1982*b*) and 2.3 nmol/mg of mitochondrial protein for exchangeable Ca^{2+} at 2.4 mM extracellular Ca^{2+} (Claret-Berthon *et al.*, 1977).

The lack of effect of alterations in the concentration of extracellular phosphate on $^{45}Ca^{2+}$ exchange was unexpected. Changes in the concentration of this anion have been shown to affect mitochondrial Ca^{2+} uptake in cells of the liver (Krell *et al.*, 1979), kidney (Borle, 1972) and heart (Langer & Nudd, 1980). However, the response of isolated liver mitochondria to changes in phosphate concentration differs from that of mitochondria from kidney or heart (Barritt, 1981). Differences between the results of Krell *et al.* (1979) and those reported here may reflect considerable differences in the experimental conditions used in the two studies.

Effect of adrenaline on kinetic parameters of Ca^{2+} transport

The results of experiments conducted at different extracellular Ca^{2+} concentrations indicate that adrenaline increases the permeability of the plasma membrane to Ca^{2+} by 40% and increases the concentration of Ca^{2+} that gives half-maximal rate (K_m) of the process (R_{32}) for the transport of Ca^{2+} from compartment 2 (cytoplasm) to compartment 3 (organelles) by 3-fold [compare with the theoretical calculations of Williamson *et al.* (1981)]. The latter result differs from conclusions reached on the basis of other studies. These have suggested that adrenaline enhances the catalytic process for the outflow of Ca^{2+} from the mitochondria and endoplasmic reticulum (Whiting & Barritt, 1982). This difference may be due to the difficulty in equating an apparent K_m value deduced from steady-state kinetic studies of isotope distribution with a change in the catalytic process for either Ca^{2+} inflow or Ca^{2+} outflow from an organelle (compare with the effects of antimycin A plus oligomycin on the fractional transfer rates).

The observation that adrenaline increases the permeability constant for the transport of Ca^{2+} across the plasma membrane, and the results of analysis of the data obtained at 20°C, are consistent with the conclusion that one of the effects of adrenaline on the liver cell is to stimulate the inflow of Ca^{2+} from the medium to the cell cytoplasm (Keppens *et al.*, 1977; Assimacopoulos-Jeannet *et al.*, 1977; Foden & Randle, 1978; Barritt *et al.*, 1981*b*). No information about the time of onset of this effect can be obtained from the present studies, although previous transient-state experiments indicate that this effect of adrenaline is slow in onset relative to the effect of the hormone on Ca^{2+} outflow from intracellular stores (Barritt *et al.*, 1981*b*).

Clear evidence that at least one metabolic effect of adrenaline (the activation of glycogen phosphorylase) can be initiated without a contribution from extracellular Ca^{2+} has been provided (Blackmore *et al.*, 1982; Reinhart *et al.*, 1982*b*). However, this does not exclude a role for stimulated Ca^{2+} inflow in the actions of adrenaline during periods of time greater than about 2 min (Barritt, 1980; Reinhart *et al.*, 1982*b*).

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