Biochimica et Biophysica Acta 903 (1987) 218–228
Elsevier

BBA 73672

Red blood cells Ca^{2+} pump is not altered in essential hypertension of humans and Kyoto rats

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(Received 3 April 1987)

Key words: Calcium ion flux; Hypertension; Ionophore A23187; Cobalt chloride; (Erythrocyte membrane)

The kinetic parameters of the Ca^{2+} pump were assessed in red blood cells of essential hypertensive subjects as compared to their respective controls. Uphill Ca^{2+} efflux was investigated in Ca^{2+}-saturated intact red blood cells using a new method recently developed for human red cells (Dagher, G. and Lew, V. J. Physiol. (London), in the press). ^{45}Ca-equilibrated cells were obtained using ionophore A23187 and Ca^{2+} efflux was assessed after addition of excess $CoCl_2$ which totally inhibits Ca^{2+} influx and thus exposes uphill Ca^{2+} extrusion by the pump. The results comprise methodological aspects of the use of this technique in rat red blood cells. The determination of the maximal velocity and the Ca^{2+} concentration for half-maximal stimulation ($K_{Ca}0.5$) did not reveal any alteration in essential hypertensives and spontaneously hypertensive rats as compared to their controls.

Introduction

Cation transport across cell membranes has been the subject of intensive investigation in animal models and humans with essential hypertension [1,2]. Abnormalities in sodium handling by different transport pathways have been described in different cell types [1,3] including red blood cells [4–6]. Recently, interest has been focussed on Ca^{2+} metabolism because of the prominent role of this ion in the regulation of smooth muscle contraction and therefore peripheral resistance. An increase in Ca^{2+} content and Ca^{2+} binding was reported in various cell types in either spontaneously hypertensive rats or essential hypertensive patients [7–10]. These alterations were thought to be the consequence of a membrane defect in calcium regulation and several studies reported disturbances in Ca^{2+} fluxes in certain cell types [10–13] but not in others [14,15]. The red blood cell has proved to be a suitable model for such a study. This cell lacks cellular organelles and its calcium content is mainly regulated by a ground permeability and an active transport mediated by the Ca^{2+} pump. A comparison of the genetically hypertensive Kyoto rat with its control revealed an increase in red blood cell Ca^{2+} content in the hypertensive strain [16], along with a reduced stimulation by calmodulin of the Ca^{2+} influx in inside-out vesicles [13]. Similar results were obtained in inside-out vesicles from red blood cells of hypertensive patients [17]. On the other hand, Vincenzi et al. [18] observed a reduced basal Ca^{2+}-ATPase activity (calmodulin-free) in red blood cells from hypertensive patients, while Vezzolli et al. [19] reported a decrease in the calmodulin-stimulated activity in red blood cell

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membrane from the genetically hypertensive Milan strain.

The activation by calmodulin of the red blood cell Ca^{2+}-ATPase has been the subject of numerous investigations. However, little is known about the state of the calmodulin-pump complex under physiological conditions. The question of whether fluctuations in Ca^{2+} at physiological levels regulate the binding of calmodulin to the Ca^{2+} pump in intact red blood cells is still a subject of controversy (for discussion see Refs. 20 and 21). On the other hand, disturbances in Ca^{2+}-dependent ATPase activity such as those reported in hypertension might reflect an alteration in the Ca^{2+} : ATP stoichiometry of the pump rather than in the Ca^{2+} efflux per se. Thus, with our current understanding of the Ca^{2+} pump regulation the question arises of whether alterations observed in broken membranes or cell-free systems could be reflected by Ca^{2+} transport across the cell membrane in intact cells.

The present study is an attempt to determine the maximal velocity of the red blood cell Ca^{2+} pump and its concentration for half-maximal stimulation by Ca^{2+} (K_{Ca} 0.5) in essential hypertension of humans and the Kyoto rat strain. A preliminary report of this work has been published elsewhere [40].

Materials and Methods

Rats

18 Okamoto spontaneous hypertensive rats were compared to their control group of 28 Wistar-Kyoto normotensive rats (rats were supplied by the centre d'élevage R. Janvier, LeGenest, France). Systolic blood pressure was 186 ± 7 and 120 ± 5 mmHg, respectively. Age-matched rats for the two groups were studied at 7, 12 and 22 weeks of age. Arterial blood was sampled under pentobarbital anaesthesia (25 mg/kg) from a catheter implanted in a carotid artery, and collected in a heparinized tube (10 IU/ml).

Patients

A group of 18 male hypertensive patients (aged 35–69 years, mean 47 years) were studied. All patients had a sustained high blood pressure without cardiac or renal insufficiency. Careful clinical investigation could not establish a cause for high blood pressure in these patients.

This group of essential hypertensives was compared to a control group of 17 normotensive subjects (aged 28–45 years, mean 38 years). Their blood pressure never exceeded 160 mmHg for systolic and 90 mmHg for diastolic pressure. These subjects were carefully selected to ensure that there were no known cases of hypertension among their close relatives.

Methods

The experimental design was based on results obtained by Tiffert et al. [22] and Dagher and Lew [23] showing that the addition of cobalt chloride in excess of calcium to a suspension of human red blood cells containing the ionophore A23187 immediately blocks all calcium movement through the ionophore without disturbing the Ca^{2+} pump-mediated fluxes. Thus, cobalt chloride can be used to assess uphill Ca^{2+} extrusion by the Ca^{2+} pump after an ionophore-induced Ca^{2+} load.

Human red blood cells were washed five times with Na^+/K^+ medium containing: 75 mM NaCl/75 mM KCl/0.2 mM $MgCl_2$/20 mM Hepes-HCl (pH 7.4 at 37° C). 0.1 mM EGTA was added to the first two washes.

Rat red blood cells were washed three times with the above medium, except that KCl was 110 mM and NaCl 40 mM. As discussed in the result section, control experiments showed that in the presence of A23187 and Ca_0, this KCl concentration is required to clamp cellular KCl at the normal concentration of 112 ± 13 mmol/l cells for rat cells.

The cells were suspended at 10% haematocrit in their respective Na^+/K^+ media with the addition of inosine 10 mM and different concentrations of $CaCl_2$ and tracer quantities of $^{45}CaCl_2$. The cell suspension was then incubated at 37° C under constant magnetic stirring.

The ^{45}Ca load was induced by the addition of A23187 (10 μM) and excess $CoCl_2$ (400 μM) was added to the cell suspension under steady-state conditions. Because of the fast Ca^{2+} efflux, the precise rate determination requires frequent sampling at brief time intervals, followed by an immediate and complete inactivation of ion trans-

port. The highest frequency attained in the pesent study was 12 per min at regular 5 s intervals. 50 μl samples of cell suspension were added to Eppendorf tubes containing 0.3 ml dibutylphthalate and 1 ml of ice-cold Na^+/K^+ medium to which 0.4 mM $CoCl_2$ was added. Samples were kept in an ice-bath (0–4°C) for a brief interval before centrifugation for 10 s at $12000 \times g$. In control experiments no loss of counts was detected up to 20 min in cold stopper solution [23]. The supernatant was then removed and the walls of the tube carefully wiped with cotton swabs. 0.5 ml trichloroacetic acid solution (6%) was added to the cell pellet and was vigorously vortexed. The cell debris were removed by centrifugation and 0.4 ml of clear supernatant was added to liquid scintillation vials and counted. To assess extracellular counts trapped in the cell pellet, 50 μl samples were taken prior to A23187 addition and added to the stopping solution. Control experiments showed that ionophore-treated red blood cells retained their initial volume, and Mg^{2+} and K^+ content, despite large changes in Mg^{2+} and K^+ permeability (see Ref. 24 and the present results).

Estimation of the Ca^{2+} concentration for half-maximal stimulation of the Ca^{2+} pump

Under our experimental conditions, after $CoCl_2$ addition, calcium is rapidly transported out of the cell, so that steady-state conditions do not prevail. The kinetic analysis of such progress curves have been previously described by Orsi and Tipton [25] and Eilam and Stein [26]. Integrated rate equations have been developed to determine the kinetic parameters of the red blood cell glucose transport pathway [27] and of several enzymatic reactions [25].

In this study, the kinetic parameters V_m, K and n were derived from a set of values of cell calcium content obtained after $CoCl_2$ addition as a function of time. The activation curve of Ca^{2+} as a function of cellular calcium (Ca_i^{2+}) can be adequately described by a simple kinetic model and obeys the following equation.

$$v = \frac{V_m}{(1 + K'/[Ca_i^{2+}])^n} \quad (1)$$

where V_{max} is the maximal Ca^{2+} efflux at saturating cellular calcium, K' the apparent dissociation constant for the Ca^{2+}-site complex, and n the minimum number of Ca_i^{2+} sites. Free cellular Ca_i^{2+} is related to total calcium content (Ca_i^T), by the equation $Ca^{2+} = \alpha Ca_i^T$ where α is the fraction of ionized calcium (see Results section and Ref. 24).

Ca^{2+} efflux can be written as dCa_i^T/dt
Eqn. 1 can then be written as

$$v = -d\,Ca_i^T/dt = V_m/(1 + K/Ca_i^T)^n \quad (2)$$

where $K = K'/\alpha$. The V_m, K and n values for each progress curve had to be determined. Values of K, n and V_m for which Eqn. 2 best fits experimental points have been obtained first by taking the natural logarithm of Eqn. 2, which is

$$\log V_m + n \log \frac{Ca_i^T}{Ca_i^T + K} = \log \frac{-dCa_i^T}{dt} \quad (3)$$

The mathematical resolution of Eqn. 3 was obtained by a direct search procedure as described in the Appendix.

The overall program, including data management, fitting, graphic and tabular output, was written in BASIC for an Apple II computer. The program is available upon request from the authors.

After the identification of V_m and paired values of K and n, Eqn. 2 was integrated using a Runge-Kutta procedure [28], with a number of experimental points varying between 8 and 14 and a step size of 1 s for curve fitting. Integration was interrupted at time points that coincided with the observed data set and simulated Ca_i^T tabulated. The relative error was then calculated using the following equation:

$$\text{relative error} = \frac{\sqrt{wi\,(Ca_i^T \text{ simulated} - Ca_i^T \text{ observed})^2}}{\sqrt{wi\,(Ca_i^T \text{ observed})^2}}$$

The calculated K and n parameters reflect the interaction of the Ca^{2+}-calmodulin-pump complex under non steady-state conditions within a range of cellular free Ca^{2+} of 0.33 to 100 μM. The interpretation of these parameters in terms of the different apparent affinity constants and minimum number of binding sites is beyond the scope

of this study. For the purpose of comparison of the behaviour of the Ca^{2+} pump as a function of cellular calcium in normotensive and hypertensive subjects, the calculated pairs of K and n parameters can be converted into one variable, the half-maximal stimulation constant ($K_{Ca^{2+}}$ 0.5). The intracellular Ca^{2+} concentration required for half maximal flux stimulation (K 0.5) can be calculated from Eqn. 2 by replacing v by $V_m/2$ and Ca_i^T by $K_{Ca^{2+}}$ 0.5. Thus

$$K_{Ca}0.5 = \frac{K}{2^{1/n} - 1}$$

The K 0.5 constant reflects therefore all pairs of K and n parameters for which Ca^{2+} efflux can be phenomelogically fitted by Eqn. 2.

Curve fitting could be achieved in all 58 cases with a number of experiment points $\geqslant 8$. The relative error never exceeded 3%. In all these cases the optimization of the paired values of K and n was achieved with the last five or six points of the progress curve, namely when the pump started unsaturating. The stability of the algorithm was tested numerically. K (α_3, see Appendix) was varied within an interval of 1 to 300 with a K step size = 1. In five cases studied, one single minimum was obtained, suggesting the uniformity of the solution. For subsequent cases a step size of 10 was chosen for K. Another mathematical resolution of Eqn. 3 was attempted using the Taylor expansion and relaxation method. It proved to be less powerful in fitting the experimental points. In 31 out of 58 curves where convergence was attained it gave similar K_{Ca} 0.5 values to the approximation method. In the remaining 27 cases either convergence was never attained or the values obtained were completely off key.

Results

The ionophore A23187 is known to induce a tightly coupled exchange of Ca^{2+} or Mg^{2+} for H^+ [29]. In red blood cells, however, shifts in the H^+ gradient are rapidly restored to normal due to the operation of the Jacobs-Stewart cycle in parallel with the efficient anion exchange pathway [30]. The addition of A23187 to a red blood cell suspension allows free Ca^{2+} distribution across the cell membrane to reach electrochemical equilibrium if the Ca^{2+} extrusion pump is inhibited or swamped by ionophore-induced fluxes [24].

Fig. 1 shows the increase in ^{45}Ca content of Wistar-Kyoto rat red blood cells after successive addition of A23187 to the external medium, followed by the addition of excess EGTA. It can be seen that after addition of 10 μM A23187 Ca^{2+} distribution across the cell membrane reaches a quasi-equilibrium state within 1–2 min. Indeed, further addition of ionophore does not modify cell ^{45}Ca content, thus suggesting that ionophore-induced leak swamps the Ca^{2+} pump fluxes. Under these conditions the steady state is indistinguishable from equilibrium conditions and free Ca^{2+} content could be estimated from external Ca^{2+} concentrations as discussed in the following section.

Relationship between cellular calcium and external calcium

Fig. 2 depicts the relationship between total cell calcium (Ca_i^{st}) and external calcium (Ca_o^{st}) at the steady state for Wistar-Kyoto red blood cells incubated with A23187. The data could be fitted with a straight line that passes close to the origin (not shown) with a slope m. As first described by Ferreira and Lew [24] the free ionized Ca^{2+} content (Ca_i^{2+}) can be estimated from the fraction of calcium that is ionized (α) and Ca_i at steady state

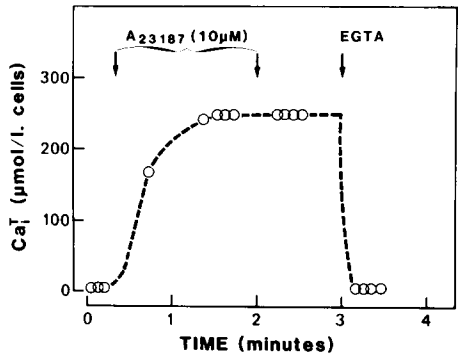

Fig. 1. The effect of two additions of ionophore A23187 (10 μM) on Ca^{2+} content of Wistar-Kyoto rat red blood cells. Cells were incubated at 10% haematocrit in Na^+/K^+ medium (KCl 110 mM) in the presence of 50 μM $CaCl_2$ (10 μM A23187 and 250 μM EGTA were added as indicated). The fraction of ionized calcium was calculated from the equilibrium condition as described in Results. α was about 0.22. One of three different experiments giving similar results is shown.

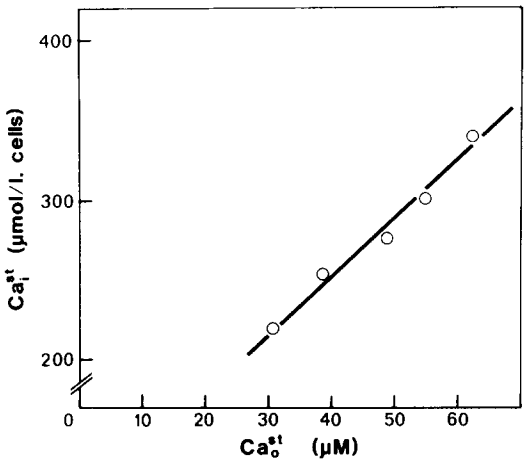

Fig. 2. Total cell Ca^{2+} content (Ca_i^{st}) as a function of external Ca^{2+} (Ca_o^{st}) under steady-state conditions in inosine-fed Wistar-Kyoto red blood cells. Experimental conditions were similar to those described in fig. 1. The calculated slope value (m) was 0.4. Thus, assuming an $r = 1.4$, the α value was calculated to be 0.45. One of four experiments giving similar results is shown.

(Ca_i^{st}) by using the following equations:

$$Ca_i^{2+} = \alpha(Ca_i^{st})$$

where

$$\alpha = r^2/m \text{ and } r = Cl_0^-/Cl_i^- = H_i^+/H_o^+$$

Under our experimental conditions, the measurement of $^{36}Cl^-$ distribution across cell membranes gave a value of $r = 1.40 \pm 0.07$ in Wistar-Kyoto red blood cells, in agreement with previous determinations [31]. This value was not different from that in spontaneously hypertensive rats red blood cells: 1.46 ± 0.08 (mean ± S.D., $n = 3$). Thus, the calculated value of α in Wistar-Kyoto red blood cells 0.34 ± 0.09 (mean ± S.D., $n = 28$) which was not different from that of spontaneously hypertensive red blood cells: 0.33 ± 0.12 (mean ± S.D., $n = 18$).

The effect of Ca^{2+} on cell volume and membrane potential

The addition of A23187 to red blood cells incubated in a plasma-like medium activates a Ca^{2+}-sensitive K^+ channel which leads to a masive K^+ loss, membrane hyperpolarization and cell shrinkage [33]. Similarly, in the presence of ionophore, the Mg^{2+} in the system would equilibrate across the cell membrane. These net cation movements can be prevented by keeping external K^+ and Mg^{2+} concentrations close to their chemical equilibrium, as first suggested by Lew and Ferreira [32].

Under our experimental conditions, rat red blood cell volume, Mg^{2+} content and $^{36}Cl_o^-/^{36}Cl_i^-$ ratio were not different before and after addition of A23187 + Ca_0. Similar results were obtained with human red blood cells, in agreement with previous observations [22,24,33].

The effect of cobalt chloride on Ca^{2+} efflux

Fig. 3 shows the effect of increasing cobalt concentrations on the Ca^{2+} efflux from Wistar-Kyoto red blood cells loaded with Ca^{2+}.

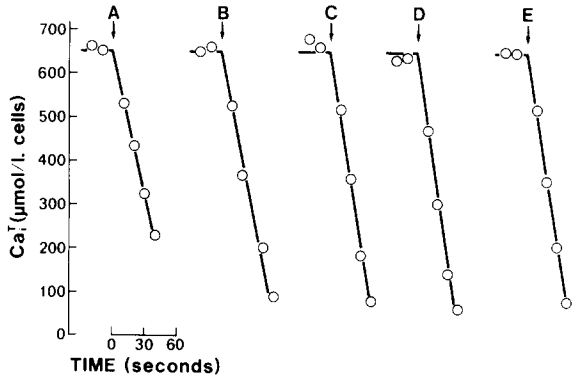

Fig. 3. Total calcium content as a function of time in inosine-fed Wistar-Kyoto red blood cells. Cells were incubated at 10% haematocrit in Na^+/K^+ medium (KCl 110 mM) in the presence of 75 μM $CaCl_2$. 10 μM A23187 was added at time zero. Transmembrane ^{45}Ca tracer distribution reached equilibrium within 2 min. Thereafter, as indicated by arrows in the figure, equal volumes of the cell suspension were distributed to separate vials incubated at 37 °C and each containing a different concentration of $CoCl_2$. A, 25; B, 100; C, 250; D, 500; E, 1000 μM. Aliquots of 50 μl were then added at regular 10 s intervals to Eppendorf tubes containing ice-cold stopping medium and processed as described in Materials and Methods. Ca^{2+} efflux was obtained by linear regression analysis of cell calcium content within the first minute after $CoCl_2$ addition. The calculated values ± S.D. were, for vial A, 38.7 ± 2; B, 53.7 ± 1.7; C, 57.5 ± 1.9; D, 58.9 ± 0.6; E, 53.0 ± 1.9 mmol/l cells per h. Tailing off cell calcium content 8 min after $CoCl_2$ addition showed similar values to that prior to A23187 addition. At equilibrium, the α value was 0.11. Similar results were obtained in two other experiments with 50 μM external calcium.

At cobalt concentrations in excess of external calcium concentation, Ca^{2+} efflux becomes maximal and is not affected by cobalt. This is in agreement with results previously obtained for human red blood cells (unpublished results). The ionophore-generated Ca^{2+} influx is estimated to be superior to 80 mmol/l cell per h. These fluxes far exceed the maximal capacity of the Ca^{2+} pump and secure rapid Ca^{2+} equilibrium.

The reproducibility of the method was tested by assessing Ca^{2+} efflux from the same rat red blood cell suspension distributed into five parallel conditions. Independent Ca^{2+} efflux determinations agreed within a range of 10%. Similar results were previously obtained in human red blood cells [23].

The effect of vanadate on Ca^{2+} efflux

Red blood cells were incubated for 30 min at 37°C in the presence of 0.5 mM metavanadate [34] prior to A23187 addition. As shown in Fig. 4, when excess $CoCl_2$ (400 μM) was added under steady-state conditions, Ca^{2+} efflux was completely inhibited by $VO_4^{=}$ and cell calcium content remained at levels comparable to steady-state conditions.

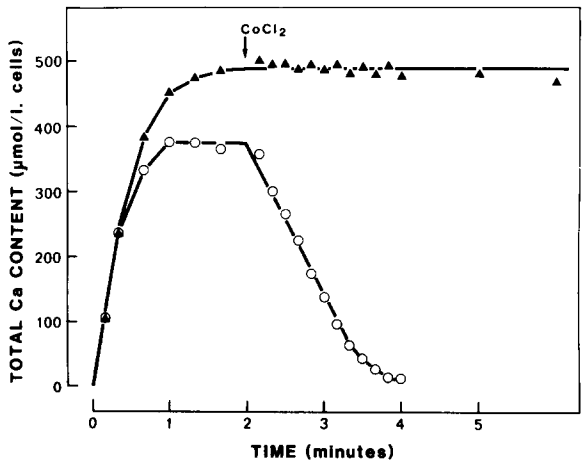

Fig. 4. Total calcium content as a function of time in human red blood cells in the absence ○ and presence ▲ of metavanadate. Inosine-fed fresh cells were incubated at 37°C at 10% haematocrit in Na^+/K^+ medium (KCl 85 mM). 0.5 mM metavanadate was added to an aliquot of the suspension (▲) 30 min prior to A23187 addition. $CoCl_2$ (400 μM) was then added under steady-state conditions to both conditions. The α value was 0.33 for control cells and 0.22 for metavanadate. Ca^{2+} efflux in control cells was 15.00 ± 0.39 mmol/l cells.

Ca^{2+}-pump-mediated efflux in Wistar-Kyoto and spontaneously hypertensive rats

A typical experiment assessing Ca^{2+} efflux mediated by the Ca^{2+} pump in spontaneously hypertensive rats and Wistar-Kyoto red blood cells is shown in Fig. 5. A23187 (10 μM) was added at time zero. ^{45}Ca content of cells increases rapidly to reach a steady state. The ionophore-induced Ca^{2+} influx was estimated to be superior to 60 mmol/l cells per h. As discussed above (Fig. 1), these fluxes far exceed the maximal velocity of the Ca^{2+} pump, and Ca^{2+} distribution at steady state is indistinguishable from equilibrium conditions. Under these conditions, the inhibition of iono-

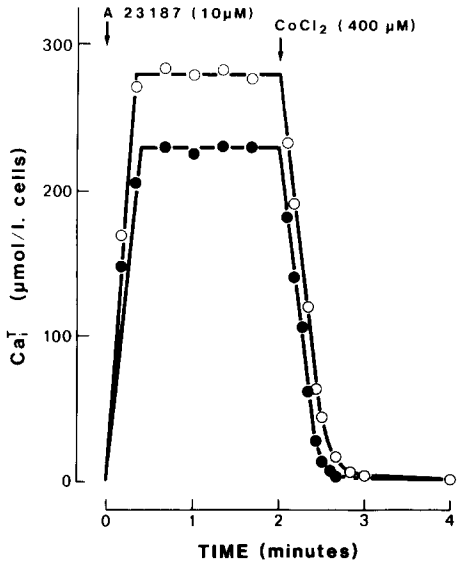

Fig. 5. Total calcium content as a function of time in inosine-fed red blood cells from Wistar-Kyoto (○) and spontaneously hypertensive rats (●) cells were incubated at 37°C at 10% haematocrit in Na^+/K^+ medium (KCl 110 mM). A23187 (10 μM) was added at time zero. Under steady-state conditions 0.4 mM $CoCl_2$ was added. Samples were then taken each 5 or 10 s and added to ice-cold stopper solution as described in Materials and Methods. The α value at equilibrium was estimated to be 0.17 for Wistar-Kyoto and 0.28 for spontaneously hypertensive rat red blood cells. Pump-mediated Ca^{2+} efflux was calculated by linear regression analysis of Ca^{2+} content as a function of time after $CoCl_2$ addition and was found to be 30.2 ± 1.0 mmol/l cells per h for Wistar-Kyoto and 29.6 ± 0.9 mmol/l cells per h for spontaneously hypertensive rats.

phore-mediated fluxes by addition of $CoCl_2$ would expose Ca^{2+} extrusion by the Ca^{2+} pump at its maximal activity. As shown 400 μM $CoCl_2$ were added to the cell suspension and samples were taken at 5 or 10 s time intervals. The fall in Ca^{2+} content was linear with time until at least 70–80% of it had been extruded. The tailing off was followed for up to 8 min, and cell Ca^{2+} content reached levels comparable to those before ionophore addition. This is a further argument suggesting a complete inhibition by $CoCl_2$ of Ca^{2+} influx through A23187. The Ca^{2+} efflux $\pm$ S.D. was calculated by linear regression analysis of Ca^{2+} content as a function of time.

No difference could be observed in the maximal Ca^{2+} efflux from red blood cells of spontaneously hypertensive rats and Wistar-Kyoto rats. This result was confirmed in a study of 18 spontaneously hypertensive rats and 28 Wistar-Kyoto rats (Fig. 4, Table I). There is a large variation in Ca^{2+} efflux from rat red cells (4–50 mmol/l cells per h) which was not correlated to either blood pressure or age and cannot be attributed to methodological parameters, since experimental reproducibility showed a maximal variation of 10%.

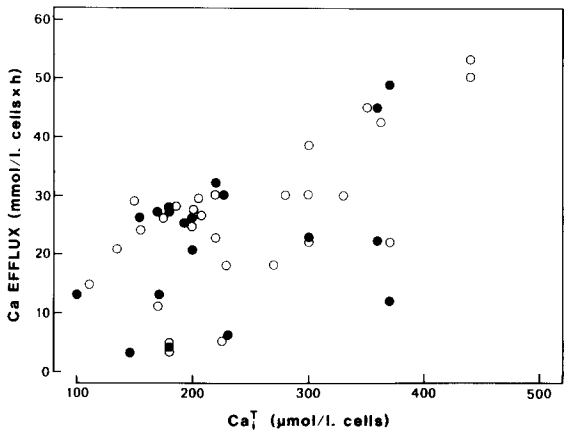

Fig. 6. Ca^{2+} efflux mediated by the Ca^{2+} pump in rat red blood cells as a function of cell Ca^{2+} content (Ca_i^T) at steady state. Open symbols (○) represent Wistar-Kyoto and filled symbols (●) represent spontaneously hypertensive rats. Assuming an average α value of 0.3, free calcium content of cells was estimated to range from 30 to 135 μM. Mean $\pm$ S.D. of Ca^{2+} efflux was 25.1 $\pm$ 12.1 mmol/l cells per h for Wistar-Kyoto and 30.0 $\pm$ 12.9 mmol/l cells per h for spontaneously hypertensive rats.

Ca^{2+}-pump-mediated efflux in human essential hypertension

The appropriate experimental conditions for the use of cobalt to assess Ca^{2+} efflux in human red blood cells are to be described elsewhere [23]. In brief, at $CoCl_2$ concentrations in excess of external calcium concentrations, Ca^{2+} efflux becomes maximal and is not affected by cobalt. On the other hand, reproducibility experiments showed a maximal variation of 8% in independent Ca^{2+} efflux determinations assessed in parallel conditions from the same cell suspension.

As shown in Fig. 5, maximal Ca^{2+} efflux measured in 18 essential hypertensive patients was not different from that of 18 normotensive controls. The variation in Ca^{2+} efflux from 8 to 30 mmol/l cells per h was not correlated to either age or blood pressure.

TABLE I

KINETIC PARAMETERS OF THE Ca^{2+} PUMP

Maximal efflux values (V_{max}) are expressed in units of mmol/l cells per h and half maximal stimulation constant values (K 0.5) in μM of free Ca^{2+}. n represents the number of cases. Statistical significance was analysed using Student's t-test.

Normotensive controls			Essential hypertensives		Wistar-Kyoto		Spontaneously hypertensives	
V_{max} (mmol/l cells per h)		K 0.5 (μM)	V_{max}	K 0.5	V_{max}	K 0.5	V_{max}	K 0.5
Range	11.7–24.3	7.7–36.5	7.8–32.2	7.4–39.2	4.3–53.7	5.3–20.6	4.0–49.7	5.2–21.5
n	17	14	18	15	28	15	18	14
Mean $\pm$ S.D.	16.9 $\pm$ 3.1	16.2 $\pm$ 7.9 *	15.6 $\pm$ 6.6	21.0 $\pm$ 11.2 *	25.1 $\pm$ 12.1	12.1 $\pm$ 4.9	30.0 $\pm$ 12.9	11.7 $\pm$ 5.3

* $P \leqslant 0.1$.

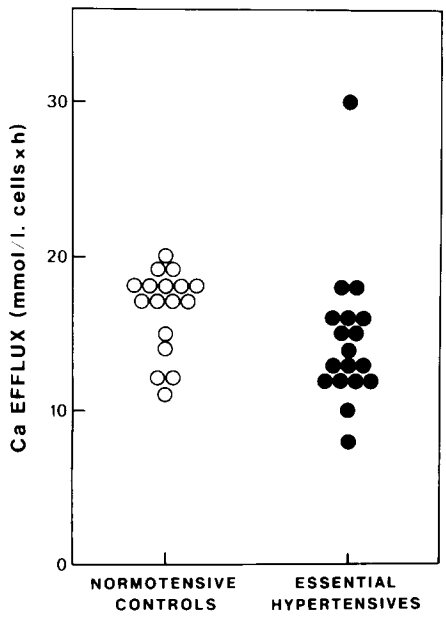

Fig. 7. Scatter diagram of Ca^{2+} pump activity in red blood cells from normotensive (○) and essential hypertensive human subjects (●). Ca^{2+} efflux was assessed under steady-state conditions. Total cell Ca^{2+} concentration varied from 200 to 300 μmol/l cells corresponding to a free calcium concentration of 60 to 90 μM with an average α of 0.3. The mean ± S.D. of Ca^{2+} pump was 16.9 ± 3.1 mmol/l cells per h for control and 15.6 ± 6.6 mmol/l cells per h for essential hypertensives.

Ca^{2+} concentration for half-maximal stimulation of the Ca^{2+} pump in essential hypertension

Results of the analysis of the Ca^{2+} concentration for half-maximal stimulation of the Ca^{2+} pump ($K_{Ca^{2+}}$ 0.5) from human and rat red blood cells is shown in Table I.

Values obtained in normotensive controls were not significantly different from those in essential hypertensives; similarly, no difference in K_{Ca} 0.5 values could be observed between spontaneously hypertensive rats and the Wistar-Kyoto rat strain.

Discussion

The present results describe for the first time the kinetic parameters of Ca^{2+} efflux mediated by the Ca^{2+} pump in intact red blood cells from Kyoto rats and humans with essential hypertension. Ca^{2+} content was increased to near equilibrium conditions by the addition of A23187 ionophore and, unlike a previous study [35] the cell inorganic ion content, volume and membrane potential were kept constant during the flux measurement as previously described for human red blood cells [23,24]. Ca^{2+} efflux was assessed after the addition of $CoCl_2$ to inhibit ionophore-mediated fluxes. Under these conditions Ca^{2+} efflux proceeds from Ca^{2+}-equilibrated cells and represents an extrusion against a concentration gradient which in red blood cells is solely mediated by the Ca^{2+} pump. This is further confirmed by the observation that Ca^{2+} efflux is inhibited by vanadate (the present results) and is sensitive to ATP depletion as previously described by Tiffert et al. [22].

The measurement of the maximal velocity of the Ca^{2+} pump in red blood cells from the Kyoto rat strain did not reveal any significant difference between the hypertensive rat and its control; similarly, no difference in the Ca^{2+} pump maximal velocity could be observed in red blood cells from hypertensive patients as compared to normotensive controls.

These findings are similar to those previously reported in broken membrane preparations. The maximal or absolute activation of the Ca^{2+}-ATPase by calmodulin was found not to be altered in red blood cell membranes from essential hypertensives [18]. Similar results were reported in other cell types such as platelets from hypertensive patients [36], and heart sarcolemmal [37] and basolateral [14] membranes from spontaneously hypertensive rats [14]. In contrast, Postnov et al. [13,17] observed, in presence of a saturating concentration of calmodulin, a decrease in the maximal activity of the Ca^{2+}-ATPase in red blood cell membrane preparations from essential hypertensives [17] and spontaneously hypertensive rats [13]. This discrepancy in the results might be a consequence of differences in membrane isolation procedures used by different groups. A reduction in hypertensive Ca^{2+}-ATPase might be induced or uncovered with the use of one particular technique. Alternatively, an alteration in the maximal Ca^{2+}-ATPase activity might be present in a subgroup of essential hypertensives which, for fortuitous reasons, were not selected in this study. Such a possibility has been previously reported in essential hypertension, where a segregation in al-

tered Na transport pathways have been observed [6,38].

Membrane preparations were also used to investigate the interaction of calmodulin with the Ca^{2+}-ATPase. The percent stimulation of this ATPase by calmodulin was found to be decreased in red blood cells [17] and platelet membranes [36] from essential hypertensives and also in red blood cells [13] and heart sarcolemmal membranes of spontaneously hypertensive rats [37]. These results suggested an altered affinity of calmodulin for the Ca^{2+} pump. Kinetic studies in native or purified Ca^{2+}-ATPase from human red blood cells showed a complex behaviour of the ATPase activity as a function of Ca^{2+}, Mg^{2+}, ATP and calmodulin [20,21]. A Ca^{2+}-induced positive cooperativity was reported in several reports, with a degree of cooperativity being modified by calmodulin and pH. This multiple effector interaction hampers the extrapolation of calculated apparent affinities obtained in isolated enzymes to intact cells.

The kinetic parameters of the Ca^{2+} pump in intact red blood cells have been previously reported. Lew et al. [24,39] postulated an apparent affinity constant of 0.5 to 4 μM with two binding sites. This corresponds to a half-maximal stimulation constant (K_{Ca} 0.5) ranging from 1.2 to 9.8 μM. These values are in agreement with the K_{Ca} 0.5 values reported in this study.

The assessment of the half-maximal stimulation constant (K_{Ca} 0.5) of the Ca^{2+} pump in intact red blood cells did not reveal any significant difference either between normotensive controls and essential hypertensive patients or between Wistar-Kyoto controls and spontaneously hypertensive rats. These results suggest that the overall ability of the red blood cells' Ca^{2+} pump to extrude calcium out of the cell is not altered in hypertension. It is tempting to postulate that the reported impairment in the affinity of the Ca^{2+} pump for calmodulin could be compensated by an increase in the cooperativity index (or number of sites) of the transporter. This would then be reflected by a normal half-maximal stimulation constant. The investigation of such a hypothesis is not possible for the present and should await further development of our understanding of the multiple interactions of the Ca^{2+} pump with its effectors in intact cells.

Acknowledgements

We are indebted to Dr. J.L. Elghozi and J.P. Grünfeld for the careful selection of patients. We wish to thank Dr. C. Galli for her skillful technical assistance and Dr. Y. Cherruault and R.P. Garay for interesting discussions. We are indebted to Dr. V.L. Lew for his helpful comments in the realisation of this study.

Appendix

In this article, the variation in cell calcium content (Ca_i^T) after $CoCl_2$ addition was studied as a function of time. As previously discussed, this decrease in calcium content is mediated by the Ca^{2+} pump, and its activity can be represented by the following equation:

$$v = \frac{V_m}{\left(1 + \frac{K}{C}\right)^n} = -\frac{dc}{dt} \tag{A-1}$$

where V_m is the maximum velocity, K the apparent dissociation constant for the Ca^{2+}-pump complex, n the minimum number of binding sites and C the total cell calcium content (Ca_i^T).

The problem is to identify V_m, K and n values for which Eqn. A-1 best fits the experimental data; these data represent a finite set of points (t_i, C_i), $i = 0, \ldots, N$, ($8 \leqslant N \leqslant 14$) where t_i represents the time and C_i the corresponding total cell calcium content (Ca_i^T).

From Eqn. A-1 we get

$$\log V_m + n \log\left(\frac{C}{C+K}\right) = \log(-\dot{C}) \tag{A-2}$$

with

$$\dot{C} = \frac{dc}{dt}$$

Let

$$\left.\begin{aligned} \alpha_1 &= \log V_m \\ \alpha_2 &= n \\ \alpha_3 &= K \end{aligned}\right\} \alpha = (\alpha_1, \alpha_2, \alpha_3)$$

and

$$F(c, \dot{c}, \alpha) = \alpha_1 + \alpha_2 \log\left(\frac{C}{C+\alpha_3}\right) - \log(-\dot{C})$$

Hence, from Eqn. A-2

$$F(C, \dot{C}, \alpha) = 0 \quad t\epsilon[0, T] \tag{A-3}$$

Let

$$J(\alpha) = \int_O^T \left(F(C, \dot{C}, \alpha)\right)^2 \mathrm{d}t$$

then the solution of Eqn. A-2 is such that

$$J(\alpha) = 0 \tag{A-4}$$

C is known for time steps $t_0, t_1, \ldots, t_N$
$J(\alpha)$ is approximated in two steps:

$$J(\alpha) = \sum_{i=0}^{N} w_i \left(F\left(C_i, \dot{C}_i, \alpha\right)\right)^2$$

with

$$w_0 = (t_i - t_o)/2$$

$$w_i = (t_{i+1} - t_{i-1})/2 \quad i = 1, \ldots, N-1$$

$$w_N = (t_N - t_{N-1})/2$$

and

$$\begin{cases} \dot{C}_0 = (C_1 - C_0)/(t_1 - t_0) \\ \dot{C}_i = (C_{i+1} - C_{i-1})/(t_{i+1} - t_{i-1}) \quad i = 1, \ldots, N-1 \\ \dot{C}_N = (C_N - C_{N-1})/(t_n - t_{N-1}) \end{cases}$$

For any fixed α_3, a best fit for α_1 and α_2 can be computed using the least-square method [28]; the value of α_3 minimising $J(\alpha)$ is computed using a standard descent method.

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