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KINETIC STUDIES ON SODIUM-DEPENDENT CALCIUM UPTAKE BY MYOCARDIAL CELLS AND NEUROBLASTOMA CELLS IN CULTURE

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Summary

Kinetic analyses were made on intracellular Na^+-dependent Ca^{2+} uptake by myocardial cells and neuroblastoma cells (N-18 strain) in culture. Cells loaded with various concentrations of Na^+ could be prepared by incubating them in Ca^{2+}-free medium containing various concentrations of Na^+. Cells pre-loaded with various concentrations of Na^+ were incubated in medium containing Ca^{2+} and ^{45}Ca. The resulting ^{45}Ca uptake by the two types of cell depended greatly on the initial intracellular concentrations of Na^+. Lineweaver-Burk plots of the initial rate of Ca^{2+} uptake against the external concentration of Ca^{2+} fitted well to straight lines obtained by linear regression ($r > 0.95$). This result shows that Ca^{2+} uptake by the two types of cell was achieved by a carrier-mediated transport system. This Na^+-dependent Ca^{2+} uptake was accompanied by Na^+ release and the ratio of Na^+ release to Ca^{2+} uptake was close to 3 : 1. A comparison of the kinetic data between myocardial cells and N-18 cells suggested that N-18 cells possess a carrier showing the same properties as that of myocardial cells, i.e.: (1) a similar dependency on the intracellular concentration of Na^+; (2) the coincidence of the apparent Michaelis constants for Ca^{2+} (0.1 mM); (3) the similarities of the K_i values for Co^{2+}, Sr^{2+} and Mg^{2+} ($Co^{2+} < Sr^{2+} < Mg^{2+}$) and (4) a similar dependency on pH. However, the maximal initial rate, V, of N-18 cells was about 1/100 that of myocardial cells. The rate of Na^+-dependent Ca^{2+} uptake by non-excitable cells was much lower than that by myocardial cells.

Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid; Bes, 2-[bis(2 hydroxyethyl)amino]ethanesulfonic acid.

Introduction

Na^+-Ca^{2+} exchange is a transport system across the cell membrane which includes both Ca^{2+} efflux coupled with Na^+ influx and Ca^{2+} influx coupled with Na^+ efflux. Baker et al. [1,2] first demonstrated this Na^+-Ca^{2+} exchange mechanism in crab nerve and squid axon. The existence of this Na^+-Ca^{2+} exchange mechanism in cardiac muscle was first demonstrated by Reuter and Seitz [3] and Glitsch et al. [4]. They also suggested that the Na^+ gradient across the cell membrane is a major energy source for Ca^{2+} extrusion, and that an Na^+-Ca^{2+} exchange system is important in determining the intracellular concentration of free Ca^{2+} [5]. These studies of Na^+-Ca^{2+} exchange were performed by measurements of mechanical responses [6,7] or tracer exchange [8,9] using intact muscle preparations. However, studies on Na^+-Ca^{2+} exchange using intact heart muscle have been complicated by the existence of an intricate extracellular space and other tissues surrounding the myocardial cells, such as nerves and blood vessels. In particular, with regard to measurement of the rate of Ca^{2+} influx, difficulties have been encountered in rapidly replacing and rapidly washing out the external medium.

Single isolated myocardial cells obtained from mammalian or chicken heart are known to beat spontaneously in culture [10—12]. Goshima et al. [13] have reported that pre-incubation of myocardial cells in Ca^{2+}-free medium containing a sufficient concentration of Na^+ causes an increase in the intracellular concentration of Na^+ in the cells, and that subsequent incubation in Ca^{2+}-containing medium causes excess uptake of Ca^{2+} by the cells. These results suggest that there is an Na^+-Ca^{2+} exchange system in cultured myocardial cells. Cultured myocardial cells are separated anatomically and functionally from nerves, connective tissue and blood vessels, and moreover they are in direct contact with the extracellular medium. Therefore, their use should greatly simplify studies on the direct effects of ions and on Na^+-Ca^{2+} exchange.

The purpose of the present work were: (1) to determine the effects of divalent cations, pH and the intracellular concentration of Na^+ on kinetic properties of Na^+-dependent Ca^{2+} uptake; and (2) to determine whether Na^+-dependent Ca^{2+} uptake also occurs in neuroblastoma cells (N-18 strain) and non-excitable cells, and if so, whether the properties of the Na^+-dependent Ca^{2+} uptake by non-myocardial cells, such as the affinities of various divalent cations for the carrier and the pH dependency, are the same as those of myocardial cells.

Materials and Methods

1. Reagents. Reagents were obtained from the following sources: fetal bovine serum from Flow Laboratories; BES (one of the Good's buffers) and EGTA from Dojindo Co.; ouabain from Sigma Chemical Co.; $^{45}CaCl_2$, $^{85}SrCl_2$, $^{58}CoCl_2$ and [^{14}C]inulin from New England Nuclear; $^{24}NaCl$ from Japan Radioisotope Association.

2. Culture of mouse heart cells, N-18 cells and other strain cells. Mouse heart ventricles were removed from 13- to 17-day-old fetuses (ddY strain) and dissociated into single isolated cells by trypsinization as described previously [14]. Cells were seeded into 35- or 60-mm diameter petri dishes (Falcon) at a den-

sity of $2 \cdot 10^5$—$4 \cdot 10^6$ cells per dish at 37°C in Eagle's minimum essential medium supplemented with 8% fetal bovine serum under a water-saturated atmosphere of 5% CO_2 in air. On incubation for 10 h, the single heart cells became attached to the surface of the petri dishes. Two different cell types, myocardial cells and fibroblast-like cells, could be easily distinguished under a phase-contrast microscope [10]. The ratio of myocardial cells to fibroblast-like cells was 4 : 1. About 80% of the single isolated myocardial cells beat spontaneously. All experiments were performed at 25 ± 1°C on myocardial cells after cultivation for 16—22 h.

Culture of the cells from mouse lung and skin was carried out similarly.

N-18 cells (a cell line from a mouse neuroblastoma) were kindly supplied by Dr. T. Amano (Institute of Life Science, Mitsubishi-Kasei). N-18 cells were maintained as monolayers in flasks (Falcon) containing culture medium consisting of 92% Eagle's minimum essential medium and 8% fetal bovine serum. The cells were incubated at 37°C in a water-saturated atmosphere of 5% CO_2 in air. Subculture was performed with 0.25% trypsin after the cultures had reached confluence. For the experiment, cells were seeded into 35- or 60-mm diameter petri dishes (Falcon) at a density of $4 \cdot 10^5$—$1 \cdot 10^6$ cells per dish, and incubated in 2—5 ml of culture medium. For all experiments, cells growing attached to the surface of petri dishes were used after cultivation for 3—5 days. B-16 mouse melanoma cells, FL cells (derived from human amnion) and L cells (derived from mouse connective tissue) were also cultured in a similar way.

3. Measurement of intracellular concentrations of Na^+ and K^+. Myocardial cells cultured in a petri dish were detached from the surface of the dish with rubber policemen. After sedimentation, the cell pellet was suspended in 1 ml of EGTA(Li)-medium * (see Table I) containing [^{14}C]methoxyinulin (0.15 μCi/ml = 5.6 kBq/ml) and unlabeled inulin (0.5 mg/ml). The cell suspension was transferred to a small tip. After centrifugation at 3000 rev./min for 5 min, the ^{14}C radioactivity in 20 μl of the supernatant was measured. Then the cell pellet was transferred to a glass tube through the tip cut, and the wet weight of the pellet was measured. Subsequently, the pellet was suspended in 1 ml of 0.1 M NaOH and the total ^{14}C radioactivity was measured. The extracellular space was calculated as the degree of dilution of the radioactivity in the supernatant to that in the cell pellet. The extracellular space of the pellet of myocardial cells was estimated to be 46 ± 13% of the total wet weight (mean ± S.D., n = 6). Myocardial cells were also detached from another dish and after sedimentation the wet weight and dry weight of the cell pellet were measured. The dry weight was estimated to be 10% of the total wet weight. The intracellular water content of the cells was then calculated as the wet weight minus the dry weight and the weight of the extracellular space.

The wet weight of another sample of detached myocardial cells was measured, then the cells were suspended in 0.1 M NaOH and an aliquot was used for measurement of the protein content by using the method of Lowry et al. [15]. From the results it was estimated that 1 mg of protein was equivalent to 5.1 mg of intracellular water.

For measurement of the intracellular concentrations of Na^+ and K^+, myocar-

* Composition: 140 mM LiCl, 0.1 mM EGTA and 10 mM Bes/Tris (pH 7.4).

dial cells were seeded into a series of 60-mm petri dishes at a constant density of $2 \cdot 10^6$—$4 \cdot 10^6$ per dish. The cells in one dish were directly suspended in 3 ml of 0.1 M NaOH and used for measurement of the protein content. The cells in other dishes were incubated in EGTA (choline-Na) medium (for compositions of reaction media, see Table I), containing various concentrations of Na^+, for 10 min. Subsequently, the cells were quickly washed four times with EGTA (140 mM Li) medium at 4°C to remove the Na^+ and K^+ in the extracellular medium, and then directly solubilized in 0.3 ml of HNO_3 solution. The Na^+ and K^+ contents of the solution were measured using a flame photometer (Hitachi, 170-30 type) as described by McDonald and DeHaan [18]. Subsequently, the intracellular concentrations of Na^+ and K^+ were first calculated as mmol per mg of protein and then as mmol per kg of cell water by assuming that 1 mg protein is equivalent to 5.1 mg of cell water. The intracellular concentrations of Na^+ and K^+ of strain cells, such as N-18 cells, were determined in a similar way.

4. Measurement of ^{45}Ca uptake. The rate of ^{45}Ca uptake by cultured myocardial cells or N-18 cells was measured as described previously [19,20]. The culture medium was removed, and the cells were washed three times with EGTA (choline-Na) medium and allowed to equilibrate with the same medium for 10 min. Then the medium was rapidly replaced by 1—2 ml of various Ca^{2+} media (Table I) supplemented with ^{45}Ca (3 μCi/ml). Uptake of radioactivity was determined by removing the radioactive medium after incubation for 5 s to 5 min, and washing the cells four times with a total of 10 ml of washing medium, consisting of 135 mM NaCl, 5 mM glucose, 1 mM $CaCl_2$ and 5 mM phosphate buffer (pH 7.4). This washing procedure took 15 s and was sufficient to remove the extracellular ^{45}Ca [19]. Almost all cells were adhering to the bottom of the petri dish throughout the experiments. The washed cells were suspended in 1 ml of 0.1 M NaOH and the radioactivity of an aliquot of the NaOH solution was measured in a liquid scintillation spectrometer (Aloka). Another aliquot of the NaOH solution was used for measurement of the protein content according to the method of Lowry et al. [15].

5. Measurement of ^{58}Co uptake and ^{85}Sr uptake. The cells were washed three times with EGTA (choline-Na) medium and incubated in the same medium for 10 min. Then the medium was rapidly replaced by 1—2 ml of Co^{2+} (choline) medium or Sr^{2+} (choline) medium supplemented with ^{58}Co or ^{85}Sr. Uptake of radioactivity was determined as described above for measurement of ^{45}Ca uptake, using a well-type gamma spectrometer.

6. Measurement of Na^+-Ca^{2+} exchange. For measurement of ^{24}Na release coupled with ^{45}Ca uptake, the myocardial cells were incubated in EGTA (choline-Na) medium containing 35 mM Na^+ and ^{24}Na (10 μCi/ml = 370 kBq/ml). The intracellular ^{24}Na activity reached a steady-state level by incubation for 5 min. After incubation in the EGTA (choline-Na) medium for 10 min, the medium was replaced by 1 ml of a reaction medium containing 280 mM sucrose, 5 mM Bes, 1 mM Ca^{2+} and ^{45}Ca (3 μCi/ml). After incubation in the reaction medium for 0—2 min, the cells were washed four times with a total of 10 ml of washing solution consisting of 280 mM sucrose and 10 mM $CoCl_2$. This washing medium did not cause a significant loss of the intracellular ^{24}Na. The values of ^{45}Ca uptake obtained by this washing procedure were exactly the same as those obtained by the washing procedure described in section 4. Radio-

TABLE I

COMPOSITIONS OF REACTION MEDIA

All media contained 5.5 mM glucose and were buffered at pH 7.4 with 10 mM Bes and NaOH, KOH or Tris. The final concentration of free Ca^{2+} or other divalent cations was adjusted with a cation-EGTA buffer system. For example, a value of $10^{7.48}$ was adopted as the apparent binding constant of the Ca-EGTA complex at pH 7.4 [16,17]. Compositions are expressed in mM.

Medium	Na^+	K^+	Li^+	Choline$^+$	Ca^{2+}	Co^{2+}, Sr^{2+} or Mg^{2+}	Cl^-	EGTA
Ca^{2+} (Na-K)	135	5	0	0	1	0	135	0
EGTA (choline-Na)	0—140	0	0	0—135	0	0	133—135	0.1
Ca^{2+} (choline)	0	0	0	135	0.01—10.0	0	133—155	0.1
Ca^{2+} (choline-Na)	0—140	0	0	0—135	0.03—1.0	0	133—137	0.1
Ca^{2+} (choline-K)	0	0—140	0	0—135	0.03—1.0	0	135—137	0.1
Ca^{2+} (choline-Li)	0	0	0—140	0—135	0.03—1.0	0	135—142	0.1
Co^{2+} (choline) Sr^{2+} (choline)	0	0	0	135	0.03—1.0	0.03—1.0	135—137	0.1
Ca^{2+}, Co^{2+} (choline) Ca^{2+}, Sr^{2+} (choline) Ca^{2+}, Mg^{2+} (choline)	0	0	0	135	0.01—10.0	0.1—3.0	136—161	0.1

activity of ^{45}Ca uptake was measured 1 week later after the ^{24}Na activity was measured, because almost all radioactivity of ^{24}Na decayed 1 week later.

Results

Alteration of the intracellular concentration of Na^+ by incubation in Ca^{2+}-free medium

For the results shown in Fig. 1, myocardial cells were incubated at 25°C in EGTA (choline-Na) medium containing various concentrations of Na^+ for 10 min, and then their intracellular concentrations of Na^+ and K^+ were measured. The intracellular concentration of Na^+ in myocardial cells increased with increase in the concentration of Na^+ in the EGTA (choline-Na) medium. The intracellular concentration of K^+ in the myocardial cells remained constant, irrespective of the concentration of Na^+ in the EGTA (choline-Na) medium. The increase in intracellular Na^+ concentration was not so marked in N-18 cells as in myocardial cells on incubation in Ca^{2+}-free medium: even when N-18 cells were incubated in EGTA (choline-140 mM Na^+) medium, the intracellular concentration of Na^+ increased to only 37 mM.

Thus, cells containing a suitable concentration of intracellular Na^+ could be prepared by incubating the cells in EGTA (choline-Na) medium containing various concentrations of Na^+. In myocardial cells it was possible to alter the intracellular concentration of Na^+ over a wide range of about 0—140 mM, while in N-18 cells it could be altered from 10 to 37 mM.

Effect of intracellular Na^+ concentration on Ca^{2+} uptake

The effect of the intracellular Na^+ concentration on Ca^{2+} uptake was examined. Fig. 2a shows the time course of Ca^{2+} uptake by myocardial cells in Ca^{2+} (choline) medium containing 0.1 mM Ca^{2+} and ^{45}Ca. Ca^{2+} uptake increased to

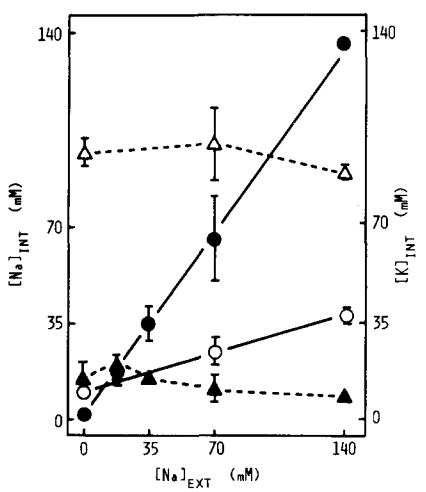

Fig. 1. Effect of Na^+ concentration (abscissa) in EGTA (choline-Na) medium containing 0—140 mM Na^+ on the intracellular concentrations of Na^+ (●) and K^+ (▲) in myocardial cells, and of Na^+ (○) and K^+ (△) in N-18 cells. The cells were incubated in EGTA (choline-Na) medium for 10 min at 25°C. Points and bars indicate means ± S.D. of values in three experiments.

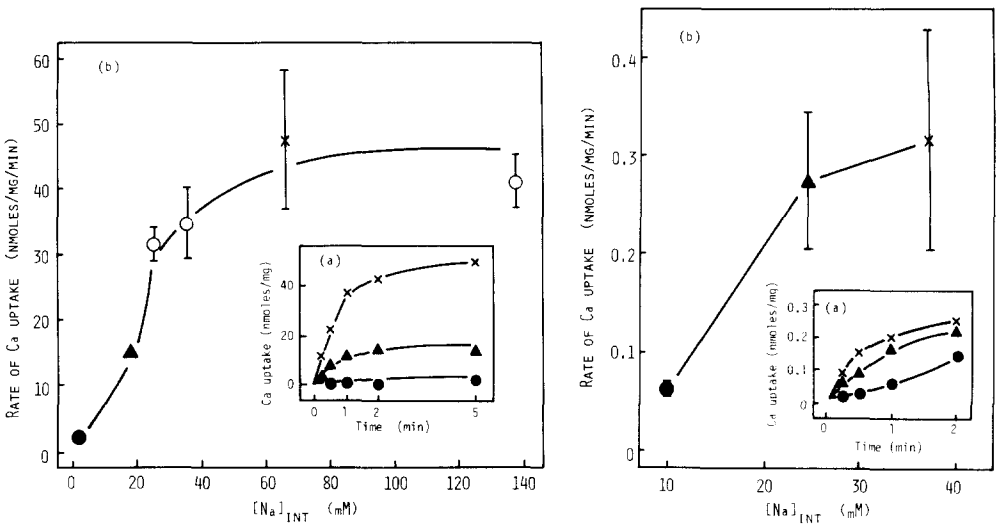

Fig. 2. Effect of the intracellular concentration of Na^+ on Ca^{2+} uptake by myocardial cells after pre-incubation in Ca^{2+}-free medium. Myocardial cells were pre-incubated in EGTA (choline-Na) medium containing 0—140 mM Na^+ for 10 min and then incubated in Ca^{2+} (choline) medium containing ^{45}Ca and 0.1 mM free Ca^{2+}. a and b represent the time course of Ca^{2+} uptake and the initial rate of Ca^{2+} uptake (in the first 15 s), respectively. Symbols in a correspond with those in b. Points and bars in b indicate means ± S.D. of values in three experiments.

Fig. 3. Effect of the intracellular concentration of Na^+ on Ca^{2+} uptake by N-18 cells after pre-incubation in Ca^{2+}-free medium. N-18 cells were pre-incubated in EGTA (choline-Na) medium containing 0 (●), 70 (▲) or 140 mM Na^+ (X) for 10 min and then incubated in Ca^{2+} (choline) medium containing ^{45}Ca and 0.1 mM free Ca^{2+}. a and b represent the time course of Ca^{2+} uptake and the initial rate of Ca^{2+} uptake (in the first 15 s), respectively. Points and bars in b indicate means ± S.D. of values in three experiments.

a saturation level with time, but was linear during the first 30 s. Thus, Ca^{2+} uptake in the first 15 s is equivalent to the initial rate. Fig. 2a shows that Ca^{2+} uptake by myocardial cells increases with increase in the intracellular concentration of Na^+: when the initial rate of Ca^{2+} uptake was plotted as a function of the intracellular concentration of Na^+, a hyperbolic saturation curve was obtained as shown in Fig. 2b. The intracellular concentration of Na^+ for the half-maximal initial rate was estimated to be about 25 mM from Fig. 2b.

The Ca^{2+} uptake by N-18 cells was also markedly stimulated by an increase in the intracellular concentration of Na^+. This indicates the existence of intracellular Na^+-dependent Ca^{2+} uptake by N-18 cells. We could not determine the affinity for the intracellular concentration of Na^+ exactly because we could not increase the intracellular concentration of Na^+ in N-18 cells to more than 37 mM, but the curve of the initial rate of Ca^{2+} uptake showed a tendency toward saturation with increase of the intracellular concentration of Na^+ to 25 mM or more (Fig. 3).

Carrier-mediated Ca^{2+} uptake

We examined the effect of the Ca^{2+} concentration on the initial rate of Ca^{2+} uptake (Fig. 4). Lineweaver-Burk plots of the experimental data fitted well a straight line obtained by linear regression (r = 0.979) and showed saturation

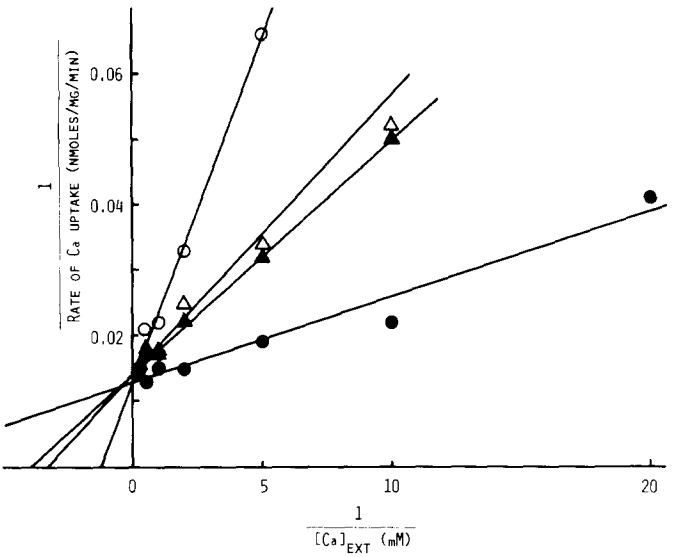

Fig. 4. Lineweaver-Burk plots of the initial rate of Ca^{2+} uptake (in the first 15 s) by myocardial cells against the external concentration of Ca^{2+}. The rate of Ca^{2+} uptake was measured using myocardial cells loaded with 37 mM Na^+, in the absence (●) and presence of 0.3 mM free Sr^{2+} (▲), 1 mM free Mg^{2+} (△) or 0.1 mM free Co^{2+} (○). Straight lines were obtained by linear regression. Correlation coefficients (r) from the data in the absence and presence of Sr^{2+}, Mg^{2+} or Co^{2+} were 0.979, 0.997, 0.996 and 0.997, respectively.

kinetics. This result suggests that Ca^{2+} uptake by myocardial cells is achieved by a transport system involving a carrier. The apparent Michaelis constant (K_m) and the maximal initial rate (V) were obtained from the x-(substrate)-axis intercept and y-(velocity)-axis intercept on the Lineweaver-Burk plot, respectively. The correlation coefficients (r) were 0.95 or more, on Lineweaver-Burk plots of all experimental data examined in the present work.

Table II gives a list of the apparent Michaelis constants and maximal initial rates. An increase in the intracellular concentration of Na^+ in myocardial cells from 10 to 37 mM caused a significant increase in the maximal initial rate without change of the apparent Michaelis constant. On the other hand, in the range of intracellular concentrations of Na^+ of 37—136 mM, the maximal initial rate of Ca^{2+} uptake became saturated. These results suggest that an increase in the intracellular concentration of Na^+ can induce more carriers to bind external Ca^{2+} and to move from outside to inside, instead of changing the affinity of Ca^{2+} for the carrier at the outer surface of the membrane.

Table II also shows that the K_m values of myocardial cells and N-18 cells at the same intracellular concentration of Na^+, 37 mM, are almost equal. This suggests that N-18 cells possess a carrier with the same affinity for extracellular Ca^{2+} as that of myocardial cells. But the maximal initial rate, V, of N-18 cells was about 1/100 of that of myocardial cells.

Ca^{2+} uptake was markedly inhibited by divalent cations such as Co^{2+}, Sr^{2+} or Mg^{2+}. Fig. 4 shows that these divalent cations competitively inhibit Ca^{2+} uptake by myocardial cells. We also observed that Ca^{2+} uptake by N-18 cells was competitively inhibited by these divalent cations (data not shown). We also

TABLE II

K_m AND V VALUES FOR Ca^{2+} UPTAKE BY MYOCARDIAL CELLS AND N-18 CELLS AT VARIOUS INTRACELLULAR Na^+ CONCENTRATIONS

Values are means ± S.D. for three to five independent experiments.

$[Na^+]_{int}$ (mM)	Myocardial cells		N-18 cells	
	K_m (mM)	V (nmol/mg per min)	K_m (mM)	V (nmol/mg per min)
10	0.12 ± 0.01	13 ± 1.4	—	—
17	0.10 ± 0.04	28 ± 1.2	—	—
35	0.09 ± 0.05	80 ± 17.3	—	—
37	0.11 ± 0.02	77 ± 4.0	0.09 ± 0.02	0.6 ± 0.2
65	0.09 ± 0.02	103 ± 5.3	—	—
136	0.11 ± 0.02	100 ± 13.1	—	—

examined the effect of monovalent cations such as Na^+, K^+ and Li^+ on Ca^{2+} uptake by myocardial cells and observed that Na^+ competitively inhibited Ca^{2+} uptake, while K^+ and Li^+ had no effect (data not shown).

Table III gives a list of the dissociation constants, K_i, of various cations. A change in the intracellular concentration of Na^+ from 37 to 65 mM did not affect these K_i values. The K_i values of these cations increased in the following order: $Co^{2+} < Sr^{2+} < Mg^{2+}$. The K_i value for extracellular Na^+ was estimated to be 30 mM, as shown in Table III. This value was almost equivalent to K_m for intracellular Na^+ (25 mM). This result suggests that the affinity for Na^+ is equal on both sides of the membrane. When N-18 cells were pre-loaded with the same concentration of Na^+ (37 mM) as myocardial cells, the K_i values of these cations roughly agreed with those for myocardial cells (Table III). This coincidence indicates that N-18 cells possess a carrier showing the same affinity for these cations as the carrier of myocardial cells. Next, we examined whether Sr^{2+} and Co^{2+} are taken up by the cells in the same way as Ca^{2+}. Uptake of Sr^{2+} was accelerated when the cells were pre-loaded with 65 mM Na^+, but not with zero Na^+. On the other hand, accelerated uptake of Co^{2+} was not observed even when the cells were pre-loaded with 65 mM Na^+. A Lineweaver-Burk plot of

TABLE III

K_i VALUES FOR INHIBITION OF Ca^{2+} UPTAKE BY MYOCARDIAL CELLS AND N-18 CELLS

Values are means ± S.D. for three to five independent experiments.

$[Na^+]_{int}$ (mM)	Cation	K_i (mM)	
		Myocardial cells	N-18 cells
37	Mg^{2+}	$5.0\ (\pm 0.7) \cdot 10^{-1}$	$8.2\ (\pm 0.3) \cdot 10^{-1}$
	Sr^{2+}	$1.7\ (\pm 0.3) \cdot 10^{-1}$	$2.3\ (\pm 1.7) \cdot 10^{-1}$
	Co^{2+}	$1.4\ (\pm 0.3) \cdot 10^{-2}$	$3.0\ (\pm 1.3) \cdot 10^{-2}$
65	Mg^{2+}	$7.7\ (\pm 2.1) \cdot 10^{-1}$	—
	Sr^{2+}	$2.2\ (\pm 0.6) \cdot 10^{-1}$	—
	Co^{2+}	$2.1\ (\pm 0.2) \cdot 10^{-2}$	—
	Na^+	30 (±9.0)	

Sr^{2+} uptake showed that the uptake of Sr^{2+} by myocardial cells was also achieved by a carrier-mediated transport system and K_m was estimated to be 0.18 mM (data not shown). The K_m value of Sr^{2+} uptake was equivalent to K_i for inhibition of Ca^{2+} uptake. This result shows that Sr^{2+} is also a substrate of the carrier for Ca^{2+}.

Effect of pH on Ca^{2+} uptake

For the results in Fig. 5, myocardial cells and N-18 cells were pre-incubated at pH 7.4 in EGTA (choline-Na) medium containing 38 and 140 mM Na^+, respectively, and then washed twice with EGTA medium containing no Na^+ at various pH values for a total period of 15 s, and transferred to Ca^{2+} (choline) medium containing ^{45}Ca. The pH value of the Ca^{2+} (choline) medium was adjusted with HCl, LiOH and Tris instead of NaOH, because Na^+ inhibits Ca^{2+} uptake, but Li^+ and Tris have no effect on Ca^{2+} uptake.

Fig. 5 shows the effect of pH on Ca^{2+} uptake by myocardial cells and N-18 cells. The curves are sigmoidal, not bell-shaped. In the pH range 5.5—8.0, the rate of Ca^{2+} uptake by both myocardial cells and N-18 cells increased with increase in the pH value. A Lineweaver-Burk plot of the effect of H^+ on Ca^{2+} uptake showed that the apparent Michaelis constant increased with decrease in the pH value, but the maximal initial rate was constant in the pH range 6.0—8.0 which was examined (data not shown). This result shows that H^+ competitively inhibits Ca^{2+} uptake. The negative logarithm of the dissociation constants for H^+, pK_H, of myocardial cells and N-18 cells were determined to be 6.3—6.5.

Intracellular Na^+-dependent Ca^{2+} uptake by non-excitable cells

As described above, intracellular Na^+-dependent Ca^{2+} uptake was observed with the excitable cells, myocardial cells and neuroblastoma cells in culture. Next, we examined whether the non-excitable cells (strain cells B-16, FL and L

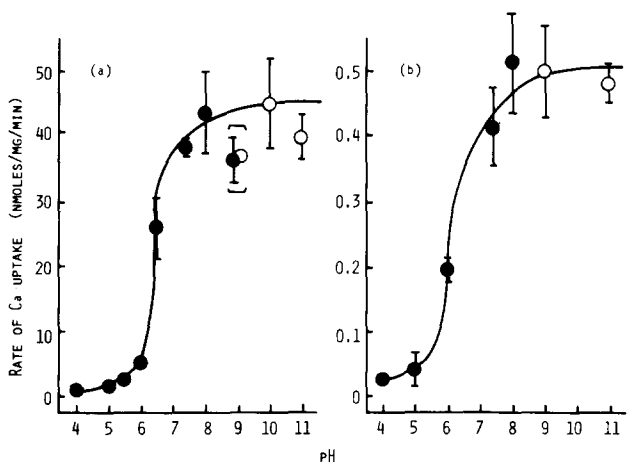

Fig. 5. Effect of pH on the rate of Ca^{2+} uptake (in the first 15 s) by myocardial cells (a) and N-18 cells (b). Experiments were carried out using the cells loaded with 37 mM Na^+ in Ca^{2+} (choline) medium containing ^{45}Ca and 0.1 mM Ca^{2+} at various pH values. Ca^{2+} (choline) medium was buffered with 10 mM Bes (●) or 10 mM Tris (○).

TABLE IV

EFFECT OF INTRACELLULAR CONCENTRATION OF Na^+ ON THE RATE OF Ca^{2+} UPTAKE BY NON-EXCITABLE CELLS

Values are means ± S.D. for three independent experiments. n.d., not detectable.

Cells	Treatment	$[Na^+]_i$ (mM)	Rate of Ca^{2+} uptake (nmol/mg per min)
Mouse heart	no addition	20 ± 4	1.6 ± 0.2
	10^{-3} M ouabain	122 ± 3	19.7 ± 0.4
B-16	no addition	19 ± 2	0.24 ± 0.02
	10^{-3} M ouabain	85 ± 2	0.53 ± 0.06
FL	no addition	5 ± 2	0.15 ± 0.01
	10^{-6} M ouabain	130 ± 1	0.13 ± 0.01
L	no addition	14 ± 1	0.32 ± 0.06
	10^{-3} M ouabain	127 ± 2	0.36 ± 0.02
Lung	EGTA (choline-0 mM Na^+) medium	n.d.	0.46 ± 0.01
	EGTA (choline-140 mM Na^+) medium	69 ± 8	3.06 ± 0.03
Skin	EGTA (choline-0 mM Na^+) medium	8 ± 6	0.64 ± 0.05
	EGTA (choline-140 mM Na^+) medium	67 ± 4	2.08 ± 0.42

cells, primary cultured cells from mouse lung and skin) also show intracellular Na^+-dependent Ca^{2+} uptake. In contrast to myocardial cells and N-18 cells, the intracellular concentration of Na^+ in strain cells could not be increased by incubating the cells in Ca^{2+}-free medium. Therefore, we enhanced the intracellular

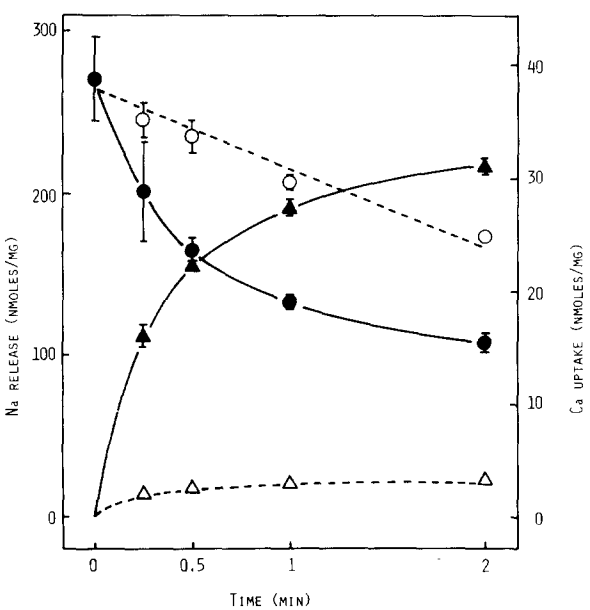

Fig. 6. Simultaneous measurements of the time courses of ^{24}Na release and ^{45}Ca uptake by myocardial cells. The experiment was carried out as described in Materials and Methods. ^{24}Na release (●) and ^{45}Ca uptake (▲) were simultaneously measured. For measurement of Ca^{2+}-dependent ^{24}Na release (○), the cells were loaded with 35 mM Na^+ and ^{24}Na, and then incubated in the medium containing 280 mM sucrose and 1 mM Co^{2+}, instead of Ca^{2+}. For measurement of Na^+-independent ^{45}Ca uptake (△), the cells were loaded with no Na^+, and then incubated in medium containing 280 mM sucrose, 1 mM Ca^{2+} and ^{45}Ca. Points and bars indicate means ± S.D. of values in three experiments.

concentration of Na^+ in these cells by adding ouabain, an inhibitor of the Na^+ pump. As shown in Table IV, the intracellular concentration of Na^+ in B-16 cells, L cells and FL cells increased on incubation for 10—12 h at 37°C in the presence of 10^{-3}, 10^{-3} and 10^{-6} M ouabain, respectively. Then the medium was replaced by medium containing ^{45}Ca and the rate of ^{45}Ca uptake was measured. The intracellular concentration of Na^+ in the cells from lung and skin could be enhanced by incubation in EGTA (choline-140 mM Na^+) medium for 10 min. Except for FL and L cells, we observed a slight activation of Ca^{2+} uptake by an increase in the intracellular concentration of Na^+ (Table IV). K_m values for the cells from lung and skin were 0.1—0.2 mM and roughly agreed with that on myocardial cells. The rate of Ca^{2+} uptake, however, by these five types of cell was much slower than that by myocardial cells.

Na^+-Ca^{2+} exchange

When myocardial cells loaded with ^{24}Na were transferred to a medium containing 280 mM sucrose and 1 mM Co^{2+}, only a slow release of ^{24}Na was observed (Fig. 6). On the other hand, when the cells loaded with ^{24}Na were transferred to medium containing 280 mM sucrose and 1 mM Ca^{2+}, instead of Co^{2+}, rapid release of ^{24}Na was observed (Fig. 6). In the experiment shown in Fig. 6, ^{45}Ca uptake by the cells was simultaneously measured. The initial rates of Ca^{2+}-dependent Na^+ release and Na^+-dependent Ca^{2+} uptake were 44.0 and 14.0 nmol/mg per 15 s, respectively. That is, this transport system mediated the exchange of three Na^+ for one Ca^{2+}.

Discussion

In this work, we showed that the initial rate of the resulting ^{45}Ca uptake increased with increase in the Na^+ concentration in the pre-incubation medium. There seem to be two possible explanations for the increase in Ca^{2+} uptake. (1) When the cells were incubated in Ca^{2+}-free medium, the membrane became more permeable to ions such as Na^+ and Ca^{2+}. Therefore, subsequent incubation in medium containing Ca^{2+} caused excess uptake of Ca^{2+} by the cells. But this possibility was excluded by the following observation. As described previously [13], even when the cells were incubated in Ca^{2+}-free medium containing no Na^+, the cell membrane became leaky, and significant K^+ loss occurred (see Fig. 1), but subsequent incubation in medium containing Ca^{2+} did not cause excess uptake of Ca^{2+} by the cells. (2) The increase in Ca^{2+} uptake was achieved by enhancement of an Na^+-Ca^{2+} exchange system of the cells. The present work and previous results support this mechanism.

An increase in the intracellular concentration of Na^+ markedly activated Ca^{2+} uptake. Particularly, at concentrations of 0—30 mM Na^+, the rate of Ca^{2+} uptake by myocardial cells increased in proportion to increase in the intracellular concentration of Na^+. This means that a slight increase (1—2 mM) in the intracellular Na^+ concentration from the physiological concentration (20 mM in the case of cultured myocardial cells) causes an appreciable increase in Ca^{2+} uptake by the cells. It is clear that what is measured in the present work is that almost all Ca^{2+} is transported across the cell membrane on account of the following observation. The radioactivity of ^{45}Ca at 0 min extrapolated from the time

course of Ca^{2+} uptake significantly increased when it was measured by washing with a solution containing no Ca^{2+} and no EGTA [21]. On the contrary, the value at 0 min was reduced to zero level by the washing procedure with a solution containing 1 mM Ca^{2+} used in this work. This shows that ^{45}Ca bound to the cell surface is effectively eliminated by substitution of the extracellular Ca^{2+} for ^{45}Ca bound. But, this result does not mean that most of the Ca^{2+} transported during the first 15 s remains in the free ionic form for a long time. Probably, during the first 15 s of Ca^{2+} uptake, considerable Ca^{2+} must be sequestrated by cellular organelles such as mitochondria and sarcoplasmic reticulum. But, we observed that the excess uptake of Ca^{2+} through Na^+-Ca^{2+} exchange caused the full contraction of myofibrils when the rate was 10 nmol/mg per min or more [13]. This suggested that the intracellular concentration of free Ca^{2+} in the cytoplasm was enhanced by the excess uptake of Ca^{2+}.

Ca^{2+} uptake was strongly inhibited at a low pH (Fig. 5). Another experiment showed that a low pH reduced the affinity of the carrier for Ca^{2+}, without changing the maximal initial rate. These results suggest that some structure near the Ca^{2+}-binding site on the carrier molecule contains residues that dissociate protons. Tanford [22] gave the following typical values for the pK_a values of several amino acid side chains in proteins: COO^-, 4.7; histidine (imidazole), 6.5; α-NH_2, 7.8; phenolic OH, 9.95; ϵ-NH_2, 10.2; guanidyl, greater than 12. We cannot determine exactly which residues of the carrier are concerned with Ca^{2+} uptake, but carboxyl or imidazole groups are likely candidates for residues near the Ca^{2+} binding site because the dissociation constant, pK_H for H^+, was determined to be 6.3—6.5 (see Fig. 5).

Recently, using sarcolemmal vesicles from dog ventricle, Pitts [23] reported that the stoichiometry of Na^+-Ca^{2+} exchange is three Na^+ for one Ca^{2+}. Our data also showed using intact heart cells that the stoichiometry of Na^+-Ca^{2+} exhange is three Na^+ for one Ca^{2+}. This result is in agreement with the model of electrogenic Na^+-Ca^{2+} exchange proposed by several authors [24—26], if this system does not transport another ion, such as H^+. On the basis of the model of electrogenic Na^+-Ca^{2+} exchange, the chemical reaction at equilibrium can be written as follows:

$$\frac{[Ca]_{ext}}{[Ca]_{int}} = \frac{[Na]^3_{ext}}{[Na]^3_{int}} \cdot \exp(-E_m F/RT)$$

where E_m, F, R and T are the membrane potential, the Faraday constant, the universal gas constant and temperature, respectively [26]. $[Ca]_{int}$ is calculated to be of the order of 10^{-6}—10^{-7} M when the values $[Na]_{int}$, $[Na]_{ext}$, $[Ca]_{ext}$ and E_m are estimated to be 20 mM, 140 mM, 1.8 mM and −50 mV, respectively. Thus, one can see that such a voltage-dependent model would sufficiently reduce the internal concentration of Ca^{2+}. However, at present, the exact value of $[Ca]_{int}$ cannot be determined. So, we cannot deny the existence of an ATP-dependent Ca^{2+}-pump system in the sarcolemmal membrane which was recently reported [27]. It is likely that the ATP-dependent Ca^{2+} pump can reduce $[Ca]_{int}$ to a lower value, 10^{-7} M or less.

Next, the kinetic data obtained for myocardial cells should be compared with those for neuroblastoma N-18 cells: (1) Ca^{2+} uptake by both myocardial

cells and N-18 cells had a similar dependency on the intracellular concentration of Na^+, at least at concentrations of 10—37 mM Na^+ (Figs. 2 and 3); (2) the apparent Michaelis constants for Ca^{2+} of the two types of cell were almost equal; (3) the K_i values for Co^{2+}, Mg^{2+} and Sr^{2+} of the two cell species were very similar; (4) Ca^{2+} uptake by the two cell species had a similar dependency on pH. These results suggest that N-18 cells possess a carrier showing the same properties as that of myocardial cells. But the maximal initial rate of Ca^{2+} uptake by N-18 cells was very much lower than that by myocardial cells. A possible explanation for the low maximal initial rate of Ca^{2+} uptake by N-18 cells is that the density of the carrier on N-18 cells is very much less than that on myocardial cells. Another possible explanation is that the velocity of movement of the carrier across the membranes is very much less in N-18 cells than in myocardial cells. We also observed that there is no Na^+-Ca^{2+} exchange system in the non-excitable cells which were examined, or that even if this system exists, the activity is much lower than that of myocardial cells (see Table IV). It would be of value to determine why only myocardial cells have a high activity of Na^+-Ca^{2+} exchange because we can understand, in part, the physiological meaning of Na^+-Ca^{2+} exchange.

In myocardial cells, extracellular Ca^{2+} flows across the cell membrane into the cytoplasm during excitation and contraction [28,29]. Thus, under physiological conditions, the movement of Ca^{2+} across the cell membrane is very active in myocardial cells, but not in non-myocardial cells. The high activity of Na^+-Ca^{2+} exchange in myocardial cells would be indispensable to physiological function in which the internal concentration of Ca^{2+} at rest is kept low by rapid extrusion of Ca^{2+} from the cytoplasm after excitation and contraction.

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References

1 Baker, P.F. and Blaustein, M.P. (1968) Biochim. Biophys. Acta 150, 167—178
2 Baker, P.F., Blaustein, M.P., Hodgkin, A.L. and Steinhardt, R.A. (1969) J. Physiol. 200, 431—458
3 Reuter, H. and Seitz, N. (1968) J. Physiol. 195, 451—470
4 Glitsch, H., Reuter, H. and Scholz, H. (1970) J. Physiol. 209, 25—43
5 Jundt, H., Porzig, H., Reuter, H. and Stugki, J.W. (1976) J. Physiol. 246, 229—253
6 Benninger, C., Einwachter, H.M., Haas, H.G. and Kern, R. (1976) J. Physiol. 259, 617—645
7 Miller, D.J. and Moisescu, D.G. (1976) J. Physiol. 259, 283—308
8 Busselen, P. and van Kerkhove, E. (1978) J. Physiol. 282, 263—283
9 Katzung, B.G., Reuter, H. and Porig, H. (1973) Experientia 29, 1073—1075
10 DeHaan, R.L. (1970) Dev. Biol. 23, 226—240
11 Goshima, K. (1969) Exp. Cell Res. 58, 420—426
12 Harary, I. and Farley, B. (1963) Exp. Cell Res. 29, 451—465
13 Goshima, K., Wakabayashi, S. and Masuda, A. (1980) J. Mol. Cell Cardiol. 12, 1135—1158

14 Goshima, K. (1977) J. Mol. Cell Cardiol. 9, 7—23
15 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
16 Schwarzenbach, G., Senn, H. and Anderegg, G. (1957) Helv. Chim. Acta 40, 1886—1900
17 Porzehl, H., Caldwell, P.C. and Ruegg, J.C. (1964) Biochim. Biophys. Acta 79, 581—591
18 McDonald, T.F. and DeHaan, R.L. (1973) J. Gen. Physiol. 61, 89—109
19 Fosset, M., DeBarry, J., Lenoir, M.C. and Lazdunski, M. (1977) J. Biol. Chem. 252, 6112—6117
20 Goshima, K., Yamanaka, H., Eguchi, G. and Yoshino, S. (1978) Dev. Growth Differ. 20, 191—204
21 Sanui, H. and Rubin, H. (1978) J. Cell. Physiol. 96, 265—278
22 Tanford, C. (1961) Physical Chemistry of Macromolecules, p. 556, John Wiley and Sons, Inc., New York
23 Pitts, B.J.R. (1979) J. Biol. Chem. 254, 6232—6235
24 Mullins, L.J. and Brinley, F.J., Jr. (1975) J. Gen. Physiol. 65, 135—152
25 Mullins, L.J. (1977) J. Gen. Physiol. 70, 681—695
26 Horackova, M. and Vassort, G. (1979) J. Gen. Physiol. 73, 403—424
27 Caroni, P. and Carafoli, E. (1980) Nature 283, 765—767
28 Niedergerke, R. (1963) J. Physiol. 167, 551—580
29 Winegrad, S. and Shanes, A.M. (1962) J. Gen. Physiol. 45, 371—394