Biochimica et Biophysica Acta 940 (1988) 188–196
Elsevier

BBA 73996

Sodium-pump activity and its inhibition by extracellular calcium in cardiac myocytes of guinea pigs

Paul Stemmer [a,*] and Tai Akera [a,b]

[a] *Department of Pharmacology and Toxicology, Michigan State University, East Lansing, MI (U.S.A.) and* [b] *National Children's Hospital Medical Research Center, Tokyo (Japan)*

(Received 2 October 1987)
(Revised manuscript received 23 February 1988)

Key words: Sodium pump; Ouabain; Calcium ion inhibition; Cardiac myocyte; (Guinea pig)

Myocardial sodium-pump activity was examined from ouabain-sensitive $^{86}Rb^+$ uptake using myocytes isolated from guinea-pig heart. Either sodium loading or the sodium ionophore, monensin, increased $^{86}Rb^+$ uptake by over 400%, indicating that the amount of Na^+ available to the pump is the primary determinant of its activity, and that the sodium pump has a substantial reserve capacity in quiescent myocytes. Moreover, the degree of the above stimulation is markedly higher than corresponding values reported with multicellular preparations, suggesting that diffusion barriers make it impossible to observe the capacity of the sodium pump in the latter preparations. Removal of extracellular Ca^{2+} increased ouabain-sensitive $^{86}Rb^+$ uptake, probably by enhancing turnover of the sodium pump rather than increasing availability of Na^+ to the pump.

Introduction

Sodium-pump activity in myocardial tissue can be estimated by monitoring a transient outward current upon exposure of sodium-loaded cells to K^+, Rb^+ or Cs^+ [1–3]. Under these conditions, however, only the maximally stimulated pump activity can be estimated. The more commonly used method of monitoring sodium pump activity from ouabain-sensitive $^{86}Rb^+$ or $^{42}K^+$ uptake allows estimation of pump activity with or without sodium loading of the cells [4,5]. In intact myocardial cells, the intracellular Na^+ activity is less than 10 mM [6,7], whereas maximal activation of isolated Na^+/K^+-ATPase [8] or the intact sodium pump [9] does not occur at Na^+ concentrations less than 100 mM. Therefore, ongoing sodium-pump activity observed without sodium loading should be distinguished from the capacity of the pump observed in sodium-loaded cells [4,5]. The difference in these two values represents the reserve capacity of the sodium pump.

It is important to estimate separately the ongoing activity and the capacity of the sodium pump, because inhibition of the pump less than the reserve capacity would appear to cause the positive inotropic effect, whereas that exceeding the reserve capacity causes toxicity [10,11]. For example, the therapeutic index of the cardiac glycoside is decreased in senescent myocardium of Fischer 344 rats probably owing to the reduced reserve capacity of the pump [12,13]. In multicellular preparations, however, the myocardial uptake of chemicals may be limited by diffusion [14]. That

* Present address: Department of Medicine, Krannert Institute for Cardiology, Indiana University School of Medicine, Indianapolis, IN, U.S.A.

Correspondence: T. Akera, The National Children's Hospital, Medical Research Center, 3-35-31 Taishido, Setagaya-ku, Tokyo 154, Japan.

such a diffusion limits availability of K^+ or Rb^+ for the sodium pump has been reported in sodium-loaded intact soleus muscle from rat [15] and in sodium-loaded Purkinje fibers from sheep heart [2]. Therefore, there are doubts as to whether the true capacity of the sodium pump can be accurately estimated in intact tissue. Isolated myocyte preparations seem to offer an advantage of being nearly free from diffusion barriers. Moreover, these preparations have the additional advantages that there is only one cell type present and that the extracellular surface of the cells can be washed quickly and efficiently, allowing accurate estimation of the specific $^{86}Rb^+$ uptake. It is still unknown, however, whether the ouabain-sensitive $^{86}Rb^+$ uptake by isolated myocytes can be stimulated by an increase in Na^+ influx or by sodium loading. Therefore, these aspects were examined in the present study. In addition, modulation of the sodium-pump activity by Ca^{2+} was studied using myocytes.

Materials and Methods

Myocytes were prepared from guinea pigs of either sex weighing approx. 400 g (albino, Mdh-A strain obtained from Michigan Department of Public Health) as previously described [16]. Dissociated myocytes were elutriated in vertical columns and were maintained in these columns up to 4 h in a solution containing 1.8 mM $CaCl_2$. 1 h before the start of $^{86}Rb^+$ uptake, the elutriation solution was changed to one containing 5 mM RbCl and 1 mM NaH_2PO_4 instead of one containing 2.8 mM KCl and 1 mM KH_2PO_4.

Viability of myocytes was assessed by pipetting 0.3 ml of a cell suspension into 2 ml of 10% formalin. Dispersion of cells in formalin resulted in their shape being maintained until samples could be examined. Cells were counted using a hemocytometer, and the rod-shaped and rounded cells were counted separately to assess viability of preparations.

Sodium-pump activity was estimated in myocytes as the difference in $^{86}Rb^+$ uptake observed in the absence and presence of 1 mM ouabain. $^{86}Rb^+$ uptake was initiated by adding cells to prewarmed (37° C) incubation tubes containing 2.0 ml of the modified Krebs-Henseleit bicarbonate buffer solution containing 5 mM RbCl and tracer amounts of $^{86}Rb^+$. Exposure of myocytes to ouabain and $^{86}Rb^+$ was simultaneous when nonspecific $^{86}Rb^+$ uptake was estimated; however, rapid inactivation of the sodium pump can be anticipated because of the high concentration of ouabain used. After 2–6 min exposure to $^{86}Rb^+$, myocytes were collected by centrifugation through a sucrose layer and then sampled for determination of $^{86}Rb^+$ and protein content. Specific radioactivity of $^{86}Rb^+$ in the incubation medium was estimated by sampling the supernatant solution after cells were removed by centrifugation.

In a series of experiments in which occupancy of the glycoside receptors on Na^+/K^+-ATPase and inhibition of the specific $^{86}Rb^+$ uptake were compared, myocytes were incubated with 0–10 μM [^{3}H]ouabain for 15 min at 37° C in a medium containing 5 mM RbCl with or without Ca^{2+}. Subsequently, aliquots of the cell suspension were assayed for [^{3}H]ouabain binding or $^{86}Rb^+$ uptake. All reactions were stopped using the centrifugation method described below.

Centrifuge tubes for sampling were prepared by pipetting 4 ml of a solution containing 280 mM sucrose, 10 mM procaine-HCl and 0.9 mM $CaCl_2$ into 15-ml polypropylene tubes (Becton-Dickinson, Parsippany, NJ), and then slowly overlaying 9 ml of a less dense solution containing 99 mM NaCl, 30 mM RbCl, 20 mM procaine-HCl (to stabilize the cell membrane), 10 mM Hepes, 2 mM $BaCl_2$, 0.9 mM $CaCl_2$, 0.1 mM ouabain and a trace of Patent Blue Violet dye to identify this layer (pH value adjusted to 7.1 with 2 M NaOH). The less dense solution was added slowly, so that at least 3 ml of the sucrose layer remained unmixed with the less dense layer. These 'sampling tubes' were placed in an NaCl/ice bath with the temperature adjusted to −2° C and allowed to stand at least 1 h before use.

At the time [^{3}H]ouabain binding or $^{86}Rb^+$ uptake reactions were to be stopped, a 0.5 ml aliquot of the incubation mixture was taken from the bottom of incubation tubes and added to a centrifuge tube prepared as described above. The decrease in temperature is calculated to be sufficient to stop virtually [^{3}H]ouabain binding or active transport of $^{86}Rb^+$. Preliminary experi-

ments showed that the amount of [^{3}H]ouabain or $^{86}Rb^+$ contained in the cells did not change significantly when centrifugation was delayed for up to 15 min after samples were added to centrifuge tubes.

Cells were collected by centrifuging the samples for 2 min at $1500 \times g$ in a refrigerated centrifuge maintained at 2°C. The less dense solution containing non-bound [^{3}H]ouabain or $^{86}Rb^+$ was aspirated from the top of the centrifuge tube leaving approx. 3 ml of sucrose solution covering the cell pellet. Tubes with cell pellets were rapidly frozen by immersing them in methanol at −30°C. Cell pellets were collected by cutting the bottom from the frozen tubes.

Samples were analyzed for $^{86}Rb^+$ without further treatment using a gamma counter. Samples for determination of [^{3}H]ouabain were dried overnight at room temperature and then wetted with 75 µl of distilled water before digesting and counting. Wetting of the samples was necessary to maintain the sucrose in solution after the addition of scintillation fluid.

Protein concentration was estimated by the method of Bradford [17] using Coomassie brilliant blue G-250 (Bio-Rad Laboratories, Richmond, CA). [G-^{3}H]Ouabain specific radioactivity, 20 Ci/mmol), ^{86}RbCl (spec. act. 5.9 mCi/mg) and tissue solubilizer (Protosol) were purchased from New England Nuclear (Boston, MA). Collagenase was obtained from Cooper Biomedical (Malvern, PA), Boehringer-Mannheim (Indianapolis, IN) or Sigma (St. Louis, MO). Hyaluronidase was purchased from Sigma.

Statistical analysis was performed using grouped *t*-test, paired *t*-test or analysis of variance as indicated. $P < 0.05$ was taken as the criterion for statistical significance.

Results

Sodium-pump activity of isolated myocytes obtained from guinea-pig heart was estimated as the difference in uptake of $^{86}Rb^+$ observed in the absence and presence of 1 mM ouabain. This concentration of ouabain causes a rapid and complete inhibition of the sodium pump (data not shown). Nonspecific uptake observed in the presence of 1 mM ouabain accounted for approx. 25% of the total uptake into quiescent cells observed in its absence. Specific $^{86}Rb^+$ uptake into quiescent myocytes was linear with respect to time for at least 15 min when it was assayed in a Krebs-Henseleit bicarbonate buffer solution containing 5 mM Rb^+ and no K^+ (data not shown). In the following studies, uptake time was limited to 6 min to ensure linearity, and was reduced to 2 min when examining the capacity of the sodium pump.

Sodium-pump activity was estimated in quiescent cells and in cells exposed to the Na^+ ionophore monensin, which has been shown to increase the rate of Na^+ influx [18]. Monensin has been shown to stimulate sodium-pump activity in contracting or quiescent preparations of cardiac muscle presumably by increasing Na^+ influx [4]. Monensin caused a concentration-dependent increase in ouabain-sensitive $^{86}Rb^+$ uptake into myocytes (Fig. 1). This effect of monensin, observed in the presence of 1.8 mM Ca^{2+}, reached an apparent peak at 30 µM. There was a 5-fold increase in sodium-pump activity, as estimated from ouabain-sensitive $^{86}Rb^+$ uptake, caused by

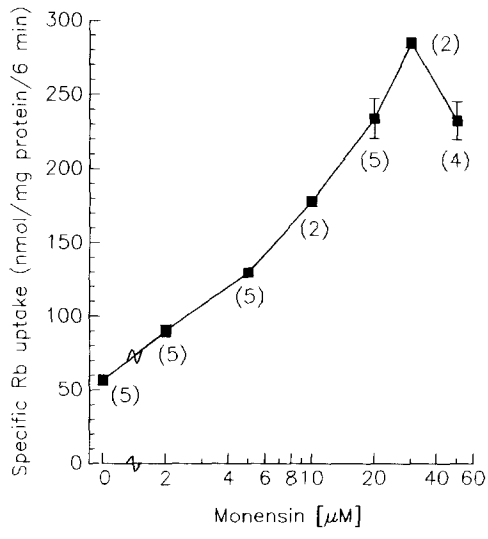

Fig. 1. Stimulation of ouabain-sensitive $^{86}Rb^+$ uptake by the sodium ionophore, monensin. Myocytes obtained from guinea-pig heart were incubated at 37°C for 6 min in a modified Krebs-Henseleit bicarbonate buffer solution containing 5 mM ^{86}RbCl, 1.8 mM $CaCl_2$ and the indicated concentration of monensin. Nonspecific uptake observed in the presence of 1 mM ouabain is subtracted. Numbers in parentheses represent the number of experiments. Vertical lines indicate S.E.

the optimal concentration of monensin in myocyte preparations.

It should be noted that the above results were obtained by exposing myocytes to monensin at the time when $^{86}Rb^+$ uptake was started. Therefore, values may represent $^{86}Rb^+$ uptake over a period of time when intracellular Na^+ concentration was increasing and the rates of $^{86}Rb^+$ uptake were also increasing. It is also possible that by the end of the 6-min incubation period, intracellular Na^+ concentration in cells exposed to each concentration of monensin had increased to the extent that the sodium pump was operating at capacity. The concentration dependence of this monensin effect might, therefore, reflect a more rapid increase in intracellular Na^+ at the higher concentrations of monensin. To test this hypothesis, myocytes were preincubated with monensin for 5 min before initiating $^{86}Rb^+$ uptake, and the uptake was estimated in a Krebs-Henseleit bicarbonate buffer solution containing 2 mM $^{86}RbCl$. This lower concentration of Rb^+ would allow a faster increase in intracellular Na^+, so that intracellular Na^+ would cease earlier to be the limiting substrate. Under these conditions, a significantly lower $^{86}Rb^+$ uptake was observed, reflecting the lower concentration of $^{86}Rb^+$, and the maximal effect of monensin was observed at 20 μM (data not shown). These results indicate that the capacity of the sodium pump may be estimated in the presence of 20–30 μM monensin.

Monensin, however, may have other effects in addition to increasing the Na^+ influx rate. Therefore, myocytes were sodium loaded to determine whether a similar stimulation of sodium-pump activity occurs when intracellular Na^+ concentration is increased. Sodium loading was accomplished by elutriating myocytes using a K^+- and Rb^+-free solution at 37 °C. The Ca^{2+} concentration of the medium used to sodium load the cells was reduced to 10 μM in an attempt to limit toxic effects of Ca^{2+}. After approx. 30 min of perfusion with the K^+- and Rb^+-free solution, all myocytes were spontaneously contracting when examined under a microscope. The contractions, however, were not discrete, but resembled a writhing movement. Upon the addition of these sodium-loaded myocytes to a Krebs-Henseleit bicarbonate buffer solution containing 1.8 mM Ca^{2+} and 3.8 mM K^+, the cells began to contract with clear cycles of contraction and relaxation.

Maximum stimulation of the specific $^{86}Rb^+$ uptake by sodium loading was achieved by a 45 min incubation of myocytes in a K^+- and Rb^+-free solution (Fig. 2). Longer incubation of cells, up to 90 min, had no additional effect on sodium-pump activity. The maximal increase in the specific $^{86}Rb^+$ uptake caused by preincubation for sodium loading in a K^+- and Rb^+-free medium was 4.8-fold.

Ouabain-sensitive $^{86}Rb^+$ uptake into the sodium-loaded cells observed in the absence of Ca^{2+} was significantly higher than the corresponding values observed in the presence of 1.8 mM Ca^{2+} (Fig. 2). The difference was significant in fully sodium-loaded myocytes, indicating that the capacity of the sodium pump is greater in the absence of Ca^{2+}.

Ouabain inhibits sodium pump activity. However, inhibition of any given percentage of

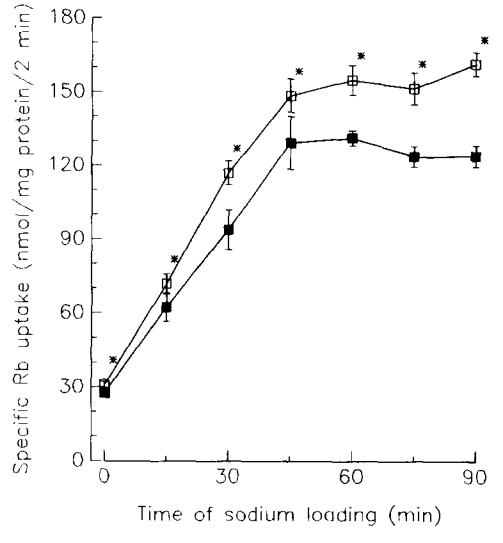

Fig. 2. Stimulation of ouabain-sensitive $^{86}Rb^+$ uptake by sodium loading. Myocytes were incubated for sodium loading at 37 °C for the time period shown on the abscissa in a K^+- and Rb^+-free solution containing 10 μM Ca^{2+}. Subsequently, $^{86}Rb^+$ uptake was estimated at 37 °C in a medium containing 5 mM $^{86}RbCl$ and either no (open symbols) or 1.8 mM $CaCl_2$ (filled symbols). Incubation period for $^{86}Rb^+$ uptake was 2 min. Ouabain-insensitive $^{86}Rb^+$ uptake is subtracted. Data represent mean for five experiments. Vertical lines indicate S.E. * Significantly different from the corresponding value observed in the presence of 1.8 mM $CaCl_2$.

Na^+/K^+-ATPase enzyme is expected to have less effect on sodium-pump activity when Na^+ influx is low and the sodium pump has a reserve capacity than when Na^+ influx is higher or when the cells are sodium loaded [19]. Therefore, the effect of increased Na^+ influx on ouabain-induced inhibition of sodium-pump activity was examined in myocytes preincubated with various concentrations of ouabain. Ouabain-sensitive $^{86}Rb^+$ uptake was 60.3 ± 4.2 and 248 ± 12 nmol/mg protein per 6 min in the absence and presence of 50 μM monensin, i.e., monensin caused a 4-fold increase in the specific $^{86}Rb^+$ uptake. Moreover, monensin shifted the ouabain concentration vs. inhibition curves to the left (Fig. 3). The concentration of ouabain needed to cause a 50% inhibition of the specific $^{86}Rb^+$ uptake was 1.25 and 0.74 μM in the absence and presence of 50 μM monensin, respectively. These results are consistent with the concept that the sodium pump has a reserve capacity which is decreased by the augmentation

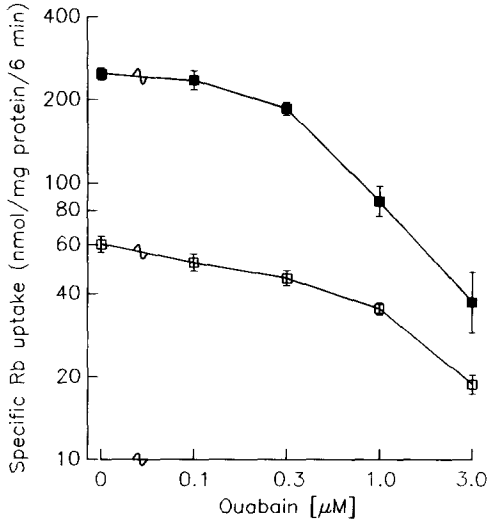

Fig. 3. Effects of monensin on ouabain-induced inhibition of the sodium pump. Myocytes were incubated at 37 °C for 60 min in a modified Krebs-Henseleit bicarbonate buffer solution containing 5 mM RbCl, 1.8 mM $CaCl_2$ and the indicated concentration of ouabain. Subsequently, cells were added to a prewarmed solution (37 °C) containing the same concentration of ouabain, 5 mM $^{86}RbCl$ and no (open symbols) or 50 μM monensin (filled symbols). Incubation time for $^{86}Rb^+$ uptake was 6 min. $^{86}Rb^+$ uptake observed in the presence of 1 mM ouabain is subtracted. Data represent mean for five experiments. Vertical lines indicate S.E. The ordinate is in logarithmic scale.

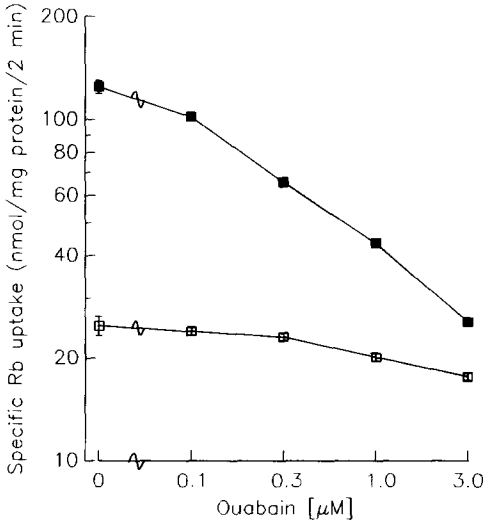

Fig. 4. Effects of sodium loading on sodium-pump inhibition caused by ouabain. Myocytes were incubated for sodium loading at 37 °C for 30 min in a modified Krebs-Henseleit bicarbonate buffer solution containing no K^+, no Rb^+ and 10 μM $CaCl_2$ (filled symbols). Control preparations were incubated for a comparable time period in a medium containing 5 mM RbCl and 10 μM $CaCl_2$ (open symbols). Subsequently, ouabain was added to the media and the mixtures were incubated for an additional 15 min period. Cells were then added to a prewarmed solution (37 °C) containing the same concentration of ouabain, 1.8 mM $CaCl_2$ and 5 mM $^{86}RbCl$. Incubation time for $^{86}Rb^+$ uptake was 2 min. $^{86}Rb^+$ uptake observed in the presence of 1 mM ouabain is subtracted. Data represent mean for five experiments. Vertical lines indicate S.E. The ordinate is in logarithmic scale.

of Na^+ influx. However, the results may be partially attributable to increased ouabain binding in the presence of monensin [16]. With either mechanism, the shifts shown in Fig. 3 and 4 are dependent on an increase in intracellular Na^+, and reflect a decrease in the reserve capacity of the pump.

Effects of intracellular Na^+ on ouabain-induced inhibition of sodium-pump activity were examined in sodium-loaded myocytes. Cells were sodium loaded for 30 min or were elutriated with a solution containing 5 mM RbCl for controls. Subsequently, the myocytes were incubated for an additional 15 min in an identical solution containing the indicated concentration of ouabain, and then assayed for $^{86}Rb^+$ uptake. The specific $^{86}Rb^+$ uptake was 24.8 ± 1.7 and 125 ± 6 nmol/mg protein per 2 min in the control and

sodium-loaded myocytes, respectively. Ouabain caused a concentration-dependent inhibition of the specific $^{86}Rb^+$ uptake in each preparation (Fig. 4). The sensitivity of myocytes to ouabain-induced inhibition, however, was markedly greater in sodium-loaded preparations. It should be noted that the ordinates of Figs. 3 and 4 are in logarithmic scale, and therefore the difference in the slope of ouabain concentration-effect curves should be identical, regardless of the initial value, if the sensitivity of the sodium pump to inhibition by ouabain is the same.

The effect of Ca^{2+} concentration in the incubation medium on the ouabain-sensitive $^{86}Rb^+$ uptake was examined without any intervention that increases sodium-pump activity. Myocytes, preincubated in a medium containing 1.8 mM $CaCl_2$, were assayed for ouabain-sensitive $^{86}Rb^+$ uptake assayed in a solution containing the indicated concentration of Ca^{2+}. Because myocytes were exposed to variable concentrations of Ca^{2+} only during the time of $^{86}Rb^+$ uptake, effects of extracellular Ca^{2+} on viability of the myocytes should

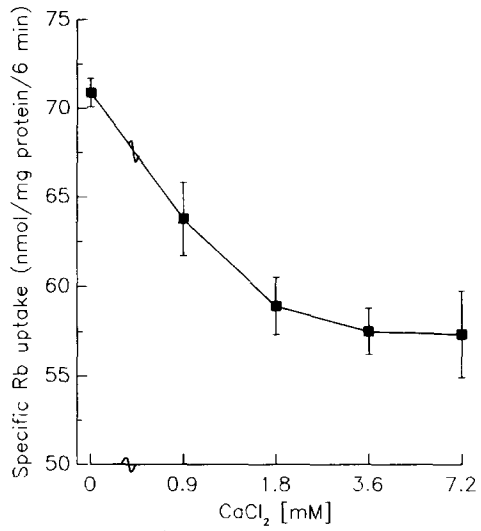

Fig. 5. Effects of Ca^{2+} on sodium-pump activity estimated from ouabain-sensitive $^{86}Rb^+$ uptake. Myocytes were incubated at 37 °C for 6 min in the medium containing 5 mM $^{86}RbCl$ and the indicated concentration of Ca^{2+}. The concentration of Ca^{2+} was adjusted by adding $CaCl_2$ to a medium containing 0.25 mM EGTA. $^{86}Rb^+$ uptake observed in the presence of 1 mM ouabain is subtracted. Data represent mean for four experiments. Vertical lines indicate S.E.

TABLE I

RELATIONSHIP BETWEEN OUABAIN BINDING AND THE SPECIFIC $^{86}Rb^+$ UPTAKE IN MYOCYTES INCUBATED WITH [^{3}H]OUABAIN IN THE PRESENCE AND ABSENCE OF Ca^{2+}

Myocyte preparations were incubated with the indicated concentration of [^{3}H]ouabain for 15 min at 37 °C in the absence or presence of 1.8 mM $CaCl_2$. At the end of the incubation period, an aliquot of cells was assayed for specific [^{3}H]ouabain binding. Another aliquot was added to an incubation mixture containing the same concentrations of Ca^{2+} and ouabain, and 5 mM $^{86}RbCl$, and assayed for ouabain-sensitive $^{86}Rb^+$ uptake. Incubation time for $^{86}Rb^+$ uptake was 2 min. Turnover rate for the sodium pump is calculated as the ratio of ouabain-sensitive $^{86}Rb^+$ uptake and the free [^{3}H]ouabain binding site concentration. Data represent mean ± S.E. for seven experiments.

$CaCl_2$ (mM)	Ouabain (μM)	Bound [^{3}H]ouabain (pmol/mg protein)	Specific $^{86}Rb^+$ uptake (nmol/mg protein per 2 min)	Turnover (min^{-1})
1.8	0	0	44.0 ± 2.1	2000
	0.1	0.46 ± 0.05	34.7 ± 3.0	1650
	0.3	1.27 ± 0.10	34.0 ± 5.1	1750
	1.0	2.69 ± 0.27	22.1 ± 2.9	1330
	3.0	4.39 ± 0.40	23.5 ± 2.2	1780
	10.0	8.10 ± 0.85	12.7 ± 3.1	2190
0 (EGTA, 0.25 mM)				
	0	0	70.4 ± 1.5	3200
	0.1	0.62 ± 0.04	61.9 ± 3.8	2980
	0.3	1.57 ± 0.12	56.9 ± 1.9	3020
	1.0	3.66 ± 0.58	48.5 ± 4.0	3300
	3.0	6.41 ± 0.27	31.0 ± 2.3	3380
	10.0	10.05 ± 0.72	15.9 ± 1.2	8300

be minimal. Increases in the extracellular Ca^{2+} concentration from zero to 0.9 mM, and then to 1.8 mM caused a significant reduction of ouabain-sensitive $^{86}Rb^+$ uptake (Fig. 5). The effect of Ca^{2+} reached an apparent plateau at 3.6 mM.

Because ouabain-sensitive $^{86}Rb^+$ uptake observed with myocytes is dependent upon the rate of Na^+ influx as well as sodium-pump activity (Figs. 1–4), the above results shown in Fig. 5 may indicate that Ca^{2+} either inhibits the sodium pump and thereby decreases its reserve capacity or it decreases sodium loading of the cells and increases the reserve capacity. Therefore, the effect of Ca^{2+} on reserve capacity of the pump was examined. We have shown previously that Ca^{2+} inhibits ouabain binding to cardiac myocytes obtained from guinea pigs [16]. Therefore, [^{3}H]ouabain binding and sodium-pump activity were examined simultaneously with myocytes incubated for 15 min at 37 °C in the absence or presence of 0.1–10 μM ouabain. The results shown in Table I confirm our previous finding that [^{3}H]ouabain binding to myocytes is enhanced in a Ca^{2+}-free incubation medium [16]. The number of ouabain binding sites (B_{max}) was estimated to be 11.0 pmol/mg protein by the analysis of Scatchard plots of the ouabain binding data observed in the absence of Ca^{2+}. This value was not significantly different in the presence of 1.8 mM $CaCl_2$. The specific $^{86}Rb^+$ uptake was significantly greater in the absence of Ca^{2+} at each concentration of ouabain, even though the number of pump sites was less. The difference in $^{86}Rb^+$ uptake observed in the presence and absence of Ca^{2+}, however, was smaller in the presence of higher concentrations of ouabain, where sodium-pump reserve capacity is reduced. The turnover rate of the remaining sodium pumps was higher in the absence of Ca^{2+} than in the presence of 1.8 mM $CaCl_2$ (Table I).

Discussion

The results demonstrate that ouabain-sensitive $^{86}Rb^+$ uptake observed in myocytes obtained from guinea-pig heart can be stimulated 400- to 500% either by the presence of the optimal concentration of monensin or by sodium loading of the cells. In a previous study with atrial muscle preparations of guinea-pig heart, the most effective means of increasing ouabain-sensitive $^{86}Rb^+$ uptake was electrical stimulation, which caused a 200% increase in observed values [4]. In that study, monensin increased ouabain-sensitive $^{86}Rb^+$ uptake; however, the magnitude of the increase was less than 50%. Greater increase in ouabain-sensitive $^{86}Rb^+$ uptake observed in the present study may be attributable to the lack of diffusion barriers. Lüllmann et al. [14] reported that a diffusion barrier restricts tissue uptake of chemicals observed in cardiac muscle preparations which are not stimulated at high frequencies. Apparently, such barriers do not exist in the myocyte preparations. Recently, Clausen et al. [15] reported that a maximum stimulation of the sodium pump could not be achieved in isolated soleus muscle from rat without a K^+ concentration of at least 85 mM to ensure that its diffusion to the Na^+/K^+-ATPase was not rate-limiting. The need for such high concentrations of the counterions is eliminated in isolated myocytes, which allow an examination of the sodium pump acting at its capacity at physiological K^+ or equivalent $^{86}Rb^+$ concentrations. Stimulation of the sodium pump by extracellular K^+ in cultured liver cells by optimal concentration of monensin reached a plateau at 4 mM K^+ [20], indicating that 5 mM $^{86}Rb^+$ used in the present study should be sufficient to maximally stimulate the sodium pump in myocytes. Non-stimulated sodium-pump activity in isolated myocytes and cultured liver cells [20] was comparable, whereas the maximum degree of sodium-pump stimulation was twice as large in the myocytes. This may reflect a difference in the maximum rate of enzyme turnover or a difference in enzyme density between the two preparations. Using myocytes, it is feasible to estimate the capacity of the sodium pump accurately in cardiac muscle.

An accurate estimate of ouabain-sensitive $^{86}Rb^+$ uptake allowed examination of the effect of Ca^{2+} on sodium-pump activity. The effect of Ca^{2+} to inhibit ouabain-sensitive $^{86}Rb^+$ uptake has not been previously reported. In the present study, ouabain-sensitive $^{86}Rb^+$ uptake was high in a Ca^{2+}-free solution, and was significantly inhibited when the extracellular $CaCl_2$ concentration was increased to 3.6 mM. This effect of Ca^{2+} was

significant in non-stimulated myocytes or in myocytes subjected to sodium loading or exposed to monensin. Possible mechanisms for this effect of Ca^{2+} are (1) a direct inhibition of Na^+/K^+-ATPase turnover [21], (2) reduction of intracellular Na^+ via the Na^+/Ca^{2+} exchange mechanism [22] or (3) a reduction of intracellular Na^+ via altered membrane permeability to Na^+ [23]. The possibility that Ca^{2+} hyperpolarizes the cells and inhibits electrogenic Na^+ extrusion may be ruled out because the rate of sodium-pump turnover has been shown to be relatively independent of membrane potentials [2,24]. Based on findings that Ca^{2+} inhibits ouabain-sensitive $^{86}Rb^+$ uptake in maximally stimulated sodium-loaded myocytes (Fig. 2), a direct inhibition of the Na^+/K^+-ATPase would appear to be the most plausible explanation. It therefore seems that 1.8 mM extracellular Ca^{2+} inhibits turnover of Na^+/K^+-ATPase in viable myocytes, and the removal of Ca^{2+} from the incubation medium increases maximal turnover rate of the sodium pump.

Ca^{2+} has been shown to inhibit isolated Na^+/K^+-ATPase, resulting from competition for either Na^+ or Mg^{2+} [21]. This inhibition, however, requires a concentration of Ca^{2+} that is significantly high (IC_{50} value = 0.5 mM) [21,25,26] compared to intracellular Ca^{2+} concentrations in viable myocardial cells [27]. Because Na^+ and Mg^{2+} act on Na^+/K^+-ATPase from the cytoplasmic side, it has been generally considered that Ca^{2+} has no direct effects on the sodium pump in intact cells [28]. More recently, however, effects of Ca^{2+} on the sodium pump have been suggested by several investigators. Among them, Powis et al. [29] reported that low concentrations of Ca^{2+} stimulate Na^+/K^+-ATPase by a mechanism which is amplified by calmodulin. Stimulation of the specific $^{86}Rb^+$ uptake by Ca^{2+}, however, was not observed in the present study.

Yingst and co-workers [30,31] reported that Ca^{2+} inhibits the sodium pump in human erythrocytes. The inhibitory effect of Ca^{2+} is mediated by a protein factor which can be extracted from human erythrocytes. In the presence of this factor, the IC_{50} value for Ca^{2+} inhibition of Na^+/K^+-ATPase activity was approx. 1 μM, i.e., the concentration that may be achieved in viable myocardial cells especially during the contraction. It is unknown, however, whether such a factor is responsible for the inhibition of sodium-pump activity observed in the present study. These results suggest that Ca^{2+} may act not only as the ultimate mediator for toxic effects of the cardiac glycosides [32], but may also exacerbate the toxicity by reducing the reserve capacity of the sodium pump and in so doing be partially responsible for the low therapeutic index of the glycosides. Alternatively, however, extracellular Ca^{2+} may modulate sodium-pump activity indirectly via its effect on membrane lipids. Mansier and LeLievre [33] reported that a perfusion of Langendorff preparations obtained from rat heart with a Ca^{2+}-free medium modifies ouabain sensitivity of the sarcolemmal Na^+/K^+-ATPase. Because ouabain sensitivity of the sodium pump is intimately related to turnover of the pump, the latter might be influenced by exposure of cells to a Ca^{2+}-free medium.

In summary, sodium-pump activity can be increased in cardiac myocytes by incubation with a sodium ionophore or by sodium loading. The increases are large compared to those observed in atrial or ventricular muscle preparations. Removal of Ca^{2+} from the incubation medium increases sodium-pump activity and its reserve capacity.

Acknowledgements

This work was supported by U.S. Public Health Service grants, HL-16052 from the National Heart, Lung, and Blood Institute and AG-02398 from the National Institute on Aging. The authors thank Ms. Aizhen Yao and Ms. Cindy Clingan for expert technical assistance.

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