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SODIUM INFLUX RATE AND OUABAIN-SENSITIVE RUBIDIUM UPTAKE IN ISOLATED GUINEA PIG ATRIA

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Summary

1. Ouabain-sensitive $^{86}Rb^+$ uptake by tissue preparations has been used as an estimate of Na^+ pump activity. This uptake, however, may be a measure of the Na^+ influx rate, rather than capacity of the Na^+ pump, since intracellular Na^+ concentration is a determinant of the active Na^+/Rb^+ exchange reaction under certain conditions. This aspect was examined by studying the effect of altered Na^+ influx rate on ouabain-sensitive $^{86}Rb^+$ uptake in atrial preparations of guinea pig hearts.

2. Electrical stimulation markedly enhanced ouabain-sensitive $^{86}Rb^+$ uptake without affecting nonspecific, ouabain-insensitive uptake. Paired-pulse stimulation studies indicate that the stimulation-induced enhancement of $^{86}Rb^+$ uptake is due to membrane depolarizations, and hence related to the rate of Na^+ influx.

3. Alterations in the extracellular Ca^{2+} concentration failed to affect the $^{86}Rb^+$ uptake indicating that the force of contraction does not influence $^{86}Rb^+$ uptake.

4. Reduced Na^+ influx by low extracellular Na^+ concentration decreased $^{86}Rb^+$ uptake, and an increased Na^+ influx by a Na^+-specific ionophore, monensin, enhanced $^{86}Rb^+$ uptake in quiescent atria.

5. Grayanotoxins, agents that increase transmembrane Na^+ influx, and high concentrations of monensin appear to have inhibitory effects on ouabain-sensitive $^{86}Rb^+$ uptake in electrically stimulated and in quiescent atria.

6. Electrical stimulation or monensin enhanced ouabain binding to ($Na^+ + K^+$)-ATPase and also increased the potency of ouabain to inhibit $^{86}Rb^+$ uptake indicating that the intracellular Na^+ available to the Na^+ pump is increased under these conditions.

7. The ouabain-sensitive $^{86}Rb^+$ uptake in electrically stimulated atria was less

sensitive to alterations in the extracellular Na^+ concentration, temperature and monensin than that in quiescent atria.

8. These results indicate that the rate of Na^+ influx is the primary determinant of ouabain-sensitive $^{86}Rb^+$ uptake in isolated atria. Electrical stimulation most effectively increases the Na^+ available to the Na^+ pump system. The ouabain-sensitive $^{86}Rb^+$ uptake by atrial preparations under electrical stimulation at a relatively high frequency seems to represent the maximal capacity of the Na^+ pump in this tissue.

Introduction

Ouabain-sensitive $^{86}Rb^+$ uptake by tissue slices has been frequently used as an estimate of Na^+ pump activity [1—6] which is responsible for the active transport of Na^+ and K^+ across the cell membrane (see Refs. 7 and 8). In these studies, tissue slices are generally incubated in a physiological buffer solution in which K^+ is replaced with Rb^+ containing tracer amounts of $^{86}Rb^+$. The difference in $^{86}Rb^+$ uptake observed in the absence and presence of ouabain is identified as the Na^+ pump activity, since this ouabain-inhibitable uptake of $^{86}Rb^+$ is coupled with active Na^+ efflux, i.e. the membrane Na^+ pump [1,3]. The assay for ouabain-sensitive $^{86}Rb^+$ uptake is performed either with Na^+-loaded slices [1,2,5,6], or without Na^+ loading [3,4]. When experiments are performed without Na^+ loading, however, the rate of ouabain-sensitive $^{86}Rb^+$ uptake may be dependent on the rate of Na^+ influx, rather than Na^+ efflux; since the intracellular Na^+ concentration is the determinant of Na^+ pump activity under certain conditions [9—11]. Biochemical data obtained with (Na^+ + K^+)-ATPase, the enzymatic representation of the Na^+ pump [7,8], support this concept. Concentrations of intracellular Mg^{2+}, ATP and extracellular K^+ are higher than the respective K_m values of this enzyme system (see Ref. 12). The intracellular Na^+ concentration (Na^+ activity), however, may be as low as 5—6 mequiv./l [13]. This value is lower than K_m values of the enzyme system for Na^+, which is estimated to be 20 mM in intact cells [14]. If the intracellular Na^+ is a primary determinant of Na^+ pump activity, then the rate of Na^+ influx, which replenishes the intracellular Na^+ pool at a site from which this cation is transported may determine the Na^+ pump activity, and hence the rate of ouabain-sensitive $^{86}Rb^+$ uptake.

In studies in which Na^+ pump activity is to be estimated, sufficient intracellular Na^+, Mg^{2+} and ATP and extracellular K^+ or Rb^+ should be available to the Na^+ pump so that the pump mechanism is turning over at a near maximal rate. Since other activators or substrates are in excess, Na^+ loading should provide this condition. It has been shown previously that Na^+ loading of thin slices obtained from left ventricular muscle of guinea pig hearts increases the rate of ouabain-sensitive $^{86}Rb^+$ uptake [15]. These studies, however, were performed with relatively short incubation periods. A preliminary study with a longer incubation time indicates that the values obtained with Na^+-loaded slices are not significantly different from those for slices without loading, indicating that Na^+ pool due to Na^+ loading might be depleted relatively rapidly. In the present studies, the possibility that the ouabain-sensitive $^{86}Rb^+$

uptake observed with relatively long incubation period is dependent on the rate of Na^+ influx rate was examined using atrial preparations of guinea pig hearts.

Methods

Ouabain-sensitive $^{86}Rb^+$ uptake was assayed as described by Ku et al. [2] with some modifications. Hearts were obtained from guinea pigs of either sex weighing 350—450 g, and were perfused (Langendorff preparations) for 5—10 min at 30°C with aerated (95% O_2/5% CO_2) Krebs-Henseleit solution of the following composition (mM): NaCl, 118.0; $NaHCO_3$, 27.2; KCl, 4.8; $MgSO_4$, 1.2; KH_2PO_4, 1.0; $CaCl_2$, 1.2 and glucose 11.1. After visible blood was removed from the tissue, the atrial muscle was excised. $^{86}Rb^+$ uptake was determined by incubating the atria with or without drugs for 35 min at 36.5°C, unless otherwise indicated, in a continuously aerated Krebs-Henseleit solution in which KCl was replaced with 2 mM RbCl and tracer amounts of $^{86}Rb^+$ (specific activity 0.15 Ci/mmol, New England Nuclear Corp., Boston MA). In a series of experiments in which the extracellular NaCl concentration was reduced, choline chloride was used as an osmotic substitute and 7.2 μM atropine sulfate was added to the medium to block the cholinergic effect [16]. When the calcium concentration was reduced, no osmotic substitute was used. $^{86}Rb^+$ uptake studies were performed with quiescent or electrically stimulated atria. In the latter preparations, atria were stimulated at an indicated rate with square-wave pulses of 4 ms duration at a voltage 50% above threshold with platinum field-stimulation electrodes using a Grass S44 stimulator (Grass Instruments Co., Quincy, MA). In the paired-pulse electrical stimulation studies, each 1 Hz stimulation was followed 90 ms later by a second stimulation of equal intensity. Under these conditions, atrial preparations consistently responded with two action potentials and a single twitch. When the incubation time for $^{86}Rb^+$ uptake was 35 min, Na^+ preloading in a K^+-free solution at 0°C failed to significantly affect either ouabain-sensitive or insensitive $^{86}Rb^+$ uptake in atrial preparations. Thus, preincubation for Na^+ loading was omitted in the present study. After incubation in the presence of $^{86}Rb^+$, atria were rinsed once in a solution containing the same ionic components, but without $^{86}Rb^+$. The atria were blotted on filter paper. After weighing the tissue, the amount of radioactivity in the tissue was assayed using a gamma scintillation spectrometer. Ouabain-sensitive $^{86}Rb^+$ uptake was calculated by subtracting the value of $^{86}Rb^+$ uptake observed in the presence of 0.3 mM ouabain from that observed in its absence.

The binding of non-labelled ouabain to (Na^+ + K^+)-ATPase in atrial preparations under various conditions was estimated from the reduction in the initial velocity of ATP-dependent [^{3}H]ouabain binding to homogenates obtained from those preparations as described by Ku et al. [2]. Left atrial preparations were incubated at 36.5°C in aerated (95% O_2/5% CO_2) Krebs-Henseleit solution in which KCl was replaced with 2 mM RbCl, in the presence of various concentrations of ouabain. After a 35 min incubation, preparations were removed from the incubation bath and were homogenized immediately at 0°C with a Polytron^R homogenizer (Brinkmann Instruments, Westbury, NY) in 1 mM

EDTA, 10 mM Tris-HCl buffer (30 mg tissue/ml of buffer). The homogenates (final concentration 3 mg tissue/ml) were incubated with 10 nM [^{3}H]ouabain (specific activity 16.4 Ci/mmol, Amersham, Arlington Heights, IL) in a medium containing 200 mM NaCl, 5 mM $MgCl_2$, 5 mM Tris-ATP and 50 mM Tris-HCl buffer (pH 7.5) at 37°C for 2 min. Preliminary experiments indicated that the ATP-dependent [^{3}H]ouabain binding to atrial homogenates was linear at least during the first 2 min of incubation at 37°C under the present experimental conditions. The binding reaction was started by adding the homogenate into a prewarmed incubation mixture and was terminated by filtering aliquots of the mixture through nitrocellulose filters (pore size, 0.8 μm, Millipore Corp., Bedford, MA) which separated bound and unbound [^{3}H]ouabain. The filter was washed once with 5 ml of an ice-cold solution containing non-radioactive ouabain (0.3 mM) and 50 mM Tris-HCl buffer (pH 7.5). Filter was dissolved in ethyleneglycol monomethylether before counting. The radioactivity was estimated with a liquid scintillation spectrometer. Counting efficiency (approximately 25%) was monitored by the external standard channel ratio method which was occasionally calibrated with internal standards. ATP-dependent [^{3}H]ouabain binding (specific binding) is the difference in the binding observed in the presence and absence of ATP.

Ouabain (ouabain octahydrate), and choline chloride were purchased from Sigma Chemical Co., St. Louis, MO. Monensin sodium was obtained from Lilly Laboratories, Eli Lilly and Co., Indianapolis, IN. α-Dihydrograyanotoxin II and grayanotoxin I were kindly supplied by Dr. Junkichi Iwasa of Okayama University, Okayama, Japan. Other chemicals were of reagent grade.

Statistical analyses were performed by Student's *t*-test. Criterion for statistical significance was a P value of less than 0.05.

Results

Effects of electrical stimulation on ouabain-sensitive $^{86}Rb^+$ uptake

The influence of electrical stimulation on ouabain-sensitive $^{86}Rb^+$ uptake was examined first. Atrial preparations were incubated at 36.5°C with or without electrical stimulation in a modified Krebs-Henseleit solution in which KCl was replaced with 2 mM RbCl containing a tracer amount of ^{86}RbCl. During a 60 min incubation period, ouabain-sensitive $^{86}Rb^+$ uptake increased almost linearly with time of incubation (data not shown). Preliminary experiments indicate that a longer incubation time resulted in a more accurate estimate of the ouabain-sensitive $^{86}Rb^+$ uptake, since the difference between the total and non-saturable uptake, observed in the absence and presence of 0.3 mM ouabain, respectively, increased with time. The variance of the data, however, were minimal with a 30—40 min incubation as a longer incubation resulted in an occasional large variation in the observed value, probably reflecting the deterioration of the part of tissues. Thus, a 35 min incubation period was employed in the following studies. When the incubation for $^{86}Rb^+$ uptake was carried out for 35 min, a 30 min Na^+ preloading in a cold (0°C) K^+-free solution failed to significantly affect the ouabain-sensitive $^{86}Rb^+$ uptake in atrial preparations (data not shown). Therefore, preincubation for Na^+ loading was omitted in the subsequent series of experiments.

Ouabain-sensitive $^{86}Rb^+$ uptake of quiescent atria was 16 nmol/mg tissue per 35 min (Table I). This value was approximately 3—4 times greater than corresponding values reported for ventricular slices of guinea pig hearts (approximately 0.95 nmol/mg tissue per 8 min) [2,5,6]. The ouabain-sensitive $^{86}Rb^+$ uptake was markedly enhanced by electrical stimulation of the atrial preparations (Table I). The degree of enhancement was proportional to the frequency of electrical stimulation: 29 ± 21, 118 ± 9.4 and 201 ± 26% increases (mean ± S.E. of 4—11 experiments) above non-stimulated preparations were observed at 0.5, 1.5 and 3.0 Hz, respectively. The nonspecific $^{86}Rb^+$ uptake, observed in the presence of 0.3 mM ouabain, was 3—4 nmol/mg tissue per 35 min, and was unaffected by electrical stimulation.

The enhancement of ouabain-sensitive $^{86}Rb^+$ uptake by electrical stimulation might be due to membrane depolarization or some changes associated with muscle contraction. In order to choose between these possibilities, experiments were performed using paired-pulse electrical stimulation which increases the number of membrane depolarizations without altering the number of contractions [17]. Under the present experimental conditions, atrial preparations consistently responded to the paired-pulse with two action potentials and a single twitch when the interval between the paired stimuli was 90 ms (data not shown). As shown in Table II, the ouabain-sensitive $^{86}Rb^+$ uptake of atria which were contracting at 1 Hz with paired-pulse stimulation was significantly higher than that of atria stimulated at 1 Hz with single-pulse stimulations and was similar to that of atria stimulated at 2 Hz with single-pulse stimulations. In these studies, in contrast to Table I, the incubation time was only 15 min. The results clearly indicate that the increase in ouabain-sensitive $^{86}Rb^+$ uptake by electrical stimulation is dependent on the number of membrane depolarizations rather than on the number of contractions.

Effects of Na^+-influx rates on ouabain-sensitive $^{86}Rb^+$ uptake

In order to uncover the mechanism by which stimulation-induced depolarization enhances ouabain-sensitive $^{86}Rb^+$ uptake, the influence of several conditions which alter Na^+ influx was examined. Reducing the extracellular Na^+ concentration lowers the Na^+ influx rate and at the same time increases the Ca^{2+} influx in the cardiac muscle [18,19]. Since a stimulation-induced incre-

TABLE I

EFFECTS OF ELECTRICAL STIMULATION ON OUABAIN-SENSITIVE $^{86}Rb^+$ UPTAKE

Values are mean ± S.E.

Stimulation frequency (Hz)	Nonspecific $^{86}Rb^+$ uptake (nmol/mg wet tissue per 35 min)	Ouabain-sensitive $^{86}Rb^+$ uptake (nmol/mg wet tissue per 35 min)
0	3.5 ± 0.3 (n = 11)	16.0 ± 0.6 (n = 11)
0.5	3.5 ± 0.5 (n = 4)	20.6 ± 3.4 (n = 4)
1.5	3.2 ± 0.1 (n = 11)	34.8 ± 1.5 * (n = 11)
3.0	3.9 ± 0.2 (n = 5)	48.1 ± 4.1 * (n = 5)

* Significantly different from the value observed at 0 Hz (no stimulation) ($P < 0.05$).

TABLE II

EFFECT OF PAIRED ELECTRICAL STIMULATION ON THE OUABAIN-SENSITIVE $^{86}Rb^+$ UPTAKE

Incubation for $^{86}Rb^+$ uptake was carried out at 36.5°C for 15 min.

Mode of stimulation	Ouabain-sensitive $^{86}Rb^+$ uptake (nmol/mg wet tissue per 15 min)
Single pulse (1 Hz)	16.3 ± 1.1 (n = 10)
Paired pulse (1 Hz)	21.8 ± 1.2 * (n = 10)
Single pulse (2 Hz)	22.0 ± 1.0 * (n = 5)

* Significantly different from the value observed with single pulse stimulation at 1 Hz.

ment in the intracellular Ca^{2+} concentration might cause a suppression of cardiac ($Na^+ + K^+$)-ATPase [20], the concentration of Ca^{2+} in the medium was reduced simultaneously in some experiments, in attempts to maintain a normal Ca^{2+}-influx rate. A reduction in extracellular Na^+ concentrations, with or without changes in the Ca^{2+} concentration, caused a significant decrease in the ouabain-sensitive $^{86}Rb^+$ uptake (Table III). The effect of a reduced extracellular Na^+ concentration was greater in quiescent atria than in electrically stimulated preparations. Particularly, the reduction of Na^+ concentration from 145 to 100 mM failed to cause a significant change in the ouabain-sensitive $^{86}Rb^+$ uptake when atrial preparations were stimulated at 1.5 Hz. Generally, the degree of the reduction in the $^{86}Rb^+$ uptake was smaller than the degree of the reduction in extracellular Na^+ concentration, and electrically stimulated preparations were less sensitive to the changes in extracellular Na^+ concentrations.

When the Na^+ concentration was reduced from 145 to 100 mM and the Ca^{2+} concentration was kept at 1.2 mM, the force of contraction of electrically stimulated atria increased significantly (data not shown). A reduction of extracellular Ca^{2+} concentration to 0.57 mM, together with the above reduction of the Na^+ concentration, caused the atrial preparations to contract with a similar force as controls when these preparations were stimulated at 1.5 Hz. The

TABLE III

EFFECTS OF EXTRACELLULAR Na^+ CONCENTRATION ON THE OUABAIN-SENSITIVE $^{86}Rb^+$ UPTAKE

Values are expressed as the percentage of the uptake observed in the presence of 145 mM Na^+ and 1.2 mM Ca^{2+}. The 100% values are 16.3 ± 0.6 nmol/mg per 35 min (n = 22, non-stimulated) and 35.5 ± 1.5 nmol/mg per 35 min (n = 10, stimulated). Values are mean ± S.E.

Na^+ (mM)	Ca^{2+} (mM)	Non-stimulated (%)	Stimulated (1.5 Hz) (%)
145	1.2	100	100
100 *	1.2	81.4 ± 5.1 ** (n = 5)	106.4 ± 12.4 (n = 5)
100 *	0.57	79.2 ± 4.8 ** (n = 5)	104.3 ± 4.6 (n = 5)
75 *	0.32	68.0 ± 3.6 ** (n = 10)	88.4 ± 5.5 (n = 10)
50 *	0.143	63.1 ± 1.8 ** (n = 5)	—
50 *	0.357	61.9 ± 4.2 ** (n = 5)	81.7 ± 3.4 ** (n = 9)

* Choline chloride plus atropine was used as osmotic substitute.

** Significantly different from the value observed in the presence of 145 mM Na^+ and 1.2 mM Ca^{2+} ($P < 0.05$).

difference in the extracellular Ca^{2+} concentration, however, failed to affect ouabain-sensitive $^{86}Rb^+$ uptake in these or in quiescent preparations (Table III). Several investigators have reported that the force of contraction of certain types of cardiac muscle is unchanged when the extracellular Na^+ and Ca^{2+} concentrations are altered simultaneously in such a way that the ratio $[Ca^{2+}]/[Na^+]^2$ is unchanged [21,22]. When the extracellular Na^+ is reduced to 50 mM, the above formula indicates that the Ca^{2+} concentration should be 0.143 mM. Under these conditions, however, the force of contraction of atrial preparations was barely detectable. The Ca^{2+} concentration of 0.357 mM in a modified Krebs-Henseleit solution in which Na^+ is reduced to 50 mM and K^+ is replaced with 2 mM Rb^+ caused atrial preparations to contract with a similar force as controls (data not shown). In the presence of 50 mM Na^+, the ouabain-sensitive $^{86}Rb^+$ uptake of quiescent atria was similar when the extracellular Ca^{2+} was either 0.143 mM or 0.357 mM (Table III). Similarly, extracellular Ca^{2+} concentration failed to affect the ouabain-sensitive $^{86}Rb^+$ uptake by isolated atria in the presence of 100 mM Na^+. These results indicate that moderate alterations of extracellular Ca^{2+} concentrations fail to affect ouabain-sensitive $^{86}Rb^+$ uptake in either quiescent or electrically stimulated atria. The change in the force of contraction in electrically stimulated atria also fails to affect $^{86}Rb^+$ uptake.

The rate of Na^+ influx in cardiac muscle cells may be increased using a Na^+-specific ionophore, monensin [23], or grayanotoxin, a poison obtained from the leaves of Ericaceae plants [24,25]. In quiescent atria, 1—10 μM monensin increased the ouabain-sensitive $^{86}Rb^+$ uptake significantly (Table IV). The maximal effect of monensin was observed with a concentration of 2.5 μM which produced an approximately 50% increase in the ouabain-sensitive $^{86}Rb^+$ uptake in quiescent atria (Table IV). The maximal enhancement of ouabain-

TABLE IV

EFFECTS OF MONENSIN AND GRAYANOTOXINS ON THE OUABAIN-SENSITIVE $^{86}Rb^+$ UPTAKE

Values are expressed as the percentage of the uptake observed in the absence of drug. The 100% values are 17.4 ± 0.7 nmol/mg per 35 min (n = 13, non-stimulated) and 34.5 ± 2.0 nmol/mg per 35 min (n = 12, stimulated). Values are mean ± S.E.

Addition (μM)	Non-stimulated (%)	Stimulated (1.5 Hz) (%)
No drug	100	100
Monensin		
1	122.1 ± 3.6 * (n = 4)	
2.5	154.3 ± 7.0 * (n = 4)	113.6 ± 7.5 (n = 5)
5	128.4 ± 4.2 * (n = 4)	
10	120.4 ± 4.7 * (n = 9)	82.5 ± 6.3 * (n = 5)
Grayanotoxin I		
0.25	89.2 ± 3.3 (n = 4)	
2.5	40.7 ± 2.1 * (n = 4)	
10	22.0 ± 3.1 * (n = 4)	
α-Dihydrograyanotoxin II		
1	89.6 ± 6.8 (n = 5)	73.3 ± 4.6 * (n = 4)
10	68.1 ± 7.5 * (n = 5)	

* Significantly different from the value observed in the absence of drug ($P < 0.05$).

sensitive $^{86}Rb^+$ uptake induced by monensin, however, was markedly smaller than that seen with electrical stimulation (see Table I). A further increase in the concentration of monensin in the medium resulted in a smaller increase in the $^{86}Rb^+$ uptake. The ouabain-sensitive $^{86}Rb^+$ uptake of atrial preparations was significantly less sensitive to the stimulatory action of monensin when the preparations were electrically stimulated at 1.5 Hz (Table IV). Electrical stimulation at 3 Hz caused approximately a 38% increase in the ouabain-sensitive $^{86}Rb^+$ uptake above that of preparations being stimulated at 1.5 Hz (Table I). Thus, the failure of monensin to enhance the $^{86}Rb^+$ uptake in electrically stimulated preparations is unlikely to result from the fact that the $^{86}Rb^+$ uptake has already plateaued in preparations which are electrically stimulated at 1.5 Hz. At a higher concentration, monensin inhibited $^{86}Rb^+$ uptake in electrically stimulated atrial preparations. These findings suggest that the action of monensin is not limited to an enhancement of $^{86}Rb^+$ uptake, presumably resulting from an increased intracellular Na^+ concentration. In addition, monensin inhibits $^{86}Rb^+$ uptake; perhaps as a result of, or independent from, its effect on the Na^+ influx rate.

Grayanotoxin I and α-dihydrograyanotoxin II have been shown to increase the resting Na^+ permeability of the cell membrane [24,25], and not to affect (Na^+ + K^+)-ATPase activity [15]. These compounds, however, failed to enhance ouabain-sensitive $^{86}Rb^+$ uptake under the present experimental conditions and apparently suppressed it in a concentration-dependent manner (Table IV). In electrically stimulated preparations, 1 μM α-dihydrograyanotoxin II also decreased ouabain-sensitive $^{86}Rb^+$ uptake. These results indicate that monensin and grayanotoxins are both capable of inhibiting ouabain-sensitive $^{86}Rb^+$ uptake in atrial muscle. Monensin only enhanced the $^{86}Rb^+$ uptake of non-stimulated preparations at low concentrations.

Effect of temperature on ouabain-sensitive $^{86}Rb^+$ uptake

The above studies indicate that $^{86}Rb^+$ uptake in electrically stimulated preparations are less sensitive than quiescent preparations to conditions that may affect transmembrane Na^+ influx. To determine if the stimulated preparations are generally less sensitive to altered conditions, the effect of temperature on the ouabain-sensitive $^{86}Rb^+$ uptake was compared in quiescent and electrically stimulated atria. Incubation for $^{86}Rb^+$ uptake was performed at 36.5, 30 and 23°C for 35 min in quiescent atria and at 36.5 and 30°C in atria electrically stimulated at 1.5 Hz. In quiescent atria, ouabain-sensitive $^{86}Rb^+$ uptake was clearly temperature dependent; 74.3 ± 4.4% (n = 10) at 30°C and 47.0 ± 1.3% (n = 5) at 23°C of values observed at 36.5°C. In contrast, there was no significant difference in the ouabain-sensitive $^{86}Rb^+$ uptake at 36.5°C and 30°C in electrically stimulated atrial preparations; values at 30°C were 101.3 ± 4.3% (n = 7) of those observed at 36.5°C. These findings indicate that with regard to ouabain-sensitive $^{86}Rb^+$ uptake, the electrically stimulated atria is more resistant to alterations in temperature than quiescent atria.

The effect of electrical stimulation on the ouabain-induced inhibition of $^{86}Rb^+$ uptake

If intracellular Na^+, which results from Na^+ influx associated with electrical

stimulation, is available to the Na^+ pump, then the rate of ouabain binding to ($Na^+ + K^+$)-ATPase might be enhanced by the electrical stimulation of atrial preparations. This possibility was investigated.

Inhibition of $^{86}Rb^+$ uptake by ouabain was examined by incubating the atria in the presence or absence of various concentrations of ouabain. The incubation for $^{86}Rb^+$ uptake was started by the addition of the atrial preparation into a prewarmed K^+-free solution containing labelled and unlabelled Rb^+ and various concentrations of ouabain. $^{86}Rb^+$ uptake was inhibited by ouabain in a concentration-dependent manner both in quiescent and electrically stimulated atria (Fig. 1). Electrical stimulation of atria at 1.5 Hz caused a shift of the concentration-inhibition curve to the left (Fig. 1). Concentration-inhibition curve was further shifted to the left by increasing the stimulation frequency from 1.5 Hz to 3 Hz. The slope of the curves was apparently unchanged by electrical stimulation (Fig. 1). These results indicate that the apparent affinity of the Na^+ pump for ouabain increases in a manner dependent on electrical stimulation. Since the incubation time for $^{86}Rb^+$ uptake (35 min) is not enough to reach equilibrium of the ouabain-($Na^+ + K^+$)-ATPase interaction, the apparent increase in sensitivity of the Na^+ pump towards ouabain may

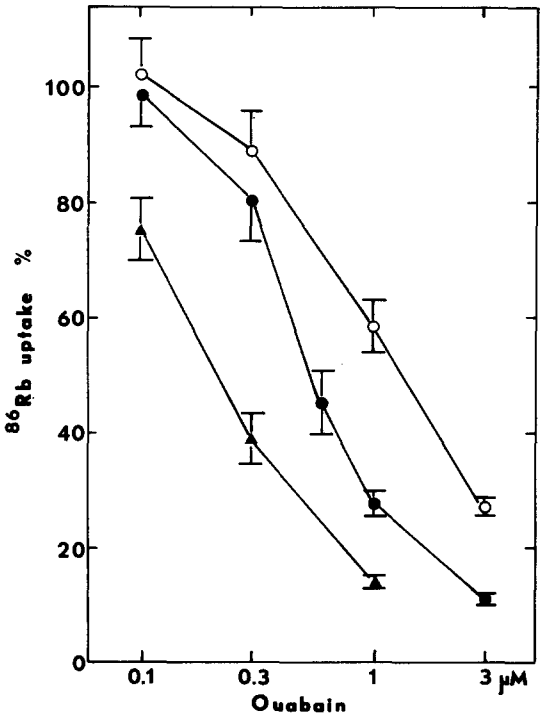

Fig. 1. Effect of the electrical stimulation on the ouabain sensitivity of $^{86}Rb^+$ uptake. Guinea pig left atria were incubated in the presence or absence of various concentrations of ouabain for 35 min at 36.5°C in a modified Krebs-Henseleit solution in which K^+ was replaced with 2 mM Rb^+ and tracer amounts of $^{86}Rb^+$. The ouabain-sensitive $^{86}Rb^+$ uptake observed in the absence of ouabain (the difference in values observed in the absence and presence of 0.3 mM ouabain) was set at 100%. ○, quiescent atria; ●, electrical stimulation at 1.5 Hz, and ▲, electrical stimulation at 3 Hz. Each point represents the mean of five experiments. Vertical lines indicate the S.E.

be due to an enhancement of the rate of ouabain binding to the enzyme brought about by electrical stimulation.

In order to ascertain whether the apparent increase in the sensitivity of Na^+ pump to ouabain under electrical stimulation is due to an enhancement of the rate of ouabain binding, the fraction of ($Na^+ + K^+$)-ATPase occupied by non-labelled ouabain was estimated by means of a reduction in the initial velocity of ATP-dependent [^{3}H]ouabain binding to homogenates obtained from atrial preparations. Atria were incubated under the same conditions as in the ^{86}Rb uptake studies in the presence or absence of ouabain. Since ATP-dependent [^{3}H]-ouabain binding to the homogenates obtained from control atria (no previous exposure to ouabain) increased linearly with time during the first 2 min of the incubation (data not shown), a 2 min incubation period was chosen for [^{3}H]-ouabain binding studies. The initial velocity of the ATP-dependent [^{3}H]-ouabain binding by homogenates obtained from control atria was set at 100% (Table V). When atrial preparations were incubated in the absence of ouabain, and the [^{3}H]ouabain binding was assayed after homogenization of the tissue, electrical stimulation of atria either at 1.5 Hz or 3 Hz had no effect on the initial velocity of the ATP-dependent [^{3}H]ouabain binding. Thus, the control data obtained from quiescent and two electrically stimulated atria were combined. Concentrations of ouabain which produced 50% inhibition of ouabain-sensitive $^{86}Rb^+$ uptake under these experimental conditions were 1.4 (quiescent), 0.5 (1.5 Hz) and 0.2 μM (3 Hz) (Fig. 1). Thus, atrial preparations were incubated for 35 min in the presence of the above concentrations of ouabain, and the initial velocity of [^{3}H]ouabain binding to homogenates obtained from these preparations were examined. The incubation of quiescent atrial preparations in the presence of ouabain caused a concentration-dependent reduction in the initial velocity of ATP-dependent [^{3}H]ouabain binding (Table V), indicating the concentration-dependent occupancy of ($Na^+ + K^+$)-ATPase by non-labelled ouabain. The concentrations of ouabain which

TABLE V

EFFECTS OF ELECTRICAL STIMULATION ON THE BINDING OF OUABAIN TO ($Na^+ + K^+$)-ATPase

Atrial preparations were incubated in the presence of various concentrations of ouabain as indicated. After the incubation, tissues were homogenized and the fractional occupancy of ouabain binding sites on ($Na^+ + K^+$)-ATPase was estimated from the reduction in the initial velocity of ATP-dependent [^{3}H]ouabain binding. The concentration of [^{3}H]ouabain for the assay of fractional occupancy by non-labelled oubain was 10 nM. Values are expressed as the percentage of the initial velocity of the binding (cpm/mg tissue per 2 min) observed with the homogenates obtained from non-ouabain-treated atria (control). The 100% value is 202 ± 3.7 cpm/mg per 2 min (n = 12). The control data (100% value) obtained from quiescent and electrically stimulated atria are combined. Percent reduction in the [^{3}H]ouabain binding indicates percent occupancy of the binding sites by non-labelled ouabain.

Stimulation frequency (Hz):	0	1.5	3
Non-labelled ouabain (μM)	Specific [^{3}H]ouabain binding (%)		
0 (control)		100	
0.2	93.9 ± 4.3 (n = 6)		73.6 ± 1.6 * (n = 8)
0.5	63.6 ± 2.8 (n = 4)	48.1 ± 4.0 * (n = 6)	
1.4	57.1 ± 1.8 (n = 4)		

* Significantly different from 0 Hz.

produced a 50% inhibition of the $^{86}Rb^+$ uptake (1.4 μM) caused a 43% reduction of the initial velocity of [^{3}H]ouabain binding. When atrial preparations were electrically stimulated at 1.5 Hz, a lower concentration (0.5 μM) of ouabain caused a 50% reduction of the initial velocity of ATP-dependent [^{3}H]ouabain binding, indicating that a similar occupancy of ouabain binding sites on ($Na^+ + K^+$)-ATPase in atrial preparations occurred at a lower concentration of ouabain when the preparations were stimulated. This concentration is similar to that which causes a 50% inhibition of $^{86}Rb^+$ uptake in atrial preparations stimulated at 1.5 Hz. When the atrial preparations were stimulated at 3 Hz, 0.2 μM ouabain produced a greater fractional occupancy of the binding sites than that observed in quiescent preparations. The degree of reduction, however, in the initial velocity of [^{3}H]ouabain binding was somewhat smaller than that of $^{86}Rb^+$ uptake under similar conditions (26% and 50%, respectively). This discrepancy may result from the overloading of the Na^+ pump associated with a high Na^+ influx rate and an enhanced inhibition of Na^+ pump. When atria were incubated at 36.5°C with 0.2 μM ouabain in a K^+-free solution containing 2 mM RbCl, and electrically stimulated at 3 Hz, toxic manifestations of ouabain became apparent after 25 min of the incubation, i.e. atrial preparations fails to follow electrical stimulations. These results, therefore,

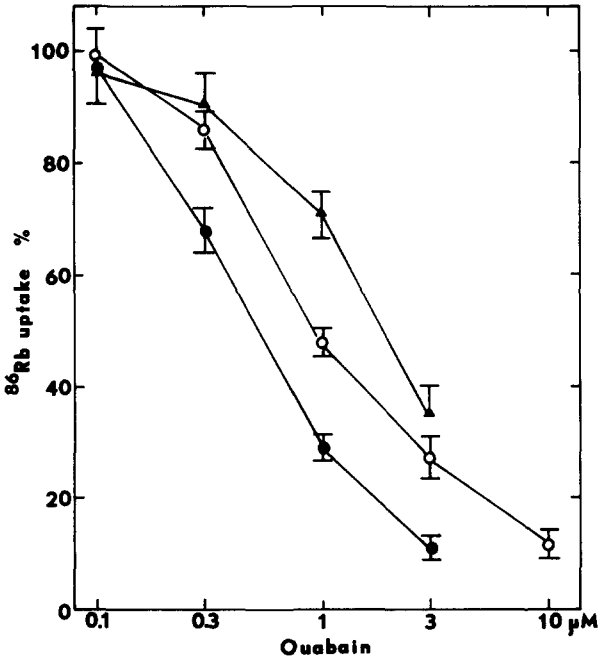

Fig. 2. Effect of monensin and α-dihydrograyanotoxin II on the ouabain sensitivity of $^{86}Rb^+$ uptake in quiescent atria. Guinea pig left atria were incubated in the presence or absence of various concentrations of ouabain for 35 min at 36.5°C in a modified Krebs-Henseleit solution in which K^+ was replaced with 2 mM Rb^+ and tracer amounts of $^{86}Rb^+$. Incubations for the $^{86}Rb^+$ uptake were performed in the presence of 2.5 μM monensin (●), 1 μM α-dihydrograyanotoxin II (▲) or in the absence of these agents (○). The ouabain-sensitive $^{86}Rb^+$ uptake observed in the absence of ouabain (the difference in values observed in the absence and presence of 0.3 mM ouabain) was set at 100%. Each point represents the mean of five experiments. Vertical lines indicate the S.E.

strongly suggest that the rate of ouabain binding is enhanced by electrical stimulation, indicating that stimulation increases the intracellular Na^+ available to the Na^+ pump.

Effects of monensin and α-dihydrograyanotoxin II on the ouabain-induced inhibition of $^{86}Rb^+$ uptake

Ouabain-sensitive $^{86}Rb^+$ uptake was enhanced by monensin and suppressed by grayanotoxins (Table IV). In order to see whether these compounds increase the intracellular Na^+ available to the Na^+ pump, the effect of these agents on ouabain sensitivity of $^{86}Rb^+$ uptake was examined in quiescent atria. As shown in Fig. 2, 2.5 μM monensin shifted the concentration-inhibition curve for ouabain-induced inhibition of $^{86}Rb^+$ uptake to the left without altering the slope of the curve. The magnitude of the shift in dose-response curve was similar to that induced by 1.5 Hz electrical stimulation (Fig. 1). Thus, it appears that monensin increases intracellular Na^+ available to the Na^+ pump. On the other hand, α-dihydrograyanotoxin II failed to shift the dose-response curve to the left but apparently shifted it to the right (Fig. 2). The above finding suggests that α-dihydrograyanotoxin II fails to increase intracellular Na^+ available to the Na^+ pump under the present experimental conditions.

Discussion

Ouabain-sensitive $^{86}Rb^+$ uptake is widely used as a means of estimating Na^+ pump activity [1—6]. The intracellular Na^+ concentration, however, is suggested as the determinant of Na^+-K^+ exchange reaction under certain conditions [9,11], and therefore, the rate of ouabain-sensitive $^{86}Rb^+$ uptake might be dependent on the rate of Na^+ influx, which provides Na^+ available to the Na^+ pump.

When Na^+ loading enhances the rate of $^{86}Rb^+$ uptake, and the assays are performed under these enhanced conditions, one may assume that the availability of intracellular Na^+ is not limiting the ability of Na^+ pump to transport Rb^+ in exchange with Na^+. When Na^+-loaded ventricular slices are incubated for a relatively short time period for $^{86}Rb^+$ uptake, this condition is probably satisfied [2,5,6]. After a long incubation period in atrial preparations, however, the ouabain-sensitive $^{86}Rb^+$ uptake was unaffected by Na^+ preloading. Our preliminary studies indicate that even in ventricular slices, a preliminary incubation (Na^+ preloading) in a K^+-free solution at 0°C for 30 min failed to affect the value of ouabain-sensitive $^{86}Rb^+$ uptake observed with a long incubation period (data not shown). These findings indicate that either intracellular Na^+ is adequate without Na^+ preloading or Na^+ preloading fails to provide the intracellular Na^+ concentration available to Na^+ pump for such a long time period. Preliminary microelectrode studies indicate that the transmembrane potential recovers rapidly in Na^+-loaded atrial preparations. This finding suggests that the transmembrane gradient of K^+, and perhaps also that of Na^+, establishes rapidly, well before the average intracellular Na^+ and K^+ concentrations return to normal levels. Thus, even in Na^+-loaded cells, Na^+ may be the determinant of $^{86}Rb^+$ uptake after a relatively long incubation period. If the intracellular Na^+ concentration is insufficient to fully activate the Na^+ pump, conditions

which increase transmembrane Na^+ influx should stimulate $^{86}Rb^+$ uptake. One way to increase Na^+ influx is electrical stimulation [26]. It is reported that the transmembrane Na^+ flux increases in proportion to the frequency of the stimulation. Electrical stimulation markedly increased the ouabain-sensitive $^{86}Rb^+$ uptake by isolated atrial preparations. The increase was proportional to the frequency of stimulation. Although electrical stimulation also changes transmembrane Ca^{2+} flux and causes the muscle to contract, ouabain-sensitive $^{86}Rb^+$ uptake was influenced neither by a moderate change in extracellular Ca^{2+} concentration nor by a change in the force of contraction. Moreover, the experiments using paired-pulse electrical stimulation demonstrate that the enhancement of Na^+ pump activity is dependent on the number of membrane depolarizations rather than on the number of contractions.

Membrane depolarizations may enhance ouabain-sensitive $^{86}Rb^+$ uptake by several mechanisms. These include increased Na^+ influx rate, changes in the properties of cell membrane, or elimination of the electrical gradient against which Na^+ has to be transported. Ouabain-sensitive $^{86}Rb^+$ uptake, however, was reduced under the condition which reduces Na^+ influx, namely when the extracellular Na^+ concentrations were decreased. The effects of altered extracellular Na^+ concentration on $^{86}Rb^+$ uptake was different with or without electrical stimulation; electrically stimulated atria being less sensitive to reduced extracellular Na^+ concentrations than quiescent atria. These findings indicate that factor(s) other than the alteration of intracellular Na^+ concentration which occurs as a result of depolarization may also play an important role in regulating Na^+ pump activity. Horowicz and Gerber [27] observed that partial depolarization induced by sodium azide stimulates Na^+ pump activity in frog sartorius muscle. While it is tempting to speculate that a reduced resistance for Na^+ transport by a reduction in the transmembrane electrical gradient results in an enhancement of the transport rate, Brinley and Mullins [28] demonstrated that the rate of Na^+ and K^+ transport is independent of the membrane potential in squid axon.

Concentrations of monensin which increase Na^+ influx in electrically stimulated Purkinje fibers of dog heart without causing an overt membrane depolarization [23], increased ouabain-sensitive $^{86}Rb^+$ uptake in quiescent guinea pig atrial preparations. These findings are consistent with a previous report by Clausen and Hansen [29] who demonstrated that veratrine, an agent which increases Na^+ influx, stimulated the ouabain-sensitive $^{42}K^+$ influx. The efficacy of monensin to enhance ouabain-sensitive $^{86}Rb^+$ uptake was substantially smaller compared to electrical stimulation (Tables I and IV). One can speculate that high concentrations of monensin exert some kind of inhibitory effect on ouabain-sensitive $^{86}Rb^+$ uptake. The inhibitory effect was predominant in electrically stimulated preparations in which the rate of Na^+ influx is already increased. These inhibitory effects may even be greater with the grayanotoxins which failed to stimulate ouabain-sensitive $^{86}Rb^+$ uptake in quiescent atrial preparations. These inhibitory effects may account for the failure of monensin to enhance ouabain-sensitive $^{86}Rb^+$ uptake as much as electrical stimulation does (Table IV). Alternatively, Na^+ which enters the cell via the fast Na^+ channel increases the Na^+ available to the Na^+ pump more effectively than either the Na^+ ionophore- or grayanotoxin-induced Na^+ influx in guinea

pig atrial muscles. Another possibility is that some unidentified factor(s) associated with membrane depolarization may affect the rate of Na^+ pump activity. The present data with grayanotoxins are at variance with a previous report by Ku et al. [15], in which grayanotoxins are demonstrated to increase the ouabain-sensitive $^{86}Rb^+$ uptake in ventricular slices of guinea pig hearts. It is unknown whether the discrepancy is due to the difference in the type and source of the tissue preparation, the length of the incubation period, or other factor(s).

Does electrical stimulation or monensin really increase Na^+ available to ($Na^+ + K^+$)-ATPase? The question may be answered by studies on the ouabain-induced inhibition of $^{86}Rb^+$ uptake. The $^{86}Rb^+$ uptake was inhibited by ouabain in a concentration-dependent manner. Apparently, the inhibition is due to the binding of ouabain to the Na^+ pump system. The binding of ouabain to isolated ($Na^+ + K^+$)-ATPase, an enzymatic representation of Na^+ pump, occurs in the presence of specific combinations of ligands [30]. Such a binding observed in vitro in the presence of ATP, Na^+ and Mg^{2+} represents the binding of cardiac glycosides to ($Na^+ + K^+$)-ATPase in beating hearts [31]. The binding is enhanced by Na^+ [30]. In intact cells, the Na^+ pump is stimulated by the intracellular Na^+ [32,33]. Therefore, the binding of ouabain to ($Na^+ + K^+$)-ATPase and ensuing Na^+ pump inhibition would be enhanced by increasing intracellular Na^+ concentration. Electrical stimulation or monensin caused a shift of concentration-inhibition curve for ouabain to the left, indicating that electrical stimulation or monensin increases the sensitivity of Na^+ pump towards ouabain. Moreover, [^{3}H]ouabain binding studies (Table V) demonstrate that the stimulation-induced shift of the concentration-inhibition curve is due to the enhancement of ouabain binding to ($Na^+ + K^+$)-ATPase. Since intracellular Na^+ enhances the binding of ouabain to the Na^+ pump, either electrical stimulation or monensin increases the availability of intracellular Na^+ for the Na^+ pump or ($Na^+ + K^+$)-ATPase. Under the present experimental conditions, however, grayanotoxins failed to enhance Na^+ available to the Na^+ pump and to stimulate $^{86}Rb^+$ uptake in atrial preparations.

In conclusion, the intracellular Na^+ concentration seems to be the major determinant of the ouabain-sensitive $^{86}Rb^+$ uptake in quiescent atria. Electrical stimulation appears to increase the $^{86}Rb^+$ uptake apparently by effectively increasing the intracellular Na^+ available to the Na^+ pump. Thus, the ouabain-sensitive $^{86}Rb^+$ uptake by atrial preparations which are electrically stimulated at a relatively high frequency seems to represent the maximal capacity of the Na^+ pump in this tissue.

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