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CALCIUM UPTAKE IN MITOCHONDRIA AND VESICLES OF HEART AND SKELETAL MUSCLE IN PRESENCE OF POTASSIUM, SODIUM, *k*-STROPHANTHIN AND PENTOBARBITAL*

H. DRANSFELD, K. GREEFF, A. SCHORN and B. T. TING

Institute of Pharmacology, University of Dusseldorf, Germany

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Abstract—$^{45}Ca^{++}$ uptake in mitochondria and vesicles of heart and skeletal muscle was measured in the presence of a low concentration of calcium. The following results were obtained. (1) In mitochondria there was a rapid and brief Ca^{++} uptake if succinate was present; when ATP was added, Ca^{++} uptake was slow and prolonged; cyanide prevented the rapid Ca^{++} accumulation and azide inhibited the slow Ca^{++} uptake. In vesicles ATP was required for Ca^{++} uptake and this was increased by oxalate, phosphate, or an ATP regenerating system. (2) *k*-Strophanthin (10^{-8}–10^{-4} M) had no significant effect on Ca^{++} uptake in mitochondria or vesicles. (3) Pentobarbital (1–6 mM) inhibited Ca^{++} uptake in mitochondria of heart and white and red skeletal muscle but had no effect on Ca^{++} uptake in vesicles. (4) The K^+:Na^+ ratio in the incubation medium had an effect on Ca^{++} uptake in mitochondria and vesicles of heart muscle and in mitochondria of red skeletal muscle. In mitochondria Ca^{++} uptake was minimal at the K^+:Na^+ ratio of 1, and raising the K^+:Na^+ ratio up to 10 approximately doubled Ca^{++} uptake. In vesicles the substitution of Na^+ for K^+ in the incubation medium diminished Ca^{++} uptake, if Ca^{++} uptake was not increased by oxalate, phosphate, or an ATP regenerating system.

EVIDENCE that Ca^{++} storage in vesicles and mitochondria is influenced by the intracellular K^+:Na^+ ratio is relevant to the understanding of the physiological coupling of the K^+ and Na^+ exchange with the changes in intracellular Ca^{++} concentration and of the mechanism of action of the digitalis glycosides. Calcium is assumed to play a central role in the contraction cycle of the muscle cell. A rise in Ca^{++} concentration in the cytoplasm leads to activation of the myofibril ATPase and thus to muscle contraction,[1–3] and a fall in Ca^{++} concentration enables the muscle to relax.[4] This raises the question: how is the Ca^{++} concentration in the cytoplasm controlled? While it had earlier been assumed that there merely occurs an exchange of Ca^{++} at the cell membrane during the contraction cycle, newer studies have shown that Ca^{++} can be bound and inactivated intracellularly.[5–8] Several mechanisms are being discussed for this phenomenon:[9] (1) binding to protein, (2) accumulation in vesicles of the sarcoplasmic reticulum, and (3) accumulation in mitochondria. It is evident that we have to know more about the kinetics of the Ca^{++} exchange in vesicles and mitochondria.

The present results show that the uptake of Ca^{++} by isolated mitochondria and vesicles depends decisively on the concentrations of Na^+ and K^+. An inflow of Na^+

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through the cell membrane and thus a change in the Na^+:K^+ ratio in the cytoplasm is known to occur prior to the muscle contraction. This makes it conceivable that the K^+ and Na^+ exchange at the cell membrane is coupled with the Ca^{++} uptake in vesicles and mitochondria.

METHODS

Dissection of the muscles

The hearts from freshly killed rabbits were excized as rapidly as possible and placed on ice cubes. Cattle hearts, back muscles of rabbits and chest muscles of pigeons were also used. Further operations were done in the cold room at 4°. The tissue was homogenized and diluted tenfold, the medium having a final concentration of 0·25 M saccharose and 50 mM imidazole buffer (pH 6·8).

Isolation of the mitochondria

The muscle was further homogenized in the Potter–Elvehjem apparatus with Teflon pestle at 3000 rpm and centrifuged for 5 min in the Christ junior III KS at 3300 rpm (approx. 700 *g*). The supernatant was centrifuged for 10 min in the Spinco L (rotor 30) at 9000 rpm (approx. 10,000 *g*). The residue was taken up in saccharose–imidazole solution to give a protein concentration of 1–2 mg/ml.

Isolation of the vesicles

First method. The supernatant obtained during the isolation of the mitochondria was centrifuged for 2 hr in the Spinco L (rotor 30) at 25,000 rpm (approx. 30,000 *g*) and the residue was taken up in saccharose–imidazole solution. The protein concentration was 1·5–2·5 mg/ml.

Second method. The homogenate was centrifuged for 10 min at 3800 rpm (approx. 1000 *g*) and the residue was resuspended and centrifuged again. Both supernatants were combined and centrifuged at 9000 *g* for 10 min, and their supernatant was centrifuged for 45 min at 78,000 *g*. The residue was resuspended in the same solution and centrifuged at 78,000 *g* a second time. The residue was suspended in a solution of 0·25 M saccharose and 3 mM histidine–imidazole buffer (pH 6·8) to give a protein concentration of 1–2 mg/ml.

Third method. The muscle mash was homogenized in saccharose–imidazole solution using the Bühler cutting homogenizer or Starmix, depending on the amount of muscle. The first method of fractional centrifugation was used to isolate the vesicles.

Fourth method. Homogenization was carried out in 0·3 M saccharose with 0·2 mM ascorbic acid (pH 7·0), using the cutting homogenizer of Bühler. The vesicles were separated by centrifugation at 60,000 *g* for 90 min (method of Briggs, Gertz and Hess)[10] and suspended in chloride (80 mM) oxalate (5 mM) solution.

That the mitochondria and vesicles were in good condition is shown by the observation that they took up Ca^{++}. Mitochondria lose the ability to take up Ca^{++} if they are kept for 4 hr at 4°, although respiration and the ability to hydrolyze ATP are not lost and might even be increased. We have used Fenn and Rahn's[11] method to evaluate the condition of freshly isolated mitochondria. It uses the ratio of ATP hydrolysis in the presence and absence of 2,4-DNP as a measure of the condition of the mitochondria. Cardiac mitochondria are supposed to have a ratio of 5–6. Our freshly isolated mitochondria had a ratio of 5·6. We did not determine Ca^{++} uptake and ATP

hydrolysis simultaneously, as we and other authors (e.g. Palmer and Posey,[12] using vesicles) did not find a valid relationship between the two.

The uptake of calcium in vesicles as shown in Table 2 is low; however, incubation medium and extent of calcium uptake are similar to those of Palmer and Posey.[12] Carsten[13] found a ten times greater calcium uptake, but he used five times more calcium and four times less protein in the medium.

TABLE 1. CALCIUM UPTAKE IN CARDIAC AND RED AND WHITE SKELETAL MUSCLE MITOCHONDRIA IN THE PRESENCE OF *k*-STROPHANTHIN AND PENTOBARBITAL*

	$^{45}Ca^{++}$ uptake (μM Ca^{++}/g protein ± S.E.)		
	Cardiac muscle	Red skeletal muscle	White skeletal muscle
—	78·4 ± 15·2 (6)	64·9 ± 16·3 (6)	49·5 ± 9·2 (6)
k-Strophanthin, 10^{-4} M	73·4 ± 14·7 (3)	52·1 ± 20·7 (4)	47·5 ± 13·0 (5)
k-Strophanthin, 10^{-5} M	77·7 ± 9·3 (9)	60·3 ± 13·8 (11)	50·0 ± 7·5 (12)
k-Strophanthin, 10^{-6} M	80·2 ± 10·1 (9)	59·5 ± 13·3 (12)	46·1 ± 7·3 (11)
k-Strophanthin, 10^{-7} M	80·5 ± 10·6 (8)	59·1 ± 13·1 (11)	48·1 ± 6·5 (12)
k-Strophanthin, 10^{-8} M	75·1 ± 18·3 (3)	64·6 ± 28·0 (3)	43·3 ± 13·1 (5)
Pentobarbital, 10 mM	—	15·3 (2)	5·6 (2)
Pentobarbital, 6 mM	31·1 ± 7·4 (6)	18·2 ± 25·5 (8)	23·0 ± 4·6 (8)
Pentobarbital, 3 mM	68·6 ± 15·7 (6)	61·1 ± 15·8 (8)	24·9 ± 7·1 (8)
Pentobarbital, 2 mM	78·5 ± 20·2 (3)	88·4 ± 23·0 (3)	44·8 (2)
Pentobarbital, 1 mM	85·6 ± 13·2 (6)	67·2 ± 20·4 (6)	55·6 ± 11·1 (8)

* Incubation medium: imidazole buffer (40 mM, pH 6·8), succinate (2 mM), ATP (3 mM), $MgCl_2$ (3 mM), $CaCl_2$ (0·06 mM), KCl (80 mM), Na^+ (10 mM). Time of incubation 10 min, temperature 25°. the number of experiments is in parentheses.

TABLE 2. CALCIUM UPTAKE IN VESICLES OF HEART MUSCLE IN THE PRESENCE OF SODIUM, POTASSIUM, PHOSPHATE, AN ATP REGENERATING SYSTEM AND *k*-STROPHANTHIN*

Incubation medium			% of total $^{45}Ca^{++}$ in the medium taken up by the vesicles ± S.E.	Ca^{++} uptake μM Ca^{++}/g protein ± S.E.	% Change of Ca^{++} uptake in the presence of *k*-strophanthin
Phosphate (mM)	K^+ (mM)	Na^+ (mM)			
—	110	—	7·6 ± 0·4 (12)	4·6 ± 0·2 (12)	+ 0·5 (5)
—	—	110	3·6 ± 0·8 (12)	3·3 ± 0·4 (12)†	+ 0·1 (5)
1·0	110	—	13·6 ± 1·5 (7)	7·8 ± 0·6 (7)	+ 1·1 (3)
1·0	—	110	9·2 ± 1·6 (7)	5·2 ± 0·8 (7)‡	+ 2·0 (3)
3·0	110	—	17·7 ± 4·4 (3)	10·1 ± 1·5 (3)	− 1·0 (3)
3·0	—	110	17·4 ± 4·0 (3)	10·0 ± 1·0 (3)	± 0 (3)
10·0	110	—	20·1 ± 3·6 (7)	11·3 ± 1·3 (7)	± 0 (3)
10·0	—	110	21·7 ± 1·8 (7)	12·5 ± 0·3 (7)	+ 1·6 (2)
ATP regenerating system, no phosphate	110	—	32·8 ± 3·1	19·0 ± 2·3	+ 1·4 (5)
	—	110	33·8 ± 4·0	20·3 ± 2·8	+ 2·9 (5)

* The number of experiments is in parentheses. Time of incubation: 10 min. ATP regenerating system: creatine phosphate and creatine phosphokinase.
† $P < 0·001$, compared to K^+ alone.
‡ $P < 0·01$, compared to K^+ alone.

Incubation

Mitochondria were incubated at 25° in a solution of 40 mM imidazole buffer (pH 6·8), 3 mM $MgCl_2$, and 0·06 mM $CaCl_2$ ($^{45}Ca/^{40}Ca = 1/200$). If not stated otherwise, 2 mM Na_2-succinate, 3 mM Na_2-ATP and 80 mM KCl were present. The protein concentration was 0·2–0·5 mg/ml.

Vesicles prepared according to the first and third methods were incubated at conditions identical with those for mitochondria. Three mM Na_2- or K_2-oxalate was added instead of 2 mM Na_2-succinate. Vesicles prepared according to the second method were incubated according to Palmer *et al.*[12] at 37° with 3 mM imidazole–histidine buffer (pH 6·8), 0·02 mM $CaCl_2$, 110 mM KCl or NaCl, 6 mM creatine phosphate, and 0·1 mg/ml creatine phosphokinase (C. F. Boehringer & Soehne), without oxalate. Vesicles prepared according to the fourth method were incubated according to Briggs *et al.*[10] with 18 mM imidazole-HCl buffer (pH 7·0), 1·8 mM oxalate, 5 mM $MgCl_2$, and 5 mM ATP.

Determination of Ca^{++} uptake

During the incubation 1·5 ml was withdrawn at intervals and the mitochondria or vesicles were separated within 4 sec by filtration on millipore filters (0·80 and 0·22 μ). The radioactivity of the dissolved filters was determined in the Packard liquid scintillation counter. Per cent Ca^{++} uptake means per cent of total ^{45}Ca in the medium taken up.

RESULTS

Ca^{++} uptake in mitochondria

(1). Dependence on succinate and ATP. When mitochondria were incubated without succinate or ATP, Ca^{++} uptake was brief, and after not more than 10 min the Ca^{++} content of the mitochondria was approximately the same as that of the incubation medium. Mitochondria preincubated for 10 min at 25° did not take up Ca^{++} in the absence of ATP and succinate.

When mitochondria were incubated with succinate (4 mM), Ca^{++} uptake reached a maximum in a few minutes and then fell and was only half of its highest level after 8 min (Fig. 1).

In the presence of both ATP (1 and 3 mM) and succinate (4 mM) Ca^{++} uptake was slow and reached a maximum in 10–20 min (Fig. 1).

(2). Effects of cyanide and azide. These are shown in Fig. 2. To demonstrate effects on Ca^{++} uptake in the absence and presence of ATP, ATP was added 3 min after the start of the incubation period. It is evident that cyanide inhibited the fast Ca^{++} uptake that occurred in the presence of ATP.

(3). Effects of *k*-strophanthin and pentobarbital. As seen in Fig. 3 and Table 1, *k*-strophanthin (10^{-8}–10^{-4} M) had no significant effect on Ca^{++} uptake in mitochondria from heart muscle and red and white skeletal muscle when ATP and succinate were present.

Pentobarbital inhibited Ca^{++} uptake in mitochondria from heart muscle and red and white skeletal muscle in the presence of ATP and succinate (Fig. 3 and Table 1).

(4). Effects of changes in the K^+:Na^+ ratio. In the previous experiments, the K^+ concentration of the incubation medium was high (80 and 120 mM) and the Na^+ concentration was low (10 or 14 mM). When mitochondria from heart muscle were

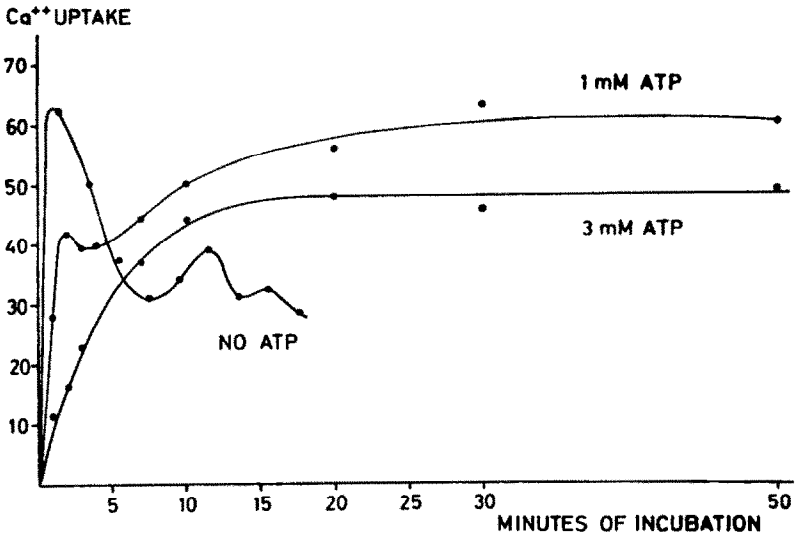

FIG. 1. Effect of ATP on Ca^{++} uptake in heart muscle mitochondria in the presence of succinate (4 mM). Per cent Ca^{++} uptake means the amount of $^{45}Ca^{++}$ as per cent of the total $^{45}Ca^{++}$ in the incubation medium.

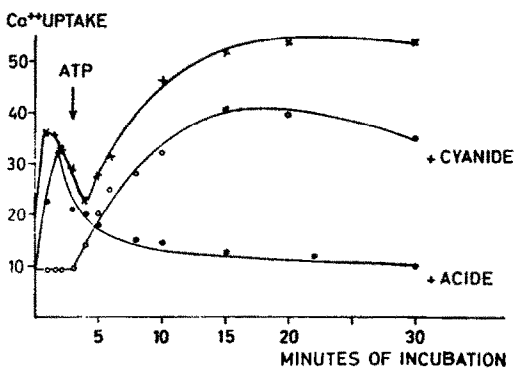

FIG. 2. Effects of cyanide (0·3 mM) and azide (0·1 mM) on Ca^{++} uptake in heart muscle mitochondria in the presence of succinate (4 mM). ATP (3 mM) was added at arrow.

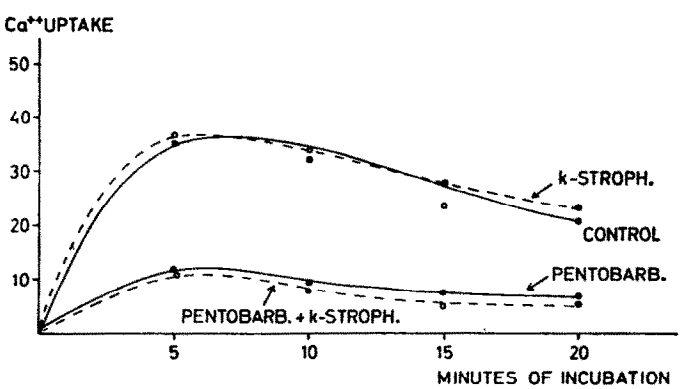

FIG. 3. Effects of *k*-strophanthin (0·01 mM) and pentobarbital (6 mM) on Ca^{++} uptake in heart muscle mitochondria.

incubated in the presence of higher Na^+ (50 mM) and lower K^+ (40 mM) levels, Ca^{++} uptake was low and it was further depressed by pentobarbital (Fig. 4). Results obtained with mitochondria from pigeon red skeletal muscle were similar (Fig. 5). However, substitution of 50 mM Na^+ and 40 mM K^+ for 10 mM Na^+ and 80 mM K^+ did not inhibit Ca^{++} uptake in rabbit white skeletal muscle mitochondria (Fig. 6).

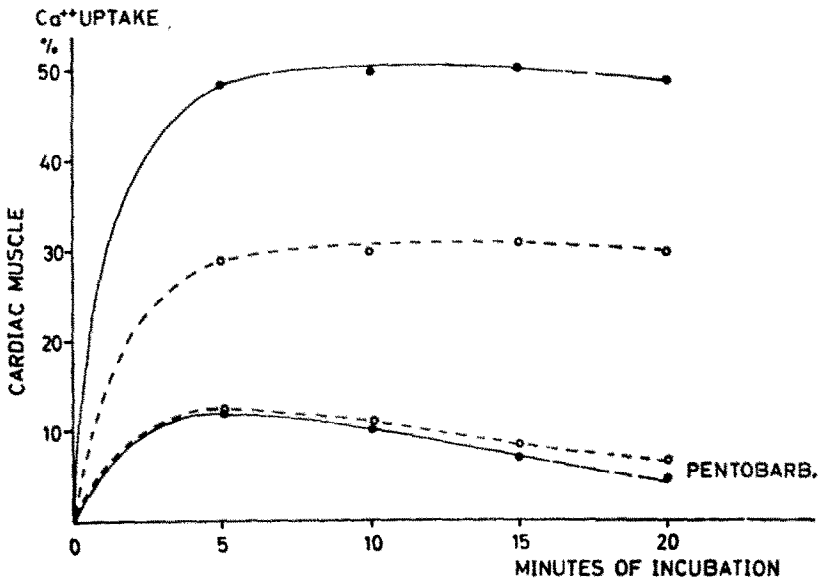

FIG. 4. Ca^{++} uptake in mitochondria of rabbit heart muscle in the presence of 80 mM K^+ and 10 mM Na^+ (solid line) and 40 mM K^+ and 50 mM Na^+ (broken line) and its inhibition by pentobarbital.

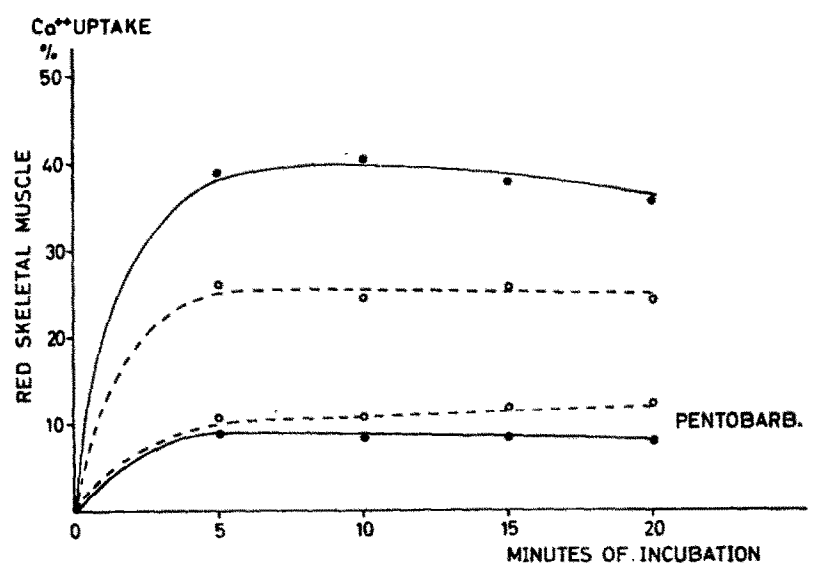

FIG. 5. Ca^{++} uptake in mitochondria of pigeon red skeletal muscle in the presence of 80 mM K^+ and 10 mM Na^+ (solid line) and 40 mM K^+ and 50 mM Na^+ (broken line) and its inhibition by pentobarbital.

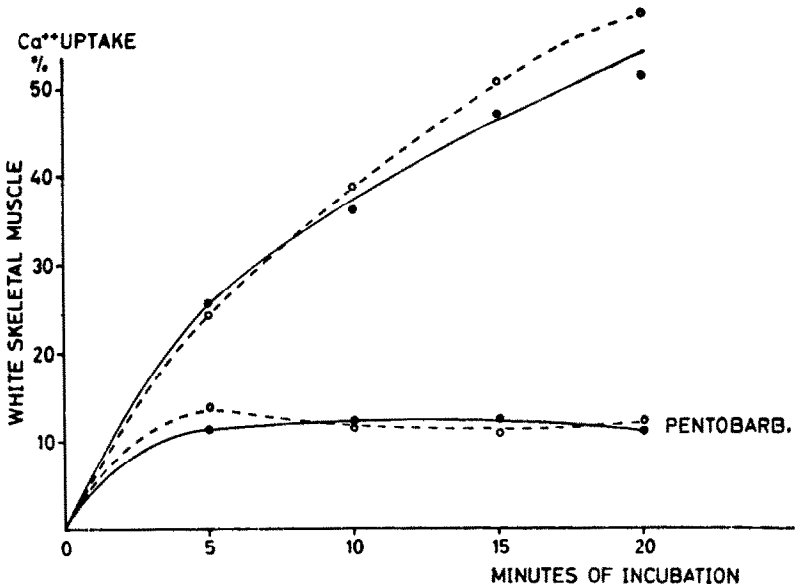

FIG. 6. Ca^{++} uptake in mitochondria of rabbit white skeletal muscle in the presence of 80 mM K^+ and 10 mM Na^+ (solid line) and 40 mM K^+ and 50 mM Na^+ (broken line) and its inhibition by pentobarbital.

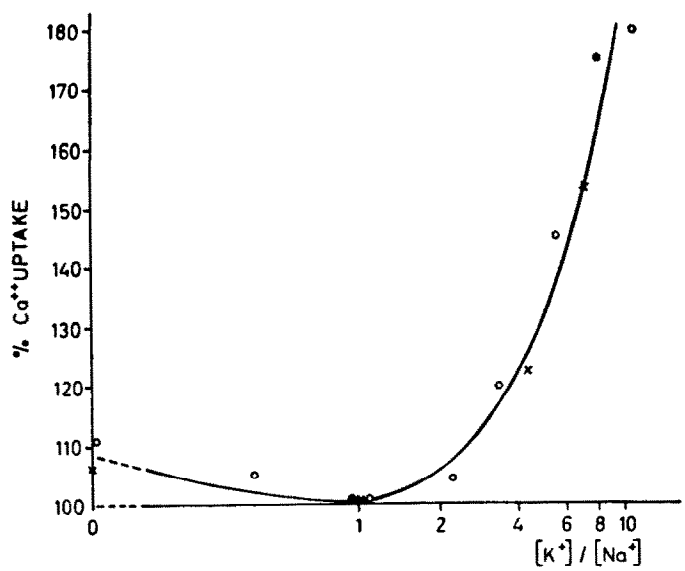

FIG. 7. Effect of the K^+:Na^+ ratio in the incubation medium on the Ca^{++} uptake in heart muscle, mitochondria. × : rabbit, total K^+ plus Na^+, 90 mM; ○: rabbit, total K^+ plus Na^+, 130 mM ●: cattle total K^+ plus Na^+, 90 mM.

Figure 7 shows additional experiments on the correlation of the K^+:Na^+ ratio with the Ca^{++} uptake. A rise in the K^+:Na^+ ratio increased the uptake of Ca^{++} in heart muscle mitochondria from rabbits and cattle.

Ca^{++} uptake in vesicles

ATP must be present for the vesicles to take up significant amounts of Ca^{++}. In the presence of 110 mM K^+, Ca^{++} uptake was higher than in the presence of 110 mM Na^+ (Table 2). Ca^{++} uptake by vesicles was increased when oxalate, phosphate, or an ATP regenerating system was added.

In the presence of oxalate, Ca^{++} uptake in vesicles from heart muscle was much slower than in vesicles from skeletal muscle (Fig. 8). Homogenization of skeletal muscle in the Starmix or Bühler homogenizer led to a faster and much greater Ca^{++} uptake than homogenization in the Potter apparatus (Fig. 8). Changing the K^+:Na^+ ratio from 80:10 to 40:50 mM did not significantly alter the results (Fig. 8). Both, *k*-strophanthin (0·01 mM) and pentobarbital (6 mM) had no effect on Ca^{++} uptake of vesicles from heart and skeletal muscle, whether incubated at a Na^+:K^+ ratio of 50:40 or 10:80 mM (Fig. 9).

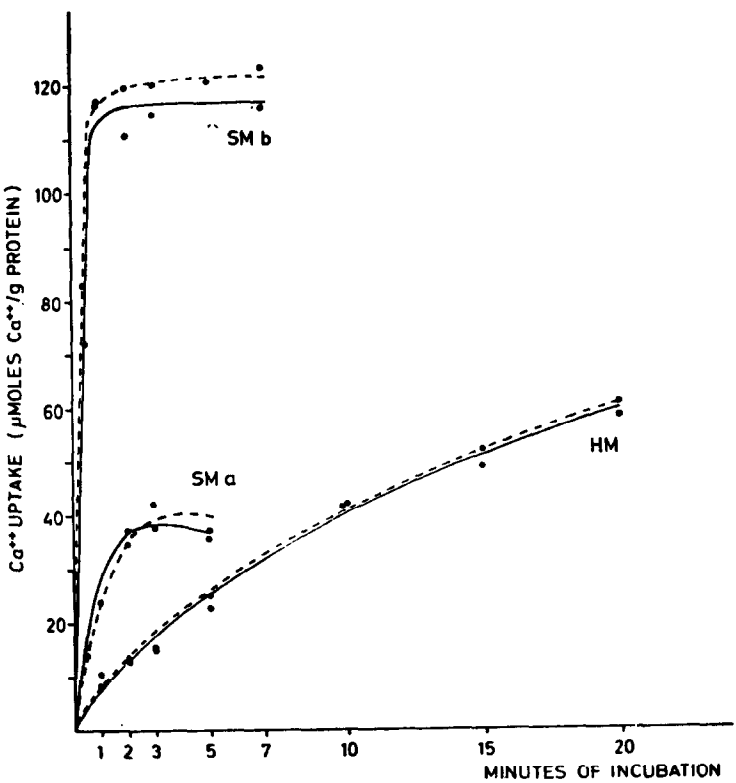

FIG. 8. Ca^{++} uptake by vesicles from heart (HM) and skeletal muscle (SM) in the presence of 80 mM K^+ and 10 mM Na^+ (solid line) and 40 mM K^+ and 50 mM Na^+ (broken line). SMa: skeletal muscle homogenized using a Potter–Elvehjem grinder (first method). SMb: skeletal muscle homogenized in the Starmix or Bühler homogenizer (third method).

In the presence of phosphate (1–10 mM) Ca^{++} uptake was 3–5 times increased (Table 2). While in the absence of phosphate, Ca^{++} uptake was significantly lower when the vesicles were incubated with 110 mM Na^+ compared with 110 mM K^+, substitution of Na^+ for K^+ did not have this effect in the presence of phosphate (Table 2). *k*-Strophanthin had no significant effect on Ca^{++} uptake (Table 2).

The presence of an ATP regenerating system (creatine phosphate and creatine phosphokinase) increased Ca^{++} uptake four to six times (Table 2). In the presence of the ATP regenerating system, substitution of Na^+ for K^+ or addition of *k*-strophanthin had no significant effect on Ca^{++} uptake (Table 2).

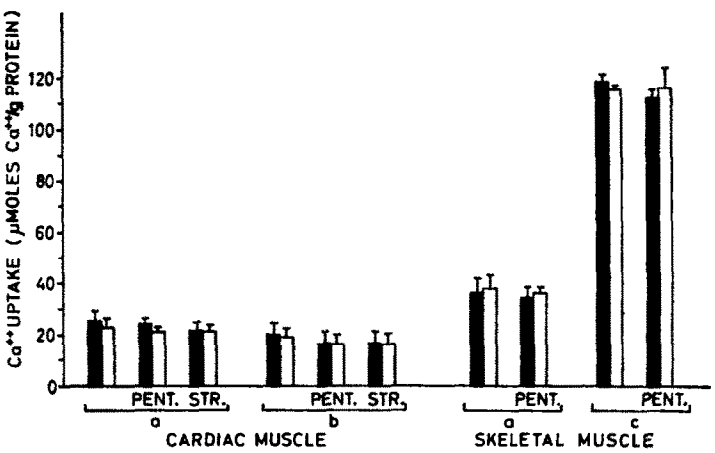

FIG. 9. Ca^{++} uptake by vesicles from heart and skeletal muscle in the presence of pentobarbital (6 mM, PENT.) and *k*-strophanthin (0·01 mM, STR.). a: vesicles prepared according to Potter (first method); b: vesicles prepared according to Briggs (Starmix, fourth method); c: vesicles prepared according to third method, homogenized in the Starmix and treated as in a. Solid bars: 50 mM Na^+ and 40 mM K^+; open bars: 10 mM Na^+ and 80 mM K^+. In a and c 3mM of oxalate was presents in b 1·8 mM. Cardiac muscle vesicles were incubated for 5 min and skeletal muscle vesicles for 2 min.

DISCUSSION

Our studies show that the Ca^{++} uptake by mitochondria is greatly influenced by the K^+:Na^+ ratio of the incubation medium. At the high K^+:Na^+ ratio of 10 the Ca^{++} uptake was approximately twice as high as that at the ratio of 1. This means that with the fall in the K^+:Na^+ ratio the ability of the mitochondria to take up Ca^{++} falls or that Ca^{++} is released. It is of particular interest that this dependence was most significant when the K^+:Na^+ ratio was high, as is the case inside the cell.

There was, strangely, a significant difference between the mitochondria of heart and red flying muscle, on the one hand, and those of white skeletal muscle on the other. This could be due to the fact that mitochondria from white skeletal muscle require different conditions for incubation or to the fact that Ca^{++} uptake and Ca^{++} exchange in mitochondria are needed as a means to control the intracellular Ca^{++} distribution only in heart and flying musculature, which has to contract regularly over long periods of time. These muscles contain a high amount of mitochondria owing to their greater energy requirements.

Vesicles of the sarcoplasmic reticulum were dependent for Ca^{++} uptake on the K^+:Na^+ ratio in a way similar to mitochondria of heart muscle if no oxalate or no

phosphate was present. However, when Ca^{++} uptake was increased by addition of oxalate or phosphate, the K^+:Na^+ lost its significance. Some recent publications also show that the Ca^{++} uptake of vesicles is greater when K^+ instead of Na^+ is present in the incubation medium.[14, 15] In experiments of Palmer[12] this effect is significant only in the absence of oxalate. Thus the results agree with our conclusion that a fall in the K^+:Na^+ ratio lowers the Ca^{++} uptake.

Strophanthin had no direct effect on Ca^{++} uptake of mitochondria or vesicles in our studies. However, digitalis glycosides have been reported to increase ionized cytoplasmic calcium.[16, 17] Therefore, we may explain the action of the digitalis glycosides as follows: Initially the inhibition of the transport ATPase in the cell membrane lowers the intracellular K^+:Na^+ ratio or slows its restoration to normal. With the diminished intracellular K^+:Na^+ ratio the ability of the mitochondria and vesicles to bind Ca^{++} is reduced, making more Ca^{++} available for the activation of the myofibril ATPase. The following observations support these assumptions. (1) The K^+ and Na^+ transport and the K^+- and Na^+-activated ATPase of the cell membrane are inhibited by digitalis.[18–22] (2) The Na^+- and K^+-stimulated transport ATPase of the heart muscle is much more sensitive to digitalis than that of skeletal muscle,[23, 24] which might explain why heart muscle is particularly sensitive to digitalis. (3) Potassium is lost from the heart not only after toxic doses but also following small doses of cardiac glycosides which increase contractility.[25, 26] (4) The sensitivity of the membrane ATPase of the heart of different animal species to the cardiotonic steroids is correlated with their potency on the heart.[27, 28]

Changes in the intracellular K^+:Na^+ ratio could be a fundamental principle in the regulation of the work of the heart. Sarnoff *et al.*[25] found that under various conditions potassium is lost from the heart when the work of the heart was increased. This occurred after raising heart rate, when aortic resistance was increased, and when acetylstrophanthidin was administered. This mechanism of adaptation, which Sarnoff calls the homeometric autoregulation, should lead to a decreased intracellular K^+:Na^+ ratio, to a diminished Ca^{++} accumulation in mitochondria and vesicles, to an increase in intracellular ionized calcium, to a greater activation of myofibril ATPase and to an increase in the force of contraction.

Pentobarbital inhibited Ca^{++} uptake in mitochondria but not in vesicles. This makes it possible to separate fractions of mitochondria and vesicles by their function and could be useful for the characterization of certain preparations. Our result is confirmed by the fact that, unlike vesicles, mitochondria require for Ca^{++} uptake not only ATP but also succinate or another substrate of the respiratory chain. It is further confirmed by our observation that cyanide inhibits Ca^{++} uptake by mitochondria.

In contrast to our results, Briggs *et al.*[10] found that Amytal inhibits Ca^{++} uptake in the vesicles of dog heart and that this is prevented by strophanthin. The authors conclude that the antagonism of barbiturates (depression of contractility) and strophanthin (stimulation of contractility) observed in the heart is based on a common site of action in the vesicles. Our results make it more probable that the inhibition of Ca^{++} uptake by barbiturates observed by Briggs *et al.*[10] was due to contamination of the vesicle fraction with mitochondria. Also the concept of a common site of action of barbiturates and strophanthin can not be reconciled with the fact that barbiturates inhibit the electron transport of the respiratory chain and thus act similar to cyanide, whereas digitalis glycosides appear to have no effect on the respiratory chain.

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