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THE INHIBITION OF Ca UPTAKE IN CARDIAC MEMBRANE VESICLES BY VERAPAMIL

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Abstract—Cardiac membrane vesicles take up Ca^{2+} in response to Na^+ gradient (high inside) and negative inside membrane potential. Both components of the Ca^{2+} uptake, the Na^+ gradient dependent uptake and the membrane potential dependent uptake are inhibited by verapamil; the action is dose-dependent and the concentrations of verapamil required to inhibit the Ca^{2+} uptake to 50% of its maximal value are 50 and 60 μM respectively. In the concentration ranges tested (50–750 μM Ca^{2+}), the inhibitory effect of verapamil could not be antagonized by increasing the Ca^{2+} concentration of the medium. Introducing verapamil into the vesicles by rapid freezing and slow thawing of the vesicles had the same inhibitory effect as adding the same concentration of verapamil on the outside of the vesicles. Adding verapamil to both sides of the vesicle membrane led to higher inhibition of Ca^{2+} uptake. It is proposed that addition of verapamil can cause a change in cardiac membranes which is manifested by a decrease in the driving membrane potential and Ca^{2+} transport.

Verapamil (Iproveratril) is a synthetic papaverine derivative widely employed in treatment of various forms of cardiac disease. Its major clinical effects include vasodilation of both peripheral and coronary blood vessels [1], an antianginal action [2] and reduction of heart rate, oxygen consumption and cardiac contractility [3, 4].

On the cellular level, the action of verapamil has been linked to inhibition of the slow Ca^{2+} and the Na^+ currents in the heart [5], inhibition of Ca^{2+} fluxes in muscle [6, 7], inhibition of low affinity Ca^{2+} binding to sarcolemma [8] and inhibition of veratridine activated (fast) Na^+ channels in cultured heart cells and neuroblastoma [9]. Other effects of verapamil include inhibition of Ca^{2+} dependent catecholamine release from the adrenal medulla [10] and Ca^{2+} dependent neurophysin and vasopressin release from the neurohypophysis [11]. Verapamil was also shown to inhibit Na^+ dependent high affinity neurotransmitter re-uptake into synaptosomes [12] Na^+ gradient dependent Ca^+ uptake and Na^+ coupled γ-aminobutyric acid uptake by synaptic plasma membrane vesicles [13].

On the molecular level, very little is known about verapamil's mechanism of action. It has been suggested [14] that verapamil acts as a Ca^{2+} antagonist since its effects could be reversed by addition of excess Ca^{2+}. Kinetic analysis [15] of the relationship between external Ca^{2+} concentration and the action of verapamil on the slow Ca^{2+} conductance of rat ventricular muscle showed a non-competitive type of inhibition. On the other hand, comparing the inhibitory action of verapamil at different Ca^{2+} concentrations by measuring the number of open Ca^{2+} channels showed [16] that the percentage of unblocked Ca^{2+} channels increased at higher Ca^{2+} concentration. In addition, some effects of verapamil were entirely independent of the presence of calcium ions [12].

In this work we examined the effects of verapamil on two types of Ca^{2+} transport: (1) the Na^+–Ca^{2+} antiport system (Na^+–Ca^{2+} exchanger) derived from heart sarcolemmal vesicles; (2) a negative inside membrane potential driven Ca^{2+} uptake. The Na^+–Ca^{2+} exchanger in the heart plays a major role in regulating cardiac activity. It transports Na^+ and Ca^{2+} ions across cardiac membrane in an electrogenic fashion. The proposed stoichiometry [17] suggests that 3–5 Na^+ ions are transported for each Ca^{2+} ion. The overall direction and magnitude of the Na^+–Ca^{2+} exchange process depends on the relative Na^+ and Ca^{2+} gradients across the membrane and the magnitude of the membrane potential [17].

The experimental preparation we chose to use in this investigation were cardiac sarcolemmal vesicles [18]. This preparation has the advantage of permitting the study of the Ca^{2+} transport systems in an isolated membrane preparation, and under well-defined internal and external ionic conditions, which do not change due to cellular metabolism.

MATERIALS AND METHODS

Cardiac membrane vesicles were prepared essentially as described by [18], except that we used hearts obtained from freshly slaughtered calves. The vesicles were characterized by electron microscopy. No intact cells or mitochondria were visible in the preparation.

Mitochondrial contamination of the cardiac sarcolemmal vesicle preparation was determined from the detectable specific activity of glutamic acid dehydrogenase GLDH (EC 1.4.1.3). GLDH activity was measured by the α-ketoglutarate coupled oxidation of NADH at 340 nm, in a reaction mixture containing: 0.1 M imidazole buffer pH 7.9, 0.00025 M NH_4-acetate, 0.012 M NADH, 0.0025 M EDTA, 0.002 M ADP, 0.012 M α-ketoglutaric acid and about

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50 μg membrane protein in the presence or in the absence of 0.1% Triton ×100. In none of the vesicle preparations tested could more than 5% mitochondrial contamination be detected. This estimate is based on comparing the specific activity of GLDH activity in heart mitochondria prepared as in [19] and the cardiac membrane vesicles both measured as such, or after Triton solubilization. In addition, heart mitochondria treated in an identical fashion to cardiac membrane vesicles did not exhibit Na^+ gradient dependent or membrane potential dependent Ca^{2+} transport activity.

Ca^{2+} transport studies were done on cardiac membrane vesicles pre-equilibrated by incubation at 37° for 20 min with a solution containing either 0.15 M Na-phosphate buffer pH 7.4 (Na^+ loaded vesicles) or 0.15 M K-phosphate buffer pH 7.4 (K^+ loaded vesicles) or 0.15 M NaCl–0.01 M Tris–HCl, pH 7.4 or 0.15 M KCl–0.01 M Tris–HCl, pH 7.4. The loaded vesicles were concentrated by centrifugation at 27,000 *g* for 20 min and suspended into a small amount of the same medium as used for preincubation. Ionic gradients were formed by diluting 3 μl of these vesicles into 250 μl of a $^{45}CaCl_2$ containing external medium, the composition of which is given in the legends of the appropriate figures.

The reaction was terminated by rapid filtration through the BA 85 0.45 μM Schleicher and Schuell filters, followed by two washes of the filter with 0.15 M KCl.

The filters were dried and counted in a liquid scintillation counter. Zero time determinations were done and subtracted from the results obtained.

[^{3}H]TPP$^+$ (tetraphenylphosphonium bromide) uptake was done as described by [20]. Schleicher and Schuell cellulose acetate filters (OE67 0.45 μM) were used to separate the vesicles from the reaction medium. The average internal volume of the sarcolemmal vesicles was calculated from the ratio of [^{14}C]polyethylene glycol (measures extravesicular space) and ^3H_2O (measures intra and extravesicular space) as described by [21] and this value was found to be 10.8 μl/mg protein. Each of the experiments presented in this paper has been repeated several times.

Protein was determined by the method of Lowry *et al.* [22].

$^{45}CaCl_2$ was purchased from NEN (New England Nuclear, Mass.), and [^{3}H]TPP$^+$ (tetraphenylphosphonium bromide) was purchased from the Nuclear Research Center, Negev, Israel). Verapamil was a generous gift from Prof. Dr D. Lenke and G. Scherrer of Knoll AG Ludwigshafen, Federal Republic of Germany. Biochemicals were purchased from Sigma, Israel. The chemicals used in this study were all analytical grade reagents.

RESULTS

Properties of the Ca^{2+} transport in cardiac membrane vesicles

In the absence of ATP, two types of Ca^{2+} transport can be demonstrated in cardiac membrane vesicles: Na^+ dependent calcium uptake and Na^+ independent uptake. The Na^+ dependent Ca^{2+} uptake was measured by preloading cardiac membrane vesicles in 0.15 M NaPi (Na-phosphate buffer)—see Methods—and diluting them rapidly into medium without Na^+ ions and containing Ca^{2+}. Figures 1a and b show such an experiment. In Fig. 1b, the NaPi preloaded

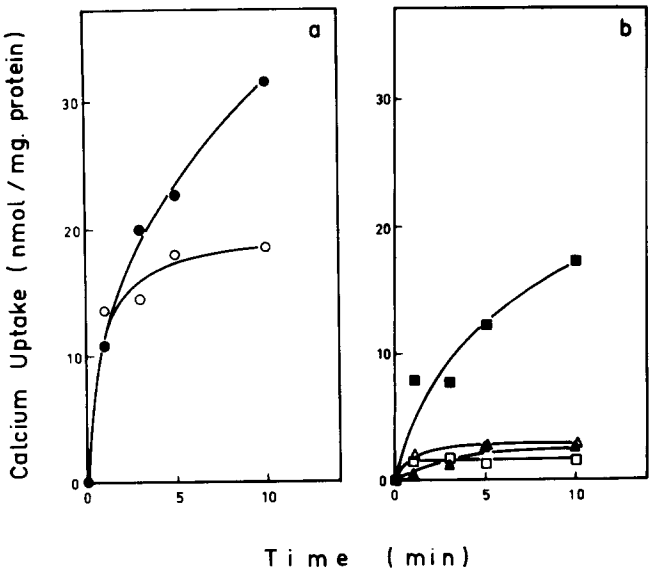

Fig. 1. Ca^{2+} uptake in sarcolemmal vesicles. (a) 3 μl (about 20 μg protein) sarcolemmal vesicles preloaded with 0.15 M Na-phosphate buffer at pH 7.4 (●—●—●) or with 0.15 M K-phosphate buffer at pH 7.4 (○—○—○) were diluted into 250 μl of an external medium containing 0.3 M sucrose, 0.01 M Tris–HCl pH 7.4, 50 μM $^{45}CaCl_2$ (0.1 μCi). The reaction was terminated as described in Methods. (b) 3 μl sarcolemmal vesicles (about 20 μg protein) preloaded with 0.15 M Na-phosphate buffer (■—■—■) or 0.15 M K-phosphate buffer (□—□—□) were diluted into 250 μl of external medium containing 0.15 M KCl, 0.01 M Tris–HCl pH 7.4, 50 μM $^{45}CaCl_2$ (0.1 μCi). Also shown are 3 μl vesicles containing 0.15 M Na-phosphate buffer pH 7.4 (▲—▲—▲) or 0.15 M K-phosphate buffer (△—△—△) which were diluted into 250 μl of medium containing 0.15 M NaCl, 0.01 M Tris–HCl pH 7.4, 50 μM $^{45}CaCl_2$ (0.1 μCi).

vesicles were diluted into iso-osmotic buffered KCl (filled squares). The preformed sodium gradient (high inside) thus created, served as a driving force for the Ca^{2+} uptake which reached in 10 min a value of 17 nmoles/mg vesicle protein. In the absence of a sodium gradient (Fig. 1b, filled triangles), when the NaPi preloaded vesicles were diluted into buffered iso-osmotic NaCl or when the direction of the Na^+ gradient was reversed by using KPi preloaded vesicles diluted into buffered NaCl (Fig. 1b, open triangles), very small amounts of Ca^{2+} ions were taken up into the cardiac vesicles. This very small amount of Ca^{2+} uptake (in the absence of Na^+ gradient) is due to an inward oriented Ca^{2+} gradient. Ca^{2+} uptake should cease only when $[Ca^{2+}]^{in} = [Ca^{2+}]^{out}$.

External K^+ is not obligatory for Ca^{2+} uptake in cardiac membrane vesicles. NaPi preloaded vesicles diluted into buffered iso-osmotic sucrose solution (Fig. 1a, filled circles) take up 31.5 nmoles of Ca^{2+}/mg protein/10 min.

Considerable amounts of Ca^{2+} are taken up by the cardiac vesicles in the complete absence of Na^+ when KPi preloaded vesicles were diluted into the buffered iso-osmotic sucrose solution containing Ca^{2+} (Fig. 1a, open circles). This Na^+ gradient independent Ca^{2+} uptake reaches 18.5 nmoles/mg protein/10 min. It should be noted that the Na^+ gradient dependent component of the Ca^{2+} uptake by cardiac membrane vesicles in the presence of external sucrose solution is similar to the Na^+ gradient dependent Ca^{2+} uptake in the presence of external KCl—namely 13 nmoles of Ca^{2+}/mg protein/10 min, and 14.3 nmoles of Ca^{2+}/mg protein/10 min respectively. These values were calculated by subtracting the Ca^{2+} taken up by the KPi preloaded vesicles (Na^+ gradient independent Ca^{2+} uptake) diluted into the sucrose or KCl solutions in each case, from identical vesicles preloaded in NaPi solution (total Ca^{2+} uptake). When NaPi preloaded vesicles were diluted into buffered KCl, the Na^+ gradient was almost the sole driving force for the Ca^{2+} uptake. The Ca^{2+} taken up by either NaPi or KPi preloaded cardiac membrane vesicles represents true transport, since addition of the Ca^{2+} ionophore A23187 to the vesicles leads to immediate release of the Ca^{2+} previously taken up (results not shown).

We examined the possibility that the membrane potential may serve as an additional driving force for Ca^{2+} uptake, besides the magnitude and direction of the Na^+ gradient. Since sarcolemmal vesicles are too small for direct electrophysiological measurements with microelectrodes, we used the distribution of the lipophilic cation TPP^+ (tetraphenylphosphonium bromide) as an index for membrane potential measurement. Figure 2 shows the uptake of [^{3}H]-TPP^+ into NaPi or KPi preloaded vesicles. In Fig. 2a, NaPi preloaded vesicles were diluted into an iso-osmotic sucrose solution (filled circles) or KCl solution (filled squares). In order to calculate from the [^{3}H]TPP^+ uptake values, the membrane potential under these conditions, the zero membrane potential has to be established. This has been done by preloading the vesicles in KPi (Fig. 2b), diluting them into an iso-osmotic KCl solution and addition of valinomycin. Since under these conditions the membrane is presumably freely permeable to K^+ ions, we can assume that no charge can accumulate on either side of the membrane and the amount of TPP^+ associated with the sarcolemmal vesicles represents the TPP^+ solubilized within the lipid phase of the membranes. This value (Fig. 2b, filled triangles) is in fact almost identical to the TPP^+ taken up by the

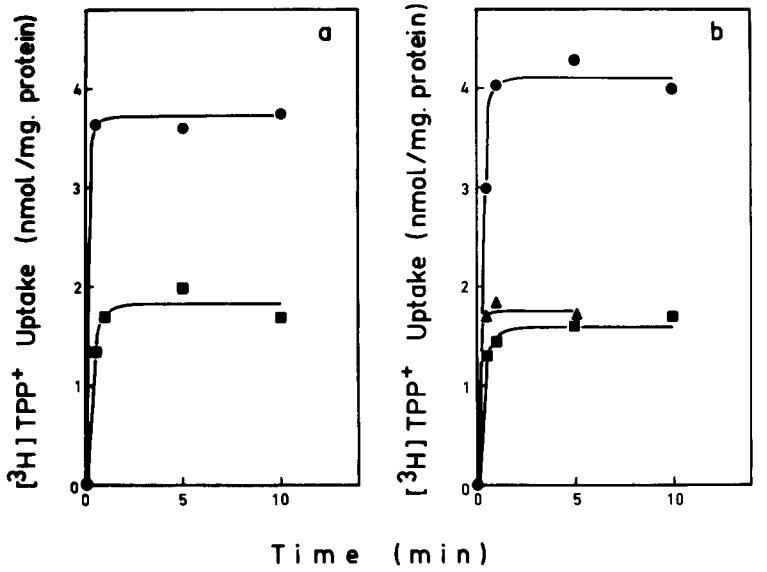

Fig. 2. [^{3}H]TPP^+ uptake into sarcolemmal vesicles. (a) 3 μl sarcolemmal vesicles (about 20 μg protein) preloaded with 0.15 M Na-phosphate buffer pH 7.4 were diluted into 250 μl of external medium containing 0.01 M Tris–HCl pH 7.4, 50 μM $CaCl_2$, 5.5 μM [^{3}H]TPP^+ (4 nCi) and 0.3 M sucrose (●—●—●) or 0.15 M KCl (■—■—■). (b) 3 μl sarcolemmal vesicles (about 20 μg protein) preloaded with 0.15 M K-phosphate buffer pH 7.4 were diluted into 250 μl of 0.01 M Tris–HCl pH 7.4, 50 μM $CaCl_2$, 5.5 μM [^{3}H]TPP^+ (4 nCi) and 0.3 M sucrose (●—●—●) or 0.15 M KCl (■—■—■), or 0.15 M KCl, 5 μM valinomycin (▲—▲—▲).

membranes, when KCl is the external medium (Fig. 2a, b, filled squares). The amounts of TPP^+ taken up by sarcolemmal vesicles are not significantly different whether Ca^{2+} is included or absent from the reaction medium. Thus, as also in other physiological systems, the membrane potential measured reflects mainly other ionic conductances and not the contribution of the Na^+–Ca^{2+} exchanger.

Since we have measured the average internal volume of the sarcolemmal vesicles (see Methods) to be 10.8 μl/mg vesicle protein, we can calculate the TPP^+ concentration within the sarcolemmal vesicles. Employing the Nernst equation and using the ratio of TPP^+ concentration outside the vesicles and within them, a potential difference of −88 mV (inside negative) was calculated, when the NaPi or KPi preloaded vesicles are diluted into buffered sucrose solution. This negative charge within the vesicles relative to the outside is probably responsible for the calcium uptake in KPi preloaded vesicles and part of calcium uptake in NaPi preloaded vesicles when sucrose is the external medium. The additional calcium uptake in NaPi preloaded vesicles is presumably due to the Na^+ gradient (high inside). In KPi preloaded vesicles diluted into the KCl solution, no potential difference exists between the inside of the vesicles and its outside and the TPP^+ associated with the vesicles probably represents only the amount of TPP^+ solubilized in the membrane. Therefore it is understandable that almost no Ca^{2+} is taken up into the vesicles under these conditions (Fig. 1b, open squares). Thus, in the absence of a negative charge inside the membranes (and in the absence of ATP) the Na^+ gradient is the sole driving force for Ca^{2+} uptake.

The effect of verapamil on the Ca^{2+} uptake in sarcolemmal vesicles

Figures 3a and b show the effect of verapamil on the Ca^{2+} uptake into sarcolemmal vesicles. In Fig. 3a, NaPi (open circles) or KPi (open squares) preloaded vesicles were diluted into the sucrose solution. Addition of 200 μM verapamil (Fig. 3a, filled circles) caused a 66% inhibition of Ca^{2+} transported by NaPi preloaded vesicles (see also Fig. 5). The inhibition caused by verapamil was immediate and its extent did not change upon preincubation of the drug with the vesicles for up to 1 hr (experiment not shown). Not only the Na^+ gradient dependent Ca^{2+} uptake by sarcolemmal vesicles was inhibited by verapamil, but also the membrane potential dependent Ca^{2+} uptake obtained in KPi preloaded vesicles (Fig. 3a, open squares) was inhibited by verapamil (Fig. 3a, filled squares).

In Fig. 3b, the inhibition by verapamil of the Na^+ gradient dependent component of the Ca^{2+} uptake is shown. The curve was obtained by subtracting the Ca^{2+} uptake obtained in KPi preloaded vesicles diluted into the sucrose solution (Fig. 3a, open squares) from the Ca^{2+} uptake obtained in NaPi preloaded vesicles diluted into sucrose (Fig. 3a, open circles). The upper curve (Fig. 3b, open circles) shows the net Na^+ gradient dependent Ca^{2+} uptake and the lower curve (Fig. 3b, filled circles) shows the net inhibition of verapamil on this uptake.

The effect of verapamil on [^{3}H]TPP^+ uptake by

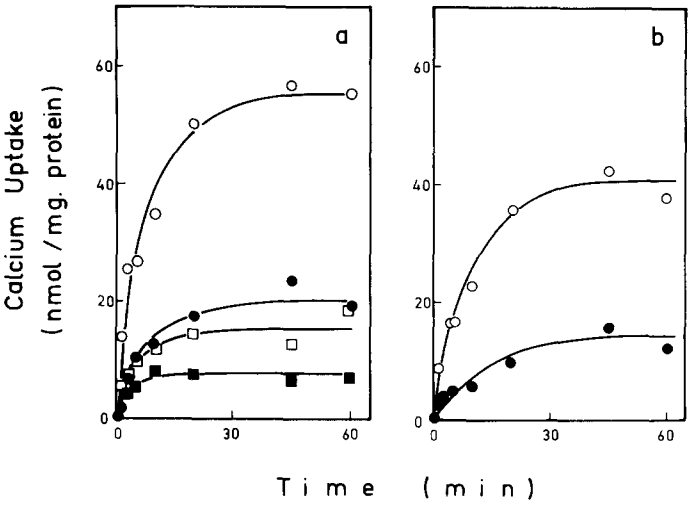

Fig. 3. (a) Effect of verapamil on Ca^{2+} uptake in sarcolemmal vesicles. 3 μl sarcolemmal vesicles (about 20 μg protein) preloaded with 0.15 M Na-phosphate buffer pH 7.4 were diluted into 250 μl of external medium consisting of 0.3 M sucrose, 0.01 M Tris–HCl pH 7.4, 50 μM $^{45}CaCl_2$ (0.1 μCi), with (●—●—●) or without (○—○—○) 200 μM verapamil. Also shown are 3 μl sarcolemmal vesicles preloaded with 0.15 M K-phosphate buffer pH 7.4 diluted into 250 μl of the above external medium, with (■—■—■) or without (□—□—□) 200 μM verapamil. (b) The effect of verapamil on the Na^+ dependent Ca^{2+} uptake in sarcolemmal vesicles. The Na^+ dependent Ca^{2+} uptake was calculated from Fig. 3a by subtracting the Ca^{2+} uptake obtained in 0.15 M K-phosphate buffer preloaded vesicles from the Ca^{2+} uptake obtained in 0.15 M Na-phosphate preloaded vesicles (○—○—○). The effect of verapamil on the Na^+ gradient dependent component (●—●—●) was calculated in the same manner from Fig. 3a by subtracting the Ca^{2+} uptake obtained in the presence of 200 μM verapamil in K-phosphate preloaded vesicles from Na-phosphate preloaded vesicles.

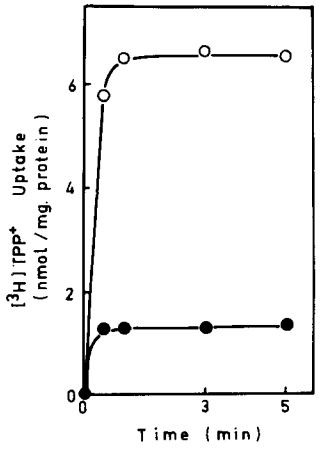

Fig. 4. Effect of verapamil on $[^3H]TPP^+$ uptake in sarcolemmal vesicles. 3 μl sarcolemmal vesicles (about 20 μg protein) preloaded with 0.15 M Na-phosphate buffer pH 7.4 were diluted into 250 μl of external medium containing 0.3 M sucrose 0.01 M Tris–HCl pH 7.4, 50 μM $CaCl_2$, 5.5 μM $[^3H]TPP^+$ (4 nCi) with (●—●—●) or without (○—○—○) 200 μM verapamil.

sarcolemmal vesicles was examined in Fig. 4. NaPi preloaded vesicles were diluted into sucrose solution containing $[^3H]TPP^+$. The uptake of $[^3H]TPP^+$ was measured in the absence (Fig. 4, open circles) and in the presence (Fig. 4, filled circles) of 200 μM verapamil. Verapamil thus inhibited both the Ca^{2+} uptake and the $[^3H]TPP^+$ uptake into the vesicles. Identical inhibition was obtained whether the cardiac membrane vesicles were preloaded in NaPi (Fig. 4) or in KPi (not shown).

In Fig. 5, we show the results of the experiments in which the inhibition by verapamil of cardiac vesicle Ca^{2+} transport has been examined at different verapamil concentrations. Figure 5a shows the inhibition of Ca^{2+} transport in both NaPi (filled circles) or KPi (open circles) preloaded vesicles diluted into buffered iso-osmotic sucrose solution. Verapamil inhibited Ca^{2+} transport (both the Na^+ gradient dependent and the Na^+ gradient independent) in a concentration dependent fashion, starting at 20 μM. The inhibition of Ca^{2+} transport increased rapidly with increasing verapamil concentrations until 50 μM verapamil, and then, further increase in verapamil concentration had smaller additional inhibitory effects on the Ca^{2+} transport, until about 400 μM verapamil. Figure 5b shows that similar effects of verpamil are manifested on the $[^3H]TPP^+$ uptake by NaPi (filled circles) or KPi (open circles) preloaded sarcolemmal vesicles. Here a similar picture emerges. There is a residual TPP^+ content of 1.2 nmoles/mg protein out of 5.2 nmoles/mg protein, which is insensitive to added verapamil. This portion of TPP^+ probably represents the fraction of the lipophilic cation solubilized within the phospholipid membrane itself, since it is equal to the TPP^+ associated with the KPi loaded vesicles in the presence of external

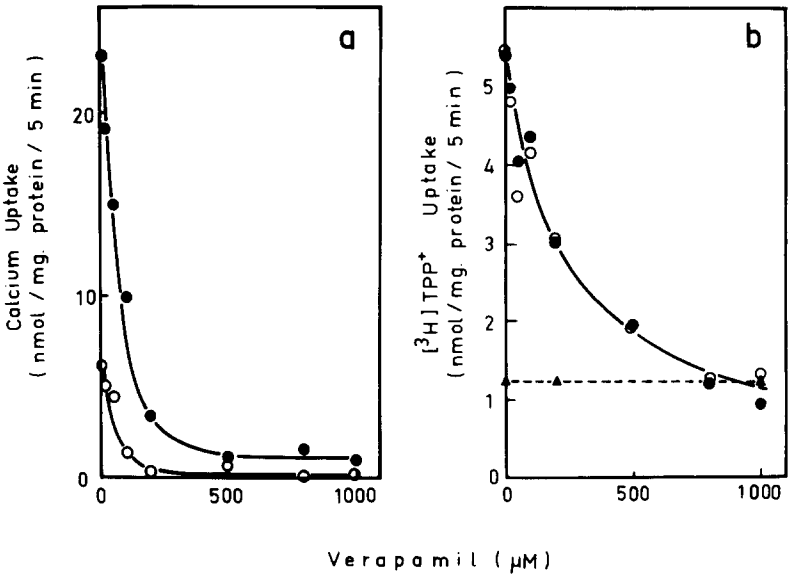

Fig. 5. (a) Effect of different concentrations of verapamil on the Ca^{2+} uptake in sarcolemmal vesicles. 3 μl sarcolemmal vesicles (about 20 μg protein) preloaded with 0.15 M Na-phosphate buffer pH 7.4 (●—●—●) or 0.15 M K-phosphate buffer (○—○—○) were diluted into 250 μl of external medium containing 0.3 M sucrose, 0.01 M Tris–HCl pH 7.4, 50 μM $^{45}CaCl_2$ (0.1 μCi) and different concentrations of verapamil ranging from 0 to 1000 μM. (b) Effect of different concentrations of verapamil on the $[^3H]$-TPP^+ uptake. 3 μl (about 20 μg protein) sarcolemmal vesicles preloaded with 0.15 M Na-phosphate buffer pH 7.4 (●—●—●) or 0.15 M K-phosphate buffer (○—○—○) were diluted into 250 μl of external medium containing 0.3 M sucrose, 0.01 M Tris–HCl pH 7.4, 50 μM $CaCl_2$, 5.5 μM $[^3H]TPP^+$ (4 nCi) and verapamil in concentrations between 0 and 1000 μM. Also shown is the uptake into 3 μl of 0.15 M K-phosphate buffer preloaded vesicles which were diluted into 250 μl of external KCl medium, with the addition of 5 μM valinomycin (▲—▲—▲).

KCl and valinomycin, when no potential is expected to develop across the membrane.

It is interesting to note that the concentration of verapamil yielding 50% inhibition of the Ca^{2+} transport obtained in the KPi preloaded vesicles or the NaPi preloaded vesicles is very similar. This value is reached around 50 μM verapamil for NaPi preloaded vesicles, and 60 μM for the KPi preloaded ones. The 50% TPP^+ uptake, however, is reached at higher verapamil concentrations (150 μM).

Can the inhibition of Ca^{2+} transport by verapamil be antagonized by increasing the Ca^{2+} concentration?

We examined whether there is a relation between the calcium ion concentration in the reaction medium and the extent of inhibition obtained by verapamil. Figure 6 shows such an experiment. NaPi preloaded vesicles (open circles) were diluted into an iso-osmotic sucrose solution containing varying concentrations of calcium ions. The extent of inhibition by verapamil of the Ca^{2+} transported was measured (filled circles). The same experiment was repeated also with KPi preloaded vesicles (open squares) to measure the Na^+ gradient independent Ca^{2+} uptake at various calcium ion concentrations in the absence or in the presence (filled squares) of verapamil. Between 5 and 500 μM calcium, the calcium concentration did not affect the extent of inhibition obtained by verapamil (both in the presence and in the absence of the Na^+ gradient). Kinetic analysis of the reciprocal of the apparent initial velocity of the negative inside membrane potential driven Ca^{2+} uptake ($1/V$) versus the reciprocal of the Ca^{2+} concentration (Lineweaver–Burke plot) is presented in Fig. 7. This analysis shows that in the Ca^{2+} concentration range 50–750 μM, the apparent K_m of the uptake (416 μM) is not altered by the presence of verapamil. The apparent maximal reaction velocity is decreased from 31.25 nmoles/mg protein/15 sec to 15.62 nmoles/mg protein/15 sec. No kinetic analysis of the Ca^{2+} uptake in NaPi preloaded vesicles diluted into the buffered iso-osmotic sucrose solution is available at present since under these conditions, at least two separate Ca^{2+} uptake processes occur: (1) Na^+ gradient driven Ca^{2+} uptake; (2) negative inside membrane potential driven Ca^{2+} uptake—each of which has a different time course of the initial velocity of Ca^{2+} uptake. Thus, it seems that not simple competition on binding sites plays a role in the inhibition obtained by verapamil of the calcium transport in sarcolemmal vesicles when the external Ca^{2+} concentration does not exceed 750 μM.

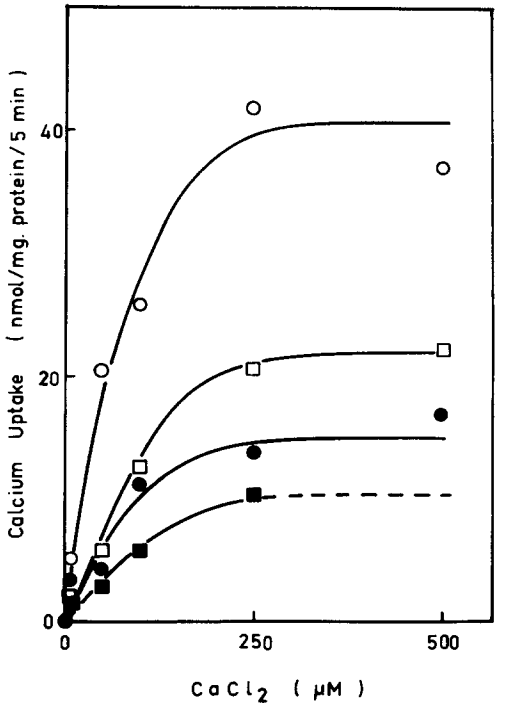

Fig. 6. The effect of [Ca^{2+}] on the extent of inhibition by verapamil of Ca^{2+} uptake. 3 μl (about 20 μg protein) of sarcolemmal vesicles preloaded with 0.15 M Na-phosphate buffer pH 7.4 were diluted into an external medium containing 0.3 M sucrose, 0.01 M Tris–HCl pH 7.4 and $^{45}CaCl_2$ in the concentration range between 5 and 500 μM, in the presence (●—●—●) or absence (○—○—○) of 200 μM verapamil. Also shown are 3 μl of 0.15 M K-phosphate preloaded vesicles which were assayed under identical conditions in the presence (■—■—■) or absence (□—□—□) of 200 μM verapamil.

The sidedness of the inhibitory action by verapamil

We examined whether the inhibitory action of verapamil could be increased by introducing it into the sarcolemmal vesicles. No labelled verapamil was available to us to measure directly its entry into the cardiac membrane; therefore we employed a technique of rapidly freezing the vesicles in liquid N_2 in the presence of verapamil, followed by slowly thawing them. Presumably, upon freezing the sealed membranes open and when they are allowed to thaw slowly, they reseal and entrap the verapamil within them. The results of these experiments are presented

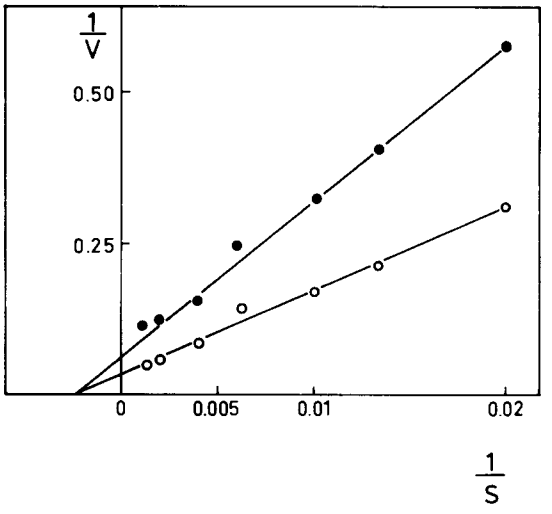

Fig. 7. The initial velocity of the negative inside membrane potential driven Ca^{2+} uptake as a function of Ca^{2+} concentrations. 3 μl K-phosphate preloaded vesicles (30–50 μg protein) were diluted into 250 μl medium containing: 0.3 M sucrose, 10 mM Tris–HCl pH 7.4 and 50–750 μM $^{45}CaCl_2$ (0.1–0.5 μCi). Ca^{2+} uptake was measured after 15 sec (○—○—○). The same experiment was also performed in the presence of 200 μM verapamil (●—●—●).

in Fig. 8. Figure 8a presents NaPi preloaded vesicles diluted into buffered sucrose solution subjected to rapid freezing and slow thawing. Calcium transport without added verapamil to the inside or to the outside is shown in the upper curve (open circles). It can be seen that freezing and thawing did not damage the capacity of cardiac membrane vesicles to take up Ca^{2+} in response to the Na^+ gradient. Three different possibilities regarding the inhibition of Ca^{2+} transport by verapamil were explored in these experiments:

(1) The inhibitory effect of verapamil on Ca^{2+} transport when the drug was added to the external diluting medium containing Ca^{2+} (Fig. 8, filled circles—"verapamil out").

(2) The inhibitory effect of verapamil when introduced only during the freeze–thaw cycle and thus only inside the vesicles (Fig. 8, filled squares—"verapamil in").

(3) The inhibitory effect of verapamil when introduced on both sides of the membrane; the Na^+ facing side (inside) and the Ca^{2+} facing side (outside). This is achieved by adding verapamil during the freeze–thaw procedure and also into the diluting incubation medium with Ca^{2+}.

The results of these experiments show that the inhibitory effect of verapamil on the calcium transport of sarcolemmal vesicles is retained after freezing and thawing. The same extent of inhibition of Ca^{2+} transport by verapamil was obtained whether verapamil acted from the inside or from the outside of the vesicles. The inhibitory effect of verapamil was, however, higher when it was introduced on both sides of the membrane. It seems that the drug can probably inhibit the Na^+–Ca^{2+} exchanger either by interfering with the Na^+ binding site of the carrier (in our experiments the inside) or by preventing Ca^{2+} transport from the outside. Since the inhibition obtained in either of these cases was below the maximal inhibition obtainable in the system, it increased when the drug was added from the inside in addition to the outside.

DISCUSSION

Cardiac membrane vesicles (in the absence of ATP) take up Ca^{2+} in response to at least two driving forces: the Na^+ gradient and the negative inside membrane potential.

The Na^+ gradient was established by pre-equilibrating the vesicles in 0.15 M Na^+ containing media and diluting them 83-fold into a solution without Na^+ ions. Under these conditions the amount of Na^+ trapped in the intervesicular space is very small (1.8 mM [Na^+]). Hence, the determination of the effects of the Na^+ gradient are straightforward and require no further discussion. The negative inside membrane potential was probably created by the

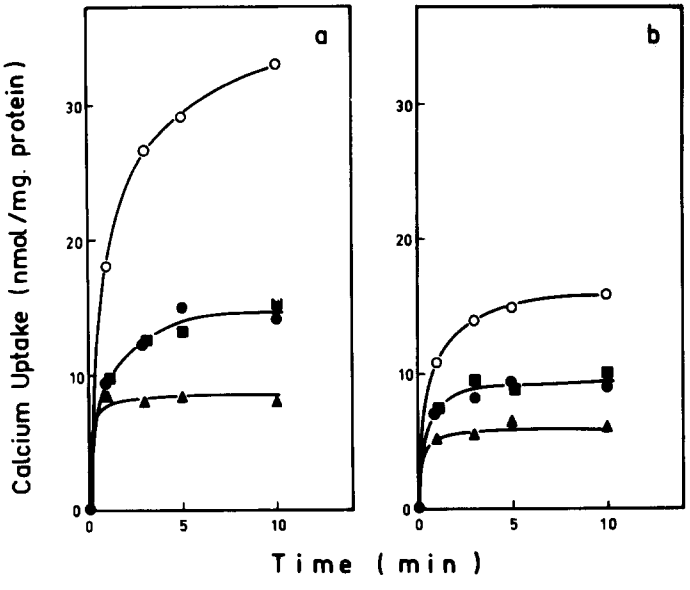

Fig. 8. Sidedness of the effect of verapamil on the Ca^{2+} uptake in sarcolemmal vesicles. (a) 200 μM verapamil was added to sarcolemmal vesicles which were preloaded with 0.15 M Na-phosphate buffer pH 7.4. The mixture was rapidly frozen in liquid nitrogen and slowly thawed in ice cold water (4°). 3 μl (about 20 μg protein) of these vesicles ("verapamil in") were diluted into 250 μl of external medium, containing 0.3 M sucrose, 0.01 M Tris–HCl pH 7.4, 50 μM $^{45}CaCl_2$ (0.1 μCi) (■—■—■; verapamil "in"), or into the same external medium containing also 200 μM verapamil (▲—▲—▲; verapamil "in" and "out"). Sarcolemmal vesicles preloaded with 0.15 M Na-phosphate buffer were frozen and thawed as above, without addition of verapamil prior to the freezing procedure. 3 μl of these vesicles were diluted into 250 μl of external medium consisting of 0.3 M sucrose, 0.01 M Tris–HCl pH 7.4, 50 μM $^{45}CaCl_2$ (0.1 μCi) (○—○—○; no verapamil) or with addition of 200 μM verapamil (●—●—●; verapamil "out"). (b) Experiment as in Fig. 8a, except that the sarcolemmal vesicles were preloaded with 0.15 M K-phosphate buffer pH 7.4. No verapamil added (○—○—○), verapamil "out" (●—●—●), verapamil "in" (■—■—■), verapamil "in" and "out" (▲—▲—▲).

combination of the relative permeability of cardiac membrane vesicles to the internal monovalent cations and their relative impermeability to internal phosphate. The magnitude of the membrane potential was measured from the distribution of the lipophilic cation TPP^+. The maximal Ca^{2+} uptake imposed by the membrane potential of −88 mV (calculated from the TPP^+ uptake after a correction for 'zero' membrane potential distribution) should produce a ratio of free Ca^{2+} ions $[Ca]^{in}/[Ca]^{out}$ of 1082 as calculated from the Nernst equation. The ratio obtained in the experiments presented here is only 120. Therefore, the movements of calcium and the action of verapamil were examined in non-equilibrium conditions, with a net inward transport of calcium. In the heart, at −88 mV there is practically no Ca^{2+} conductance [17]. It has been proposed [23, 24], that the gating properties of cardiac Ca^{2+} channels depend on cytoplasmic processes such as phosphorylation. It is therefore quite possible that in cardiac membrane vesicles, in the absence of cytosolic factors, the gating of Ca^{2+} channels will be impaired and consequently the Ca^{2+} channels will be open when they should not be. Since this negative inside membrane potential of −88 mV is a large driving force, Ca^{2+} will enter into the vesicles.

Verapamil inhibits both forms of Ca^{2+} uptake in a concentration-dependent manner (Figs. 3 and 5). The inhibition of Ca^{2+} uptake by verapamil could not be reversed by increasing the calcium concentration of the medium between 50 and 500 μM (Fig. 6). Kinetic analysis of the apparent initial velocity of the negative inside membrane potential driven Ca^{2+} uptake as a function of Ca^{2+} concentration between 50 and 750 μM Ca^{2+} (Fig. 7) showed a non-competitive type of behaviour. These findings are similar to the ones described by Payet *et al.* [15]. On the other hand, Lee and Tsien [16] found a competitive behaviour between $[Ca^{2+}]$ and verapamil between 3 and 30 mM $[Ca^{2+}]$. Preliminary experiments performed by us in cardiac membrane vesicles at higher Ca^{2+} concentrations (above 1 mM) showed a complex non-Michaelis–Menten type behaviour. Under those conditions, the inhibition by verapamil of the initial velocity of negative inside membrane potential driven Ca^{2+} uptake decreased as the Ca^{2+} concentration of the medium increased. Pang and Sperelakis [8] found that verapamil (10^{-6}–10^{-5} M) significantly inhibited low affinity Ca^{2+} binding to cardiac sarcolemma (K_d = 3 mM). The high affinity Ca^{2+} binding relevant to our work was not affected in the concentration ranges of verapamil they used. Moreover, even at higher Ca^{2+} concentrations, increase in $[Ca^{2+}]$ did not alter the extent of inhibition obtained by verapamil of the Ca^{2+} binding [8].

Verapamil inhibited TPP^+ uptake by cardiac sarcolemmal vesicles in a similar fashion to the inhibition of Ca^{2+} uptake. This inhibition was also dependent on verapamil concentration (Fig. 5b). Hence, part of the inhibitory action of verapamil on calcium transport seems to be due to its action on the membrane potential. At present, we do not know how this effect is exerted. However, this effect may be of relevance to the effects of verapamil on calcium uptake into synaptosomes [25].

It is of interest to note that the effects of verapamil can be partially reversed by either adding excess of phosphatidylcholine liposomes to verapamil inhibited membranes, or diluting them with excess medium. Therefore, it is feasible that verapamil acts also on the lipid phase of the membrane and not only on the transporting molecules.

The concentrations of verapamil (20–400 μM) used in our work are higher than the therapeutic plasma concentrations of the drug. In other *in vitro* studies, however, such as the adrenal medulla [10, 26], neurophypophysis [11], synaptosomes [12, 25] and synaptic plasma membrane vesicles [13], similar concentrations of verapamil were effectively used. In addition, since the local concentration of verapamil in its target tissues is not known, no comparison can be made between plasma concentrations of verapamil and *in vitro* studies employing isolated membranes. There is evidence that systemic levels [27] of verapamil 3 hr after administration were largely confined to liver and cardiac tissue.

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