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THE EFFECT OF VERAPAMIL ON THE Ca^{2+}-TRANSPORTING AND Ca^{2+}-ATPase ACTIVITY OF ISOLATED CARDIAC SARCOLEMMAL PREPARATIONS

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1 The effect of (±)-, (+)- and (−)-verapamil on the Ca^{2+}-binding, Ca^{2+}-transporting activity, and Ca^{2+}-dependent adenosine triphosphatase (ATPase) activity of isolated cardiac sarcolemmal preparations was studied. Enzymatic treatment was used to establish the nature of the sites facilitating [^{14}C]-(±)-verapamil binding.

2 (±)-Verapamil 1 μM inhibited the passive binding of $^{45}Ca^{2+}$. The (+)- and (−)-isomers were equiactive.

3 (±)-Verapamil 1 μM inhibited the ATP-dependent transport of $^{45}Ca^{2+}$ and the associated activation of the Ca^{2+}-sensitive ATPase. The activity resided in the (−)-isomer.

4 Lineweaver-Burk plots for the initial rates of ATP-dependent transport showed that the inhibition induced by the (−)-isomer was accompanied by a reduced K_m and V_{max}.

5 Enzymatic removal of *N*-acetyl neuraminic acid and galactose residues increased [^{14}C]-(±)-verapamil binding; removal of *N*-acetylglucosamine and treatment with phospholipase C and trypsin decreased the binding.

6 These results have been interpreted to mean that (−)-verapamil interferes with the ATP-dependent Ca^{2+}-transporting properties of the sarcolemma, and that this effect is accompanied by an altered activity of the intrinsic Ca^{2+}-sensitive ATPase. *N*-acetylneuraminic acid and galactose residues do not provide binding sites for verapamil at the cell surface.

Introduction

Verapamil (Haas & Hartfelder, 1962) is the prototype of a group of substances commonly referred to as 'Ca^{2+}-antagonist' (Fleckenstein, 1971). Other drugs usually included in this group are nifedipine, fendiline and diltiazem (Fleckenstein, 1977). These drugs share one common property. They depress the slow inward Ca^{2+} current which flows during the plateau phase of the action potential (Kohlhardt, Bauer, Krause & Fleckenstein, 1972; Kohlhardt & Mnich, 1978). In the case of verapamil this reduction in the slow inward Ca^{2+} current involves a slowed rate of recovery of the gated 'channels' or 'carriers' responsible for mediating the voltage-activated transport of Ca^{2+} across the sarcolemma (Kass & Tsien, 1975; Nawrath, Ten Eick, McDonald & Trautwein, 1977; Kohlhardt & Mnich, 1978).

The cardiac sarcolemma is complex and consists of a thick outer carbohydrate-rich glycocalyx below which is the thin, enzymatically-active, plasmalemma (Nayler, 1977; Frank, Langer, Nudd & Seraydarian, 1977). Carbohydrates known to be present in the glycocalyx include *N*-acetylneuraminic acid, galactose and *N*-acetylglucosamine (Hughes, 1975; Frank *et al.*, 1977). Some investigators have postulated that the *N*-acetylneuraminic acid residues may provide important storage sites for Ca^{2+} involved in excitation-contraction coupling (Langer, Frank, Nudd & Seraydarian, 1976; Langer, 1977).

Comparatively little is known about the mechanisms whereby verapamil interferes with the Ca^{2+}-transporting properties of the cardiac sarcolemma. Earlier studies (Nayler & Szeto, 1972) showed that concentrations of (±)-verapamil that are negatively inotropic reduce the size of the membrane-bound pool of Ca^{2+} displaceable by La^{3+}, and Williamson, Woodrow & Scarpa (1975) described an inhibitory

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effect of (±)-verapamil on the adenosine triphosphate (ATP)-independent (passive) binding of Ca^{2+} by isolated cardiac sarcolemma.

The following investigations were undertaken to establish (1) whether verapamil impedes the Ca^{2+}-binding and ATP-dependent transporting activity of isolated cardiac sarcolemma and if it does is the effect stereospecific? (2) whether the effect of verapamil is accompanied by a change in the activity of the sarcolemmal Ca^{2+}-activated ATPase enzyme, and (3) to obtain, by enzymatic treatment, data relating to the nature of the binding sites for verapamil.

Methods

Adult male New Zealand White rabbits, weighing between 2.5 and 3.0 kg were used. They were killed by a blow on the neck. The hearts were immediately excised and subfractionated as described below to provide a sarcolemmal-rich subcellular fraction.

Isolation of cardiac sarcolemma

The isolation procedure used is based on that of St. Louis & Sulakhe, (1976) and has been described in detail elsewhere (Mas-Oliva, Williams & Nayler, 1979a; Nayler, Mas-Oliva & Williams, 1979). Essentially it involves solubilization of the contractile proteins followed by differential centrifugation and separation of the remaining membrane components by density gradient centrifugation, using either swing out or zonal rotors. The fraction banding between 1.53 and 1.60 M sucrose was used as the sarcolemmal fraction, after being characterized in terms of its enzymatic properties relative to those obtained for freshly isolated sarcoplasmic reticulum and mitochondria (Table 1). The preparations were also examined by electron microscopy. Cardiac muscle sarcoplasmic reticulum was prepared as previously described (Nayler, Dunnett & Berry, 1975). Mitochondria were isolated as described by Williams & Barrie (1978). Cytochrome oxidase was measured as described by Cooperstein & Lazarow (1951), and protein by an adaptation of the method of Bradford (1976). ATPase activity was assayed as described by Matsui & Schwartz (1966) and liberated inorganic phosphate (Pi) determined by the method of Fiske & Subbarow (1925). Adenyl cyclase activity was assayed with a cyclic AMP assay kit (The Radiochemical Centre, Amersham). The cholesterol content of the fractions was measured with a cholesterol assay kit (Boehringer, Mannheim, West Germany).

Calcium binding assays

Calcium binding was measured isotopically by a rapid sampling technique followed by Millipore filtration. Passive binding refers to binding of calcium in the absence of added adenosine triphosphate (ATP). ATP-dependent binding (or transport) refers to the additional binding observed after adding ATP.

The reaction mixture used for the binding experiments contained KCl 100 mM, Tris-HCl 50 mM, $MgCl_2$ 5 mM, nitrilotriacetic acid (NTA) 2.5 mM, pH 7.4 at 25°C. Racemic (±)-, (+)- and (−)-verapamil were dissolved in this buffer and 10 µl aliquots added to the incubation media to provide a final concentration of 1 µM. Sarcolemmal protein 50 µl (250 to 350 µg) was preincubated for 5 min at 25°C in the buffer previously described and passive binding initiated by adding 25 µl of $CaCl_2$ containing approximately 2.5 µCi $^{45}Ca^{2+}$, giving a final volume of 1.675 ml and a free $[Ca^{2+}]$ of 60.0 µM. After 5, 25 and 45 s, 200 µl samples were withdrawn, immediately filtered through 0.45 µm Millipore filters (Millipore S.A. Molsheim, France) and washed with 7 ml of a buffer containing KCl 100 mM and Tris-HCl 50 mM, at pH 7.4. After 45 s of passive binding, ATP-supported calcium transport was initiated by adding 10 µl of a solution containing 2.71 µmol of ATP (pH 7.4), giving a final ATP concentration of 2.50 mM. Variations in the free calcium concentration of the reaction media caused by the addition of ATP were calculated by using an ion-complexes in solution computer programme and available stability constants (Sillén & Martell, 1971). At 5, 25, 45 and 65 s after the addition of ATP, 200 µl samples were withdrawn and filtered as described above. The filters were dried for 30 min before mixing with scintillation fluid (2 vol toluene, 1 vol Synperonic NP9 (Cargo-Fleet Co. Stockton, U.K.), 40 mg/l PPO (2,5-diphenyloxazole). 0.1 g/l PPOP [1-4 bis 2-(5-phenyl-oxazolyl]benzene). Radioactivity was counted in a Packard Tri-Carb Liquid Scintillation Spectrometer.

Calcium ATPase activity assay

The Ca^{2+}-stimulated ATPase activity of the preparation was measured in parallel with the calcium binding assays: 20 µl samples were withdrawn and added to 1 ml aliquots of boiling, de-ionized water and assayed for Pi. Ca^{2+}-stimulated ATPase activity was assayed as the difference in total ATPase activity obtained in the presence and absence of either 60 or 150 µM free Ca^{2+}.

[^{14}C]verapamil-binding

The ability of isolated fragments of sarcolemma to bind verapamil was investigated isotopically, using 1 µM [^{14}C]-(±)-verapamil and Millipore filtration. Sarcolemmal protein (0.6 to 0.8 mg) was preincubated, with continuous stirring for 30 min at 25 C, with or

without the appropriate enzymes used under optimal conditions: 10 units/ml neuraminidase, (type VI), 10 units/ml β-galactosidase (type VI), 1 unit/ml β-acetylglucosaminidase, 1 unit/ml phospholipase C (type I), and 100 μg/ml trypsin (type XI). After preincubation, the sarcolemmal suspension was centrifuged at 9000 *g* for 10 min, washed once with the enzyme-free buffer described above and recentrifuged. The resultant pellets were resuspended in 100 μl of the buffer used for the $^{45}Ca^{2+}$ binding studies. The buffer contained 60 or 100 μM free Ca^{2+} and membrane protein was added to provide a final concentration of 0.2 to 0.3 mg/ml. The reaction was started by adding [^{14}C]-(±)-verapamil. After 15 s incubation with continuous stirring at 25°C the reaction was terminated by Millipore filtration and the radioactivity of the filters counted to determine the extent of the binding. Preliminary studies established that binding of [^{14}C]-(±)-verapamil was complete within 15 s.

Mechanical function studies

To establish the relative negative inotropic potencies of (±)-, (+)- and (−)-verapamil, non-cumulative dose-response curves were obtained, using thin (diameter <0.5 mm) trabeculae excised from the right ventricles of small (2 kg) adult New Zealand white rabbits. The trabeculae were suspended isometrically in a temperature controlled (32°C), flow-through bath of 3.0 ml capacity, filled with modified Krebs Henseleit buffer solution gassed with 95% O_2 + 5% CO_2 and containing, (mM): NaCl 115.0, Na HCO_3 25.0, KCl 4.0, KH_2PO_4 0.9, Mg_2SO_4 1.1, $CaCl_2$, 2.3 and glucose 11.0. Field stimulation was effected via large platinium electrodes, delivering rectangular pulses of 5 ms duration at a frequency of 1 Hz. The stimuli originated from an SRI stimulator assembly (Scientific Research Instruments Co., Croydon, U.K.). Tension development was monitored with a Statham UC4 transducer (Stratham Instrument Co., Oxnard, California, USA), and displayed on a Devices direct writing chart recorder (MX212) at a paper speed of 1 mm/s. Each preparation was equilibrated for 60 min before any drug was added.

Statistical analysis

Unless otherwise stated, results are presented as mean ± s.e. mean of *n* experiments. Tests of significance were made by Student's *t* test.

Reagents

The enzymes were obtained from Sigma Chemical Co., Poole, Dorset. The other reagents were analytic reagent grade obtained from BDH, Poole, Dorset.

$^{45}Ca^{2+}$ (specific activity 10 to 40 mCi/mg Ca^{2+}) was obtained from the Radiochemical Centre, Amersham. ^{14}C-labelled verapamil (specific activity, 100 mCi/mmol), (±)-, (+)- and (−)-verapamil were donated by Knoll AG, Ludwigshaften, Germany. Solutions were prepared in deionized, distilled water.

Results

Characterization of the sarcolemmal preparation

Table 1 shows that the subcellular fraction used here was rich in sarcolemma and essentially free of sarcoplasmic reticulum and mitochondrial contamination. Cytochrome oxidase activity was low, adenylate cyclase and ouabain-sensitive Na^+K^+-ATPase activity was high, and the Ca^{2+}-dependent ATPase activity was azide-insensitive. The fraction was relatively rich in cholesterol and when examined under the electron microscope the membranes assumed the shape of flattened sacs, an appearance which contrasts with the small vesicles characteristically obtained for sarcoplasmic reticulum preparations.

Table 1 Distribution of marker enzymes and cholesterol in isolated fractions

Fraction	*Na^+K^+-ATPase* −ouabain	+ouabain	*Ca^{2+}-ATPase* $-NaN_3$	$+NaN_3$	*Adenylate cyclase*	*Cytochrome oxidase*	*Cholesterol*
Sarcolemma	7.12 ± 0.36	4.82 ± 0.10	5.18 ± 0.12	5.09 ± 0.13	983.0 ± 24.0	0.014 ± 0.008	86.3 ± 5.2
Sarcoplasmic reticulum	0.32 ± 0.05	0.30 ± 0.05	33.40 ± 1.36	30.35 ± 1.12	214.0 ± 80	0.040 ± 0.017	24.7 ± 3.8
Mitochondria	0.28 ± 0.04	0.29 ± 0.04	—	—	103.0 ± 4.0	1.218 ± 0.189	4.2 ± 0.7

ATPase activity is expressed as μmol Pi mg^{-1} h^{-1}, adenylate cyclase activity is pmol mg^{-1} min^{-1}, cytochrome oxidase as μmol of cytochrome oxidized mg^{-1} min^{-1} and cholesterol as μg/mg protein. Values are mean ± s.e. mean of 3 to 4 experiments. The sensitivity of the Na^+,K^+-ATPase to ouabain was established by adding ouabain as strophanthin to provide a final concentration of 1×10^{-5} M. Sodium azide (NaN_3) was added to provide a final concentration of 3×10^{-3} M. For details of assay procedures see methods section.

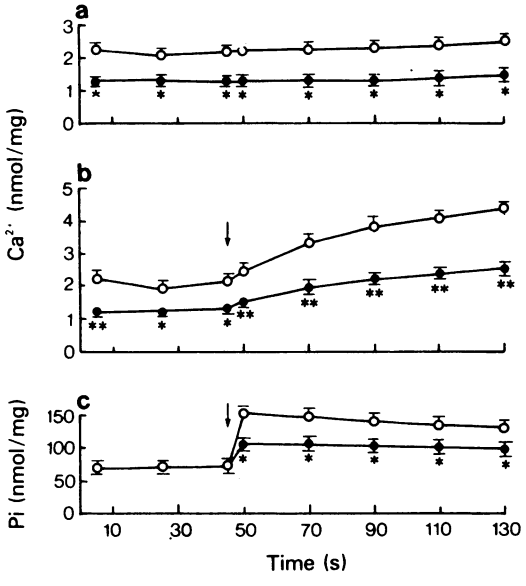

Figure 1 Effect of (±)-verapamil 1 μM on the $^{45}Ca^{2+}$ binding and Ca^{2+}-activated ATPase activity of isolated cardiac sarcolemmal fragments. $^{45}Ca^{2+}$-binding was measured in the presence of 60 μM free Ca^{2+}, in the absence (a) and presence (b) of ATP, added at the arrow. In (c) the activation of the ATPase enzyme was effected by adding ATP to provide a final concentration of 2.5 mM. Note that the apparent presence of Pi in the medium before the addition of the ATP is probably due to the presence of contaminant endogenous Pi.(○) Control: (●) (±)-verapamil. Reactions were monitored for 130 s, samples being removed for analysis at the indicated points. Each point is the mean of 5 experiments; vertical lines indicate s.e. mean. Test of significance relate to the inhibition caused by racemic verapamil: $*P < 0.025$; $**P < 0.01$.

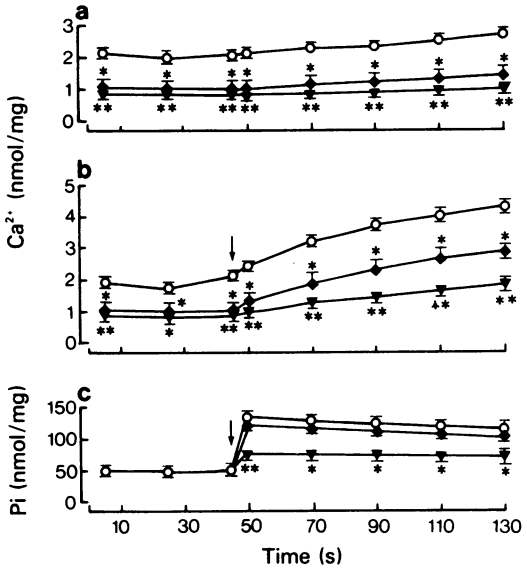

Figure 2 Effect of (+)-(◆) and (−)-isomers (▼) of verapamil on the Ca^{2+}-binding and Ca^{2+}-activated ATPase activity of isolated sarcolemma; (○) is control. The experiments were performed as described for Figure 1.

Figure 1a shows that the sarcolemmal fragments bound Ca^{2+} passively. Addition of ATP (Figure 1b) 45 s after initiating the passive binding process resulted in an increased rate of binding accompanied (Figure 1c) by an increased rate ($P < 0.025$) of ATP hydrolysis.

Effects of (±)-, (+)- and (−)-verapamil on Ca^{2+} binding and ATPase activity

Figure 1 shows that (±)-verapamil at a final concentration of 1 μM reduced the passive ($P < 0.025$ Figure 1a) and ATP-dependent ($P < 0.01$, Figure 1b) binding of Ca^{2+}. Figure 1c shows that this (±)-verapamil-induced inhibition of the ATP-dependent Ca^{2+} transport was accompanied by a slowed ($P < 0.025$) rate of ATP hydrolysis.

Figure 2a shows that the (+)- and (−)-isomers of verapamil are equally effective in inhibiting passive Ca^{2+} binding. Although the (−)-isomer of verapamil also slows the ATP-dependent transport of Ca^{2+}, the (+)-isomer is inactive (Figure 2b). Figure 2c shows that under the experimental conditions used here, only the (−)-isomer is effective in slowing the activity of the Ca^{2+}-activated ATPase enzyme.

The initial rates of ATP-stimulated Ca^{2+} transport monitored over a free Ca^{2+} range of 20 to 200 μM, in the presence and absence of (+)- and (−)-verapamil, are shown in Figure 3. The rate during the first 5 s of the reaction was taken as the initial rate. These data show an inhibitory effect for (−)-verapamil on the initial rate of ATP-dependent Ca^{2+} transport. The reciprocal plot (insert, Figure 3) shows that the inhibition is accompanied by a decreased K_m and V_{max}, indicating a mixed-type inhibition.

[^{14}C]-verapamil binding studies

Previously we have established that ^{14}C-labelled verapamil binds to isolated sarcolemmal fragments and

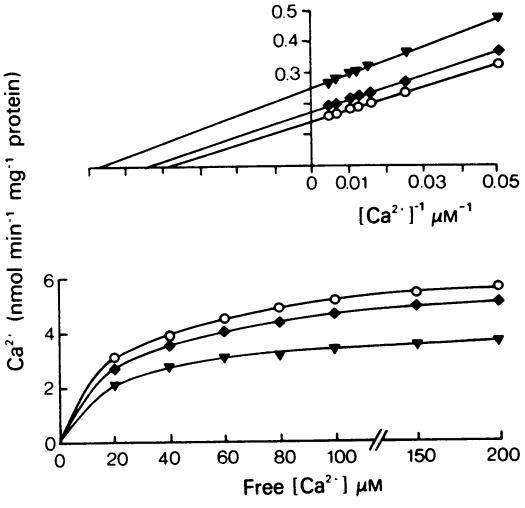

Figure 3 Effect of (+)-(◆) and (−)-isomers (▼) of verapamil on the initial rate of ATP-dependent Ca^{2+} binding, measured over a time course of 5 s after the addition of ATP; (○) is control. Each point is the mean of five different experiments (s.e. mean bars have been omitted for clarity). Insert: reciprical plot of the initial rates of Ca^{2+} binding at different free Ca^{2+} concentrations.

particularly to carbohydrate-containing residues (Nayler *et al.*, 1979). Further identification of the binding sites was attempted here by using a variety of enzymes to cleave links in the neuraminic acid-galactose-glucosamine sequence which forms part of the sarcolemmal carbohydrate complex (Figure 4). Neuraminidase (Figure 4) cleaves the link between *N*-acetylneuraminic (or sialic) acid and galactose; β-galactosidase cleaves the link between galactose and *N*-acetylglucosamine; acetylglucosaminidase cleaves the link between glucosamine and subsequent carbohydrate residues. Figure 5 shows that pretreating the sarcolemmal fragments with either neuraminidase or β-galactosidase increased [^{14}C]-verapamil binding by 21 and 14% respectively. Pretreatment with β-acetyl glucosaminidase, however, decreased [^{14}C]-verapamil binding, by 29% (Figure 5). Figure 5 also shows that preincubation of the sarcolemmal preparations with either trypsin or phospholipase C decreased [^{14}C]-verapamil binding.

Mechanical function studies

The dose-response curves obtained for the negative inotropic effect of (±)-, (+)- and (−)-verapamil are shown in Figure 6. These data show that the negative inotropic activity of racemate verapamil is due predominately to negative inotropism of the (−)-isomer.

Discussion

These results show that a concentration of (±)-verapamil that is negatively inotropic, decreases the Ca^{2+} binding and Ca^{2+}-activated ATPase activity of an isolated cardiac sarcolemmal preparation. The results also show that whereas the (+)- and (−)-isomers are equally effective in inhibiting the ATP-independent (or passive) binding of Ca^{2+}, the ATP-dependent (or active) transport and the associated hydrolysis of ATP are sensitive only to the (−)-isomer. Previous investigators have shown that the negative inotropism of (±)-verapamil is attributable almost exclusively to the activity of the (−)-isomer (Bayer, Kaufmann & Mannhold, 1975). Our dose-response curves confirm this conclusion. It follows, therefore, that if, as is

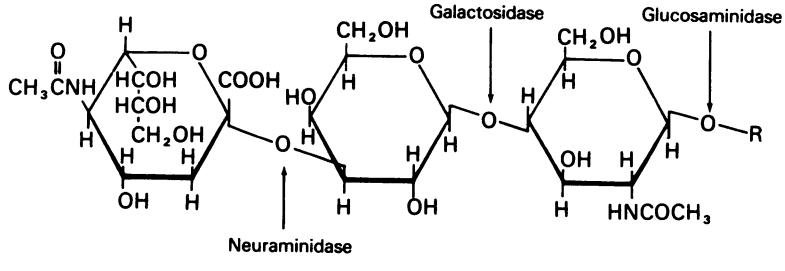

Figure 4 Schematic representation of the sites of action of neuraminidase, β-galactosidase and β-acetylglucoseaminidase.

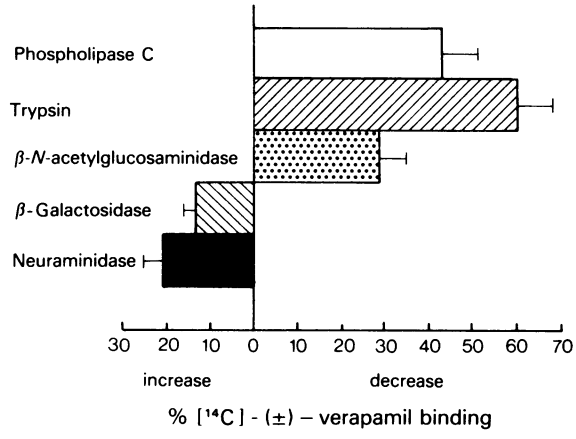

Figure 5 Effect of preincubation with phospholipase C, trypsin, β-acetyl-glucosaminidase, β-galactosidase and neuraminidase on the association of ^{14}C-labelled (±)-verapamil with isolated sarcolemmal fragments. Each point is the mean of three different experiments; horizontal lines show s.e. mean.

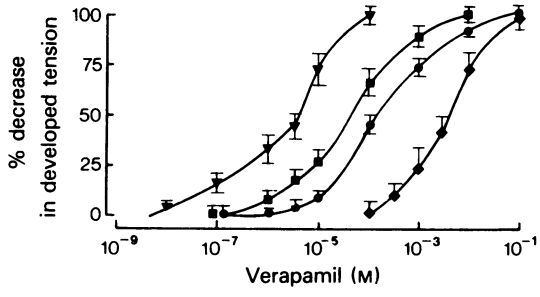

Figure 6 Non-cumulative dose-response curves for the negative inotropic activity of (±)-, (+)- and (−)-verapamil on isolated, paced rabbit trabeculae. (◆) (+)-Verapamil; (▼) (−)-verapamil; (■) (±)-verapamil; (●) diltiazem. Each point is the mean of 4 separate determinations; vertical lines indicate s.e. mean.

generally believed, the negative inotropism of verapamil involves a decreased inward transport of Ca^{2+} through the slow Ca^{2+} channels (Kohlhardt *et al.*, 1972; Ehara & Kaufmann, 1978) it is unlikely that this negative inotropism results directly from an inhibition of the ATP-independent (or passive) binding of Ca^{2+}. If this had been the case then we should have expected to find evidence of stereospecificity for the inhibitory effect of verapamil on the passive binding of Ca^{2+}. However, it is possible that this inhibition of passive Ca^{2+} binding contributes to the ability of verapamil to reduce the size of the lanthanum displaceable pool of superficially stored Ca^{2+} (Nayler & Szeto, 1972). If some of the Ca^{2+} needed for excitation-contraction coupling originates from this superficially-located 'pool', then the inhibitory but non stereospecific effect of verapamil on the passive binding of Ca^{2+} could contribute, but only indirectly, to the drug's negative inotropism. Evidence from our enzyme treatment studies suggest that an interaction between verapamil and the neuraminic acid residues which some investigators believed to be the main sites of Ca^{2+} storage within the glycocalyx (Frank *et al.*, 1977) is not involved in this inhibitory effect of verapamil on passive Ca^{2+} binding.

The sarcolemmal preparations used in the present study are relatively pure (Table 1) and consist of a mixed population of 'inside-out' and 'right side-out'

sacs (Mas-Oliva *et al.*, 1979a). The Ca^{2+}-activated ATPase enzyme is known to be located on the cytosolic surface of the plasmalemma (Dhalla, Anand & Harrow, 1976; Mas-Oliva, Williams & Nayler, 1979b) and therefore must be expected to pump Ca^{2+} outwards. The activity of this enzyme was inhibited by (−)-verapamil (Figure 2). Since mmolar amounts of verapamil are needed to interfere with the Ca^{2+}-transporting and Ca^{2+}-activated ATPase activity of sarcoplasmic reticulum-enriched preparations (Nayler & Szeto, 1972; Watanabe & Besch, 1974) it is unlikely that the inhibitory effects described here for 1 μM verapamil can be explained in terms of an inhibitory effect on any contaminant sarcoplasmic reticulum. Verapamil is lipophylic (Bondi, 1978) and therefore probably enters the hydrophobic zone of the cell membrane. It is possible, therefore, that even under *in vivo* conditions it can approach and therefore inhibit the intrinsic Ca^{2+}-ATPase of the sarcolemma. Moreover, because this inhibition is stereospecific it may be linked, perhaps indirectly, with the drug's negative inotropism.

The ^{14}C-labelled verapamil studies after enzyme treatment indicate that verapamil associates with glycoproteins and glycolipids in the sarcolemma. From gel electrophoresis studies (Nayler *et al.*, 1979) we know that the association involves predominantly the glycolipids. It does not require the presence of Ca^{2+} (unpublished data), a finding which should not be confused with the observations of Williamson *et al.* (1975) relating to the ability of (±)-verapamil to inhibit the passive binding of Ca^{2+} in a competitive manner, or with our findings relating to the effect of the (+)- and (−)-isomers on Ca^{2+} binding. The present results show that removal of the *N*-acetylneuraminic acid residues increases [^{14}C]-(±)-verapamil binding. It is unlikely therefore that the drug complexes with the *N*-acetylneuraminic acid residues of the glycoproteins and glycolipids. The same argument applies to the galactose residues. By contrast, removal of either the *N*-acetylneuraminic acid or the galactose residues appears to facilitate access of the drug to its binding sites. Removal of the *N*-acetylglucosamine residues and polar heads of phospholipids decreased the verapamil binding indicating, perhaps, that the presence of these residues contributes to the low affinity binding sites of the drug. Evidence exists suggesting that verapamil is bound at both high and low affinity sites by cardiac sarcolemma (Williamson *et al.*, 1975). Because of the hydrophobic nature of verapamil it is possible that association of the drug with the lipid component of the membrane reflects the high affinity binding sites, whilst the association with specific carbohydrate residues of the glycocalyx, demonstrated here, represents low affinity binding.

In conclusion, therefore, our results indicate that verapamil does not associate with either the *N*-acetylneuraminic acid or galactose residues of the glycocalyx. The *N*-acetylneuraminic acid residues are the terminal residues of the carbohydrate chains and since they are negatively charged it is possible that they provide storage sites for Ca^{2+} (Frank *et al.*, 1977). However, since verapamil does not bind to these residues it is unlikely that the negative intropism of the drug can be explained simply in terms of its occupancy of potential Ca^{2+}-storing sites in this region of the glycocalyx. Our results also show that verapamil interferes with the ATP-dependent Ca^{2+} transporting activities of the sarcolemma and with the associated hydrolysis of ATP by an intrinsic Ca^{2+}-activated ATPase. These inhibitions (Ca^{2+}-transport and ATPase activity) showed stereospecificity, the (−)-isomer being active. The negative inotropism of verpamil also resides in the (−)-isomer. The question arises, therefore, as to how an inhibition of an energy-dependent transport of Ca^{2+} could result in negative inotropic response, particularly when the ATPase is probably involved in the extrusion of Ca^{2+} from the cell. One possibility could be that the Ca^{2+}-activated ATPase transports calcium into a 'compartment', which might be associated with superficially located sites and from which calcium could be released by the next action potential. Such a system would contribute to a pool of rapidly recycling calcium required for the maintained functioning of the voltage-activated slow calcium channels. Additional experiments are needed to explore this possibility.

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