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Effects of angiotensin, catecholamines and cyclic AMP on calcium storage in aortic microsomes

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Summary

1. The binding of calcium observed in microsomal membranes derived from the intimal-medial layer of rabbit aorta was specifically increased by the magnesium salt of adenosine triphosphate (Mg-ATP). This calcium uptake was inhibited by potassium and sodium.

2. Angiotensin II, both in the presence and in the absence of Mg-ATP, reduced the binding of calcium and increased the release of membrane-incorporated calcium. These effects were dose-dependent. Analogues of angiotensin II devoid of intrinsic activity, failed to alter the release of calcium.

3. Dibutyryl-cyclic AMP increased the binding of calcium and reduced the rate of its release. The maximal effect was observed at the concentration of 10^{-5}M.

4. (−)-Noradrenaline reduced the binding of calcium; this effect was inhibited by phenoxybenzamine. Conversely, adrenaline like cyclic AMP, increased the calcium binding; the effect of adrenaline was suppressed by a β-adrenoceptor blocking agent.

5. These observations demonstrate the existence of membranes in rabbit aorta capable of storing calcium. Excitatory drugs seem to affect directly and specifically the binding and release of calcium in these membranes.

Introduction

In smooth muscle, as in striated muscle and myocardium, the calcium ion ultimately controls the tone of the contractile proteins (Somlyo & Somlyo, 1968; Hurwitz & Suria, 1971). The rise in free intracellular calcium (Ca^{2+}) leading to contraction may result from one or several of the following mechanisms: (i) increase in the membrane permeability to extracellular calcium which would enter the cell passively down a steep transmembrane electrochemical gradient, (ii) reduction of the active mechanism which controls the calcium efflux from the cell, (iii) translocation of calcium from cellular sites of storage. An ATP-dependent calcium uptake has been observed in the mitochondrial and microsomal fractions of uterine smooth muscle (Carsten, 1969; Batra & Daniel, 1971), of intestinal smooth muscle (Anderson, Lundholm & Mohme-Lundholm, 1972), and of the intimal-medial layer of aorta (Fitzpatrick, Landon, Debbas & Hurwitz, 1972; Baudouin, Meyer, Fermandjian & Morgat, 1972). The participation of intracellularly bound Ca^{2+} in the contraction of the aorta is suggested by the persistence of contractile responses to catecholamines and angiotensin in Ca^{2+}-free solutions (Sitrin & Bohr, 1971;

Somlyo, Devine, Somlyo & North, 1971). The transfer of calcium in microsomal preparations of smooth muscle is affected by different drugs. Adrenaline and 3',5'-cyclic monophosphoric acid (cyclic AMP) increase the uptake of Ca^{2+} in microsomes of rabbit colon (Anderson *et al.*, 1972); acetylcholine and angiotensin can release Ca^{2+} from microsomal fractions of intestinal smooth muscle and aorta (Tagaki & Uchida, 1970; Baudouin *et al.*, 1972). These observations raise the possibility that excitatory drugs may have a direct action on cellular calcium stores. The present study complements the analysis of Ca^{2+} binding in microsomes of rabbit aorta and its variations induced by angiotensin which have been reported in preliminary form (Baudouin *et al.*, 1972), and provides new information on the variations of Ca^{2+} binding induced by catecholamines and dibutyryl-cyclic AMP.

Methods

Rabbit aortae dissected free from adventitia were homogenized in all-glass Potter–Elvehjem homogenizers in ice-cold 0·33 M sucrose-0·01 M Tris maleate, pH 7·4, containing $MgCl_2$ 1 mM and KCl 1 mM. Homogenates were subjected to differential centrifugation at 4° C between 3,000 and 100,000 *g* according to the method of Verity & Bevan (1969). The microsomal pellet precipitated at 100,000 *g* from the 35,000 *g* supernatant was suspended in 10 mM L-histidine monochlorhydrate, pH 6·8, containing various cations and reagents as specified in the text.

Determinations of calcium incorporation (Ca^{2+} binding studies) were performed on 70–100 μg of microsomal protein in the presence of various concentrations of $CaCl_2$ containing 0·3 nCi ^{45}Ca in a total volume of 0·5 ml histidine buffer. The reaction was performed at 26° C, and stopped by filtration of the reaction mixture through a Millipore filter (HA 0·45 μm), followed by fast rinsing with 5 ml of chilled calcium-free medium. The radioactive Ca^{2+} retained by the microsomes was measured by liquid scintillation spectrometry, after the filters had been dissolved in methoxyethanol. Values were corrected for ^{45}Ca remaining on filters in the absence of microsomes.

The release of membrane-incorporated Ca^{2+} was examined after subjecting microsomes to 10^{-4}M $^{45}Ca^{2+}$ for a period of 10 min; the medium was then diluted by the addition of 25 volumes of Ca^{2+}-free medium, and the amount of Ca^{2+} remaining on the microsomes at different time intervals after the dilution was determined after the filtration technique by liquid scintillation (Ca^{2+} release studies). Throughout the whole study, measurements of Ca^{2+} binding to microsomes, or of Ca^{2+} remaining in the microsomes during release studies, were performed in triplicate for a given concentration of Ca^{2+}, and for a given batch of microsomes. Blank values of ^{45}Ca adsorbed on to the filters in the absence of microsomes, were also performed in triplicate. Measurements of total microsomal Ca^{2+} were carried out with a Perkin–Elmer absorption spectrophotometer and in separate experiments with deproteinized solutions containing 5% HCl and 0·5% $LaCl_3$. Microsomal protein concentration was determined by the method of Lowry, Rosebrough, Farr & Randall (1951). Cytochrome-C-oxidase activity (EC 1.9.3.1) was assayed by the spectrophotometric technique of Cooperstein & Lazarow (1951).

For electron microscopy, the 100,000 *g* pellets were fixed in 2% glutaraldehyde, followed by 2% OsO_4, dehydrated in ethanol and embedded in epoxyresin. Ultrathin sections were cut on a Reichert OmU_2 ultramicrotome, and stained with $KMNO_4$ and lead citrate for a study of vesicle morphology.

Drugs used

All chemicals used were of reagent grade. Deionized water was distilled twice in a quartz still and used to prepare solutions. Angiotensin II was [Asn^1,Val^5]-angiotensin II (Hypertensin Ciba). (−)-Noradrenaline and (−)-adrenaline (Sigma) were dissolved before each experiment in histidine solution. Dibutyryl-3′,5′-cyclic AMP (N^6,O$^{2'}$-dibutyryl adenosine 3′,5′-cyclic monophosphoric acid), GTP (guanosine 5′-triphosphate), ATP (adenosine 5′-triphosphate) and sodium azide were obtained from Sigma Chemicals. Radioactive $^{45}Ca^{2+}$ was supplied by the Radiochemical Centre of Amersham. The specific radioactivity was 2–5 Ci/g. The analogues of angiotensin II ([Asn^1,Phe^4]-A II, [Asn^1,Ala^6]-A II, [Asn^1,Ala^8]-A II) were kindly supplied by Doctors F. M. Bumpus and P. A. Khairallah (Cleveland, Ohio, USA). The α-adrenoceptor blocking agent phenoxybenzamine and the β-adrenoceptor blocking agent LB-46 (prinodolol) were obtained from Smith, Kline & French and Sandoz respectively.

Results

Electron micrographs of the 100,000 *g* pellet of rabbit aorta homogenates showed that this fraction consisted of vesicular structures. Most vesicles consisted of smooth membranes but occasional rough membrane vesicles were seen. No intact mitochondria were encountered, and the preparation was free of cytochrome-C-oxidase which served as a biochemical indicator of the presence of mitochondria.

Calcium binding

Influence of Mg-ATP

Microsomes suspended in 10 mM histidine buffer solution containing 100 mM KCl were incubated at 26° C in the presence and in the absence of Mg-ATP. The rate of Ca^{2+} binding was not influenced by Mg-ATP, the equilibrium being reached after 10 min both in the presence and absence of Mg-ATP. However, the magnitude of the Ca^{2+} binding was Mg-ATP dependent: in the presence of 5 mM

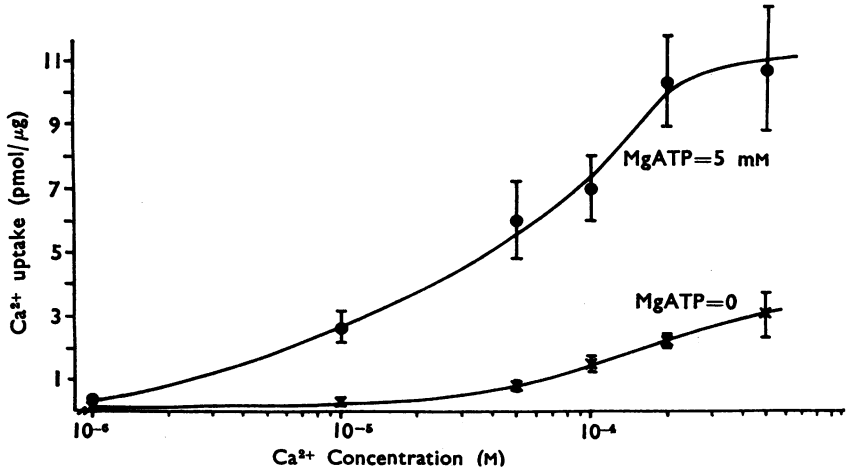

FIG. 1. Variations in the microsomal binding of Ca^{2+} in the presence and in the absence of 5 mM Mg-ATP with different Ca^{2+} concentrations in the incubation medium. Vertical bars indicate S.E.M. 5–9 experiments.

Mg-ATP, the maximal Ca^{2+} binding of 10·33 pmol/μg membrane protein, observed at a Ca^{2+} concentration of 2×10^{-4}M in the medium, was 4–5 fold higher than that observed in the absence of Mg-ATP (Fig. 1). The effect of Mg-ATP was maximal at the concentration of 5×10^{-3}M and was apparent above the concentration of 10^{-4}M. The optimal Ca^{2+} binding depended on the presence of both Mg^{2+} and ATP. Substitution of Mg-ATP by the Tris-salt of ATP reduced the Ca^{2+} binding. A reduction of Ca^{2+} binding was also observed with the Tris-salt of GTP ; Mg-GTP at the concentration of 5 mM had no potentiating effect (Table 1). Measurements of total microsomal Ca^{2+} by atomic absorption spectrophotometry indicated that the Ca^{2+} content of microsomes was 10 pmol/μg ; after 10 min of incubation in the presence of 10^{-4}M Ca^{2+} and 5×10^{-3}M $MgCl_2$ this value became 22 pmol/μg indicating that the binding measured with $^{45}Ca^{2+}$ was real and did not reflect a simple exchange between endogenous Ca^{2+} and the radioactive Ca^{2+} of the incubation medium.

TABLE 1. *Effects of cations and nucleotides on microsomal Ca^{2+} binding*

K^+	Na^+	Mg^{2+}	ATP	GTP	Ca^{2+} binding (%)
0·1	0	0	0	0	100
0·05	0	0	0	0	595
0·05	0·05	0	0	0	398
0·1	0·1	0	0	0	58
0·1	0	0·005	0	0	49
0·1	0	0·05	0	0	15
0·1	0	0	0·005	0	46
0·1	0	0·005	0·005	0	310
0·1	0·1	0·005	0·005	0	72
0·1	0	0	0	0·005	50
0·1	0	0·005	0	0·005	96

The concentration of cations and nucleotides is expressed as M. The uptake of Ca^{2+} is expressed in %, and the binding of Ca^{2+} observed in presence of 0·1 M K has been arbitrarily considered as representing 100%. Each measurement is the mean of 5–8 experiments.

TABLE 2. *Effects of angiotensin II on microsomal binding of Ca^{2+}*

Angiotensin (M)	Ca^{2+} binding (%) MgATP=0	MgATP=5 mM
0	100	100
10^{-9}	94·9	89·1±2·5
10^{-8}	73·6±3·9	85·1±5·1
5×10^{-8}	69·8±2·5	72·0±6·5
10^{-7}	57·3±5·5	62·5±6·7
10^{-6}	56·0±9·6	62·0±5·0

Values of Ca^{2+} binding are expressed in %. The Ca^{2+} binding observed in absence of angiotensin has been arbitrarily considered as 100%. Incubation time 10 min; mean±S.E.M. of 5–7 experiments.

TABLE 3. *Effect of dibutyryl 3'5'-cyclic AMP on microsomal binding of Ca^{2+}*

Dibutyryl 3'5'-cyclic AMP (M)	Ca^{2+} binding (%) MgATP=0	MgATP=5 mM
0	100	100
10^{-8}	100	100
10^{-7}	92·0±10·5	105·0±1·0
10^{-6}	106·0± 5·9	110·5±3·5
10^{-5}	125·6± 9·5	119·6±4·1
10^{-4}	123·5±11·2	119·5±0·8

Values of Ca^{2+} binding are expressed in %. The Ca^{2+} binding observed in absence of dibutyryl-cyclic AMP has been arbitrarily considered as 100%. Incubation time 10 min; mean±S.E.M. of 5–7 experiments.

Influence of cations

In the presence of ATP, the cations K^+, Na^+ and Mg^{2+} reduced the Ca^{2+} binding. The inhibitory effect of Na^+ was also apparent in the presence of 5 mM Mg-ATP (Table 1).

Effects of sodium azide and sodium oxalate

Sodium azide which is known to inhibit Ca^{2+} uptake in mitochondria at a concentration of 0·5 mM (Farnberg & Gergely, 1965), had no effect on Ca^{2+} binding either in the presence or in the absence of Mg-ATP.

Addition of 2·5 mM sodium oxalate to the incubation medium either in the presence or absence of Mg-ATP did not modify the binding of Ca^{2+}.

Effects of drugs on the binding of Ca^{2+}

Angiotensin II inhibited the binding of Ca^{2+} both in the presence and absence of Mg-ATP. The inhibitory effect was dose-dependent. The maximal inhibitory effect, representing a 40% reduction of the Ca^{2+} binding occurring in the absence of the hormone, was observed at a concentration of 10^{-7}M (Table 2). Dibutyryl-cyclic AMP increased the binding of Ca^{2+} to microsomes. A maximal increase of +25% was obtained at a concentration of 10^{-4}M (Table 3).

The effects of catecholamines are indicated in Tables 4 and 5: (−)-noradrenaline and adrenaline had inhibitory and stimulatory effects respectively; it was not possible to obtain precise dose-response curves with (−)-noradrenaline. The maximal stimulatory effect of adrenaline was obtained at a concentration of 10^{-5}M. In the

TABLE 4. *Effects of (−)-noradrenaline on microsomal binding of Ca^{2+}*

(−)-Noradrenaline (M)	Ca^{2+} binding (%) Mg-ATP=0	Mg-ATP=5 mM
0	100	100
10^{-7}	80·5±13·9	86·8±8·2
10^{-6}	74·7±10·6	85·1±6·8
5×10^{-6}	74·4±15·8	74·6±5·4
10^{-5}	79·5±10·5	89·1±6·6
5×10^{-5}	81·7±17·5	89·0±3·4
10^{-4}	80·1±12·3	87·3±5·0
5×10^{-4}	56·7± 7·4	90·8±4·2
(−)-Noradrenaline 10^{-4}+phenoxybenzamine 10^{-4}		113·5±5·1

Values of Ca^{2+} binding are expressed in %. The Ca^{2+} binding observed in absence of (−)-noradrenaline has been arbitrarily considered as 100%. Incubation time 10 min; mean±S.E.M. of 6 experiments.

TABLE 5. *Effects of adrenaline on microsomal binding of Ca^{2+}*

(−)-Adrenaline (M)	Ca^{2+} binding (%) Mg-ATP=0	Mg-ATP=5 mM
0	100	100
10^{-7}	146·1±18·5	103·7± 5·1
10^{-6}	131·3±19·1	116·2± 5·8
5×10^{-6}	106·9± 5·7	109·6± 5·9
10^{-5}	139·6± 3·9	132·2± 4·5
5×10^{-5}	112·4± 0·8	128·2± 8·8
10^{-4}	149·0± 8·3	131·2± 5·3
5×10^{-4}	143·2± 4·7	129·5±11·0
Adrenaline 10^{-4}	131·2±5·3	
+LB-46 10^{-4}	101·2±3·2	

Values of Ca^{2+} binding are expressed in %. The Ca^{2+} binding observed in absence of adrenaline has been arbitrarily considered as 100%. Incubation time: 10 min; mean±S.E.M. of 6 experiments.

presence of phenoxybenzamine, (−)-noradrenaline increased the Ca^{2+} binding. The stimulatory effect of adrenaline was suppressed by the β-adrenoceptor blocking agent LB-46.

Calcium release

Washing the microsomes after exposure to 10^{-4}M Ca^{2+} with 25 volumes of chilled Ca^{2+}-free medium did not change the amount of incorporated $^{45}Ca^{2+}$ for the first two minutes. However, washing the $^{45}Ca^{2+}$-loaded vesicles at 26° C resulted in a release of the incorporated $^{45}Ca^{2+}$ that was much faster during the first two minutes than between the second and fifth minutes. Addition of 5 mM Mg-ATP to the washing medium increased the rate of release (Fig. 2).

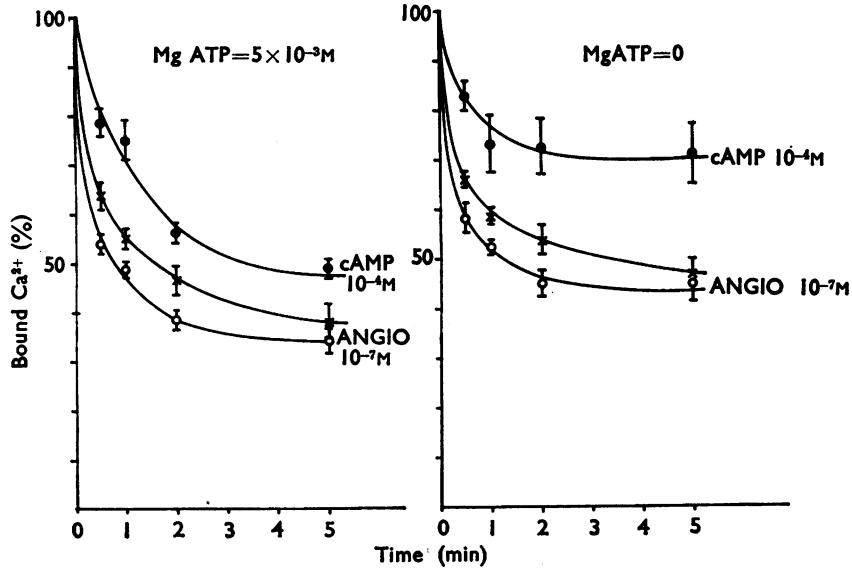

FIG. 2. Variations in the release of membrane-bound Ca^{2+} with angiotensin II (ANGIO) and dibutyryl-cyclic AMP (cAMP) in the presence and in the absence of Mg-ATP. Mean of 5–9 experiments. Vertical bars indicate the S.E.M. ×——×, Ca^{2+} release in absence of hormones; ●——●, Ca^{2+} release in presence of cyclic AMP; ○——○, Ca^{2+} release in presence of angiotensin.

Effects of drugs on the release of calcium

The releasing effect of angiotensin II was apparent both in the absence and in the presence of Mg-ATP, and was maximal at a concentration of 10^{-7}M; this result has been presented in detail in a previous communication (Baudouin *et al.*, 1972). [Phe^4]-angiotensin II, [Ala^6]-angiotensin II, and [Ala^8]-angiotensin II which have very little or no intrinsic activity on rabbit aorta had no effect on calcium release at concentrations of 10^{-5}M.

The rate of Ca^{2+} release was reduced by dibutyryl-cyclic AMP both in the presence and absence of Mg-ATP. The maximal inhibitory effect was obtained at a concentration of 10^{-4}M, and is represented in Figure 2. The effects of catecholamines on the release of Ca^{2+} were studied at the concentration of 10^{-4}M and were conducted in the presence of 5 mM Mg-ATP. The amount of $^{45}Ca^{2+}$ bound to the vesicles was determined 30 s after washing with Ca-free medium in four experi-

ments. (−)-Noradrenaline and adrenaline significantly increased and reduced the Ca^{2+} release by 14·7 and 10·9% respectively.

Discussion

The two main questions raised by these results are related to the mechanism of Ca^{2+} binding and to the significance of the variations in Ca^{2+} transfer induced by the drugs. The vesicular arrangement of the microsomal membranes is compatible with either a penetration of Ca^{2+} inside the vesicles, or to a binding of Ca^{2+} to the membranes. Sodium oxalate penetrates and precipitates Ca^{2+} inside the vesicles, increasing the Ca^{2+} uptake when the cation enters the vesicles (Katz & Repke, 1967). Since sodium oxalate had no effect in our preparation, the most likely explanation is that Ca^{2+} is bound to the membranes. The mechanism of the increase in Ca^{2+} incorporation caused by Mg-ATP may be explained by several hypotheses, which have been proposed for Ca^{2+} uptake in striated and cardiac muscle microsomes: (i) the Ca^{2+} binding induced by Mg-ATP may result from a conformational change in the membrane (Onishi & Abashi, 1964; Landgraf & Inesi, 1969); (ii) alternatively, the Ca^{2+} binding might be due to an active transport association in some way with the hydrolysis of ATP (Hasselbach, 1964). In our preliminary experiments, it was reported that a Mg-dependent ATPase was activated by Ca^{2+} in parallel with its binding in rabbit aorta microsomes (Baudouin *et al.*, 1972). This observation which supports the active transport mechanism was not constantly repeated in subsequent experiments, and further studies are needed for a definite answer.

It is tempting to suggest that the microsomal binding of Ca^{2+} may have a physiological significance since it may contribute in regulating the concentration of free cytoplasmic Ca^{2+} and therefore the contractile state of muscle fibres. The first objection to this hypothesis is raised by the low affinity of the microsomes for Ca^{2+} (about 5×10^{-5}M) which is about 100 times lower than that of the contractile proteins of smooth muscle (Filo, Bohr & Ruegg, 1965). However this discrepancy, although important, cannot be considered as a definitive argument for two reasons: firstly, one may conceive that a reduction in the affinity for Ca^{2+} may be the result of some damage occurring to the membranes during the extraction procedure; secondly, whereas the experiments on contractile proteins were conducted at 37° C our microsomal experiments were performed at 26° C in order to reduce the rate of Ca^{2+} movement and thus allowed a more accurate analysis. The variations of binding and release of Ca^{2+} induced by drugs which affect the vascular tone, suggest the hypothesis that their mechanical response could be, at least partially, mediated by a translocation of Ca^{2+} from binding stores in cellular membranes. The effects of angiotensin, and to a lesser extent, those of noradrenaline, are compatible with this proposition: the two agonists reduce the amount of Ca^{2+} incorporated within the membranes which could in turn result in an increase in the cytoplasmic free Ca^{2+} concentration. At the concentration of 10^{-7}M, angiotensin inhibits the Ca^{2+} binding by 40% corresponding to an inhibition of 4 pmol Ca^{2+} per μg of membrane protein. Throughout these experiments, it was found that the extraction process yielded 50 μg membrane protein from 100 mg of wet rabbit aorta. For simplification, using the assumption that the intracellular volume corresponds to half of the weight of the aorta (Baudouin, Meyer & Worcel, 1971), 1 μg of membrane protein corresponds to 1 μl of intracellular

water. Angiotensin at the concentration of 10^{-7}M which induces the maximal contraction, would therefore increase the cytosomal concentration of Ca^{2+} by 4×10^{-6}M. This is higher than the threshold concentration of $1{\cdot}8 \times 10^{-7}$M Ca^{2+} for contraction of glycerinated vascular smooth muscle reported by Filo *et al.* (1965). Several observations indicated that the effects of angiotensin and noradrenaline were specific: (i) analogues of angiotensin II devoid of intrinsic contractile activity on rabbit aorta failed to alter the release of calcium, (ii) we found that angiotensin II did not reduce the binding of Ca^{2+} in microsomes extracted from the rabbit oesophagus which is not contracted by angiotensin (Baudouin-Legros & Meyer, unpublished), (iii) phenoxybenzamine suppressed the effects of (−)-noradrenaline.

The opposite effects of cyclic AMP, which increased Ca^{2+} binding and decreased Ca^{2+} release, are also compatible with the physiological significance of the observed Ca^{2+} transfer, given the relaxing properties of the nucleotide (Triner, Nahas, Vulliemoz, Overweg, Verosky, Habif & Ngai, 1972). However, the variations of Ca^{2+} transfer induced by adrenaline, although specific since they were suppressed by a β-adrenoceptor blocking agent, do not support a relationship between Ca^{2+} transfer and mechanical activity. Like (−)-noradrenaline, adrenaline has a contractor effect on rabbit aorta (Furchgott, 1967), and unlike (−)-noradrenaline, adrenaline was found to increase the microsomal Ca^{2+} binding. No explanation was found for these conflicting results . However, in the presence of an α-adrenoceptor blocking compound, adrenaline has a relaxing effect on aorta (Triner *et al.*, 1972). It is possible that for some unknown reason, adrenaline has a dominating β effect in the isolated microsomal membranes. In addition, it must be noted that the membranes present in the 100,000 *g* microsomal pellet derive from plasma membrane, caveoli and sarcoplasmic reticulum (Verity & Bevan, 1969). Binding of Ca^{2+} seems to occur in plasma membrane (Sparrow & Simmonds, 1965; Wolowyk, 1971), and in sarcoplasmic reticulum (Somlyo & Somlyo, 1971). Therefore the variations observed on the crude microsomal fraction represent only a net effect, and some clarification could be obtained by further studies of the effects of the different drugs on purified membrane fractions. These studies could also provide some indication as to whether the vasoactive drugs act on the plasma membrane or on intracellular membranes. Effects on calcium binding observed in the former could suggest that drugs do not enter the cell, whereas effects observed in the latter may suggest the opposite.

In all cases, the changes in Ca^{2+} transfer induced by the various drugs were observed both in the presence and the absence of Mg-ATP. This result may indicate that the drugs were not acting through some ATP-dependent active mechanism, and that their effects were essentially secondary to 'passive' changes in the membrane permeability.

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