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Effects of Drugs on the Interaction of Calcium and Cardiac Muscle Membrane Fragments

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The effect of drugs on calcium-binding to cardiac muscle membrane fragments and its turnover rate was studied. Ouabian, acetylcholine, isoproterenol and norepinephrine did not have any effect either on calcium-binding to membrane fragments or an washout and release curves of previously bound calcium. Local anesthetics inhibited the calcium-binding. Tetracaine at concentrations of 1 and 10 mM inhibited the calcium-binding by 30% and 54%, respectively, while 10 mM lidocaine inhibited it by 17%. Propranolol, a well-known adrenergic beta-blocker, also inhibited calcium-binding at the external calcium concentrtion of 10^{-3} M. This effect of propranolol may be attributed to its local anesthetic-like action, rather than to the adrenergic blocking effect.

Key Words: Cardiac Muscle Membrane, Calcium Ion, Cardioactive Drugs.

It is currently believed that the excitation of muscle membrane results in a rise of intracellular Ca^{++} concentration which leads to the activation of actomyosin adenosine triphosphatase(ATPase) and the interaction of actin and myosin (Heilbrun and Wiercinski, 1947; Porter and Palade, 1957; Hasselbach and Makinose, 1961; Porter, 1961; Weber and Winicur, 1961; Ebashi and Lipmann, 1962; Weber and Herz, 1962; Inesi *et al.*, 1964; Lee *et al.*, 1965; Sandow, 1965; Page, 1968).

The source of Ca^{++} released upon excitation is the sarcoplasmic reticulum in the skeletal muscle. However, in cardiac muscle the development of sarcoplasmic reticulum is poor (Fawcett and Selby, 1958; Porter, 1961; Inesi et al, 1964), and some studies are available which suggest the influx of Ca^{++} into the cardiac cell during excitation. Thus, under appropriate experimental conditions there is a close relationship between the amount of calcium taken up per beat and the magnitude of tension developed by the heart muscle. This is true whether the cardiac contractile tension is increased by higher frequencies of stimulation, by higher calcium concentrations in the external medium (or high $[Ca^{++}]/[Na^{+}]^2$ ratios), or by some drugs (Klaus and Kuschinsky, 1962; Lüllmann and Holland, 1962; Winegrad and Shanes, 1962; Niedergerke, 1963; Niedergerke and Orkand, 1963; Grossman and Furchgott, 1964a, 1964b, 1964c; Govier and Holland, 1965). Also a component of the ionic current in the action potential has been found to be dependent on the external calcium concentration in the frog heart (Niedergerke and Orkand, 1963), and in mammalian cardiac muscle (Reuter, 1966;

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Reuter and Beeler, 1969; Beeler and Reuter, 1970). These findings suggest that the Ca^{++} influx through the membrane during excitation appears to be an important determinant of the cardiac contractility. It is also clear that the calcium influx must be ultimately balanced by the calcium efflux in the steady state. This suggests the presence of an active transport mechanism in the cardiac membrane, since the electrochemical potential of extracellular Ca^{++} is far greater than that of intracellular Ca^{++}.

From the above consideration it is of interest to study the effects of some important drugs which are known to influence the cardiac contractility and/or calcium movement in the heart on calcium-binding characteristics of cardiac muscle membrane. Therefore, an attempt has been made in this study to investigate the interaction of Ca^{++} and cellular membrane fragments isolated from the heart muscle as affected by cardiotonic drugs. Since local anesthetics are known to influence calcium binding of cell membrane (Feinstein, 1964; Feinstein and Paimre, 1969), some of them were also studied along with cardiotonic drugs, for comparative purposes.

METHODS

Preparation of cell membrane fragments: Cardiac muscle membrane fragments were prepared and dried on Corning cover-glass, as described in the preceding paper (Kang and Lee, 1976).

Effects of drugs on calcium-binding: Cover-glasses with dried membrane preparations were incubated with or without cardiotonic drugs for 10 minutes in the incubation mixture containing 100 mM KCl, 1 mM adenosine triphosphate (ATP, Tris), 1 mM $MgCl_2$, 20 mM Tris-maleate (pH 7.0), 0.7 uCi/ml ^{45}Ca, and 10^{-3} M Ca^{++} or 10^{-7}M Ca^{++} Ca^{++}-EGTA [ethyleneglycol-bis (beta-aminoethyl ether)N,N'-tetraacetic acid] buffer was used to make 10^{-7} M Ca^{++} solution (Weber and Winicur, 1961). Concentrations of drugs and minor modifications in incubation mixtures will be described in the section of results. After incubation, cover-glasses were taken out, drained, and washed 7 times in a large volume of 20-mM Tris buffer solution (pH 7.0) by dipping for 2 seconds. After the washing, cover glasses were dried, and were either placed in a gas-flow counter (Nuclear Chicago) for the determination of radioactivity, or introduced into counting vials along with scintillation cocktail for determination of the radioactivity in a Packard Tri-carb liquid scintillation spectrometer (Model 3320).

Effects of drugs on calcium turnover and release: For this purpose, washout curves of Ca^{++} previously bound to membrane fragments were determined. Two different kinds of wash solutions were used; 20 mM Tris, pH 7.4 (solution 1), or 1 mM ATP, 1 mM $MgCl_2$, 1 mM ethylenediaminetetraacetate (EDTA) and 20 mM Tris, pH 7.4 (solution 2), In addition, wash solutions contained a drug to be tested in the experimental group but not in the control. Sometimes, washout solutions had the same components as incubation media excluding ^{45}Ca. The choice and the time of application of the above wash solutions are described in the result section.

Drugs selected for cardiac effects are norepinephrine (NE), acetylcholine (Ach), ouabain, isoproterenol, adenosine 3',5'-cyclic monophosphoric acid (cyclic AMP) and propranolol. Local anesthetics used were tetracaine, lidocaine and procaine. Two concentrations of Ca^{++} representing extra- and intracellular concentrations (Weber and Winicur, 1961, Weber and Herz 1962), namely 10^{-3} and 10^{-7} M were selected for the incubation medium.

RESULTS

Effect of Cardioactive Drugs on Calcium Binding and Its Turnover Rate: Results on the effect of various cardiotonic drugs on the calcium binding at the two different calcium concentrations of the incubation mixture are shown in Table 1. As can be seen in this Table, ouabain, Ach, isoproterenol, cyclic AMP and NE did not influence the calcium binding to the dried membrane fragments. Only propranolol inhibited the calcium binding and this inhibition was not effected by the simultaneous presence of NE with propranolol.

Since the cardioactive drugs did not influence the final binding state of Ca^{++} to membrane fragments, the possibility that these drugs might effect the turnover rate of bound ca^{++} was explored. For this purpose, the washout curves were investigated. Typical washout curves with 10^{-5} M NE, and 10^{-5} M isoproterenol are shown in Fig. 1. As can be seen in this Fig. no effect of NE and isoproterenol was observed.

Other washout was performed in the presence or absence of ATP or EDTA which are known to influence Ca^{++} binding to the membrane fragments. First, the membrane fragments after the incubation were washout with solution 1, then switched to washing solution 2 at 12th wash. The change over to washing solution 2 is followed by another steep washout curve as can be seen in Fig. 2. The presence or absence of 10^{-5} M NE and 10^{-5} M isoproterenol in the wash solution did not make any difference in the washout curves. The similar negative results were obtained with 10^{-5} M Ach and ouabain.

Thus, all drugs tested were found to be ineffective to the initial or to the second curves in this experiment. This indicates the lack of effect of those drugs on the turnover rate of Ca^{++} bound to the membrane fragments.

Table 1-A. Effects of drugs on the binding of calcium to membrane fragments at low concentrations of calcium (10^{-7} M)

Groups	Concentration of drugs	n	CMP/mg protein average ± S.E.M.
Control		7	995 ± 38.7
Ouabain	10^{-5} M	8	1002 ± 19.7
Ach	10^{-5} M	8	1022 ± 19.2
Isoproterenol	10 M	8	1005 ± 56.8

Table 1-B. Effects of drugs on the binding of calcium to membrane fragments at high concentrations of calcium (10^{-3} M)

Groups	Concentration of drugs	n	CPM/mg protein average ± S.E.M.
Control		6	786 ±16.6
Ouabain	10^{-6} M	6	783 ±15.3
Ach	10^{-5} M	6	790 ±20.0
Cyclic AMP	10^{-5} M	6	795 ±17.5
Propranolol	10^{-3} M	4	481 ±22.0*
	10^{-4} M	4	626 ±22.8**
	10^{-5} M	4	782 ± 34.5
NE	10^{-6} M	6	793 ± 18.6
	10^{-4} M-1	4	782 ± 35.3
NE+Propranolol	10^{-4} M 10^{-3} M	4	470 ± 30.1*

Incubation was carried out for 10 minutes in a medium containing 100 mM KC1, 1 mM ATP (Tris), 1 mM $MgCl_2$, 20 mM Tris-maleate (pH 7.0), 0.7 uCi/ml ^{45}Ca and 10^{-7} M Ca^{++} adjusted by Ca-EGTA buffer (A) or 10^{-3} M Ca^{++} (B).
n : Number of samples
* : p value < 0.001
** : P value < 0.01

Effect of Local Anesthetics on Calcium Binding: Local anesthetics have been known to inhibit calcium binding to skeletal muscle membrane. Local anesthetics which were found to reduce calcium binding in the skeletal muscle membrane were investigated.

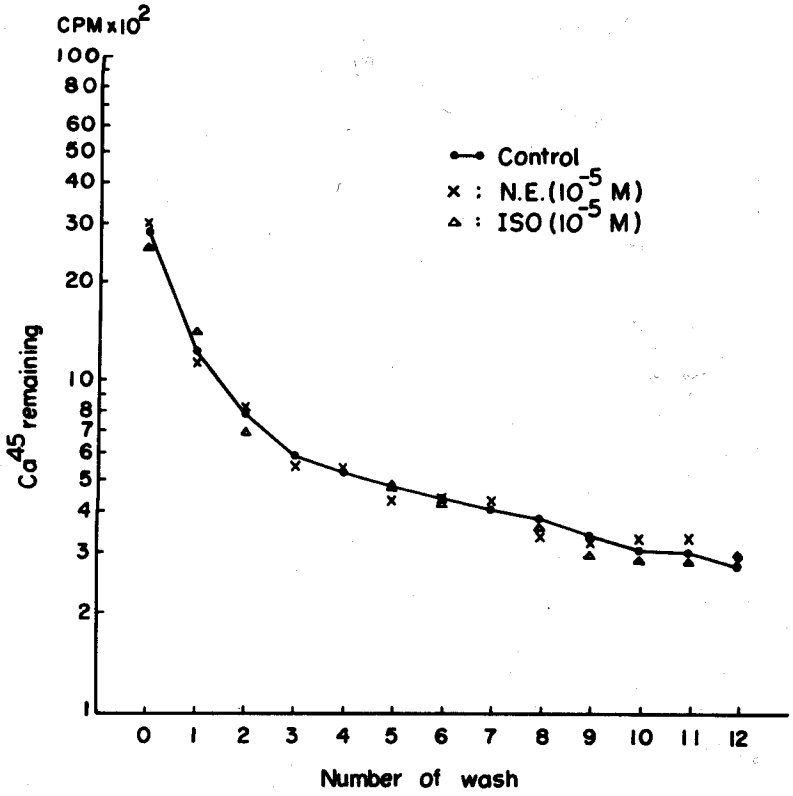

Fig. 1. Effect of norepinephrine and isoproterenol on calcium washout curve, The dried membrane fragments were incubated for 10 minutes at 37°C in a medium containing 20 mM Tris (pH 7.4), 50 mM NaCl, 1 mM ATP, 1 mM $MgCl_2$ and 0.3 mM $CaCl_2$ with 0.2 uCi/ml ^{45}Ca. The cover glasses were washed in the same incubation mixture but without ^{45}Ca (0) and plus 10^{-5} M NE (x) or 10^{-5} M isoproterenol (△). Each point respresents the average of 5 samples.

Result of this experiment is shown in Table 2. As can be seen in this Table, tetracaine caused a highly significant reduction in calcium binding at concentrations of both 1 mM and 10 mM, whereas lidocaine only at 10 mM. These results show that local anesthetics decrease calcium binding to cardiac membrane fragments and the degree of inhibition by tetracaine at concentrations used here is quite comparable to those obtained in skeletal muscle membrane(Feinstein and Paimre 1969).

The effect of procaine in a concentration of 10 mM was also studied. However, with procaine, a lower concentration of Ca^{++} (10^{-7} M and 10^{-5}M) in the media was employed. Procaine in a concentration of 10 mM also inhibits calcium binding to the membrane fragments in media containing 0.02 mM Ca^{++} as shown in Table 2B. However, the procaine effect is not seen when calcium concentration of the medium was reduced to 10^{-7} M.

DISCUSSION

Drugs which have been known to influence the cardiac contractility and "contraction-dependent calcium pool"(Grossman and Furchgott 1964a, 1964b, 1964c) are tested in the present experiment to investigate whether these drugs

Table 2-A. Effects of local anesthetics on calcium binding

	mM	n	CPM/mg protein average ± S.E.M.	Per cent inhibition	P value between control
Control		5	1055 ± 48.3		
Tetracaine	1.0	5	735 ± 27.5	30	0.001
Tetracaine	10.0	5	481 ± 20.0	54	0.001
Lidocaine	1.0	5	1018 ± 35.0	3	N.S.
Lidocaine	10.0	5	878 ± 48.3	17	0.05

Incubation was carried out for 10 minutes in a medium containing 140 mM NaCl, 10 mM KCl, 3 mM ATP (Tris), 3 mM $MgCl_2$, 1 mM $CaCl_2$, 20 mM Tris (pH 7.4) and 0.2 uCi/ml ^{45}Ca.
N.S. : Not significant; mM : Concentration of local anesthetics; n : Number of samples.

Table 2-B. Effects of procaine on calcium binding

(Ca^{++})		mM	n	CPM/mg protein average ± S.E.M.	P value of difference
2×10^{-5}M	Control		7	1445 ± 27.1	0.05
	Procacine	10	7	1323 ± 19.0	
1×10^{-7}M	Control		8	976 ± 17.0	N.S.
	Procaine	10	7	986 ± 38.3	

Incubation was carried out for 10 minutes in a medium containing 100 mM KCl, 20 mM Tris-maleate (pH 7.0), 1 mM ATP (Tris), 1 mM $MgCl_2$, 1 uCi/ml ^{45}Ca and varying concentrations of Ca^{++} (adjusted with Ca-EGTA buffer).
N.S. : Not significant; mM : Concentration of procaine; n : Number of samples.

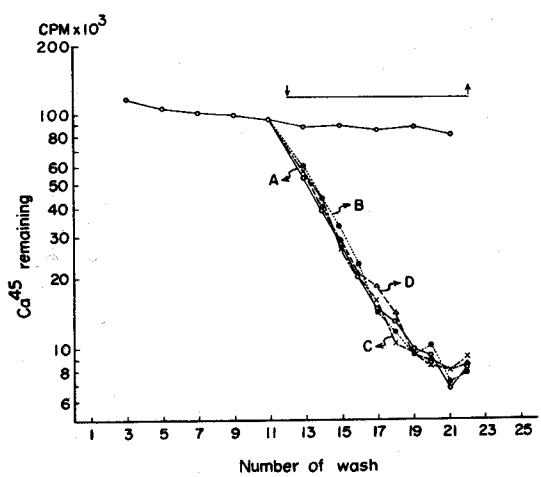

Fig. 2. Effect of norepinephrine and isoproterenol on calcium washout curve in the presence of ATP and EDTA. Incubation was carried out as described in Fig. 1, the cover glass was washed from the 12th wash in solution containing 1 mM ATP, 1 mM $MgCl_2$, 1 mM EDTA, 20 mM Tris (pH 7.4) without (A), with 10^{-6} M NE (B), 10^{-5} M NE (C) and 10^{-5} M isoproterenol (D). Each point represents the average of 5 samples. Arrows indicate a period during which cover glasses were washed with washing solution 2.

influence the affinity of the hypothetical calcium transport system in the cell membrane as was suggested by Wooley(1963) and by Nayler(1966) in the smooth muscle membrane.

As shown in Table IA and IB, no significant effect of NE, isoproterenol, Ach, and ouabain on calcium binding were found at both 10^{-7} M and 10^{-3} M of calcium concentration.

Results obtained in the washout curve experments show that no drugs tested other than local anesthetics show any influence on either binding or the turnover rate of ^{45}Ca.

In the presence of ATP and EDTA in the washing solution, a steeper washout curve was obtained as shown in Fig. 2. With EDTA, this is probably entirely due to its calcium chelating effect. However with ATP, there is a possibility that ATP may increase calcium turnover rate in the cardiac membrane through some active processes involved with transport mechanism. It is to be noted that the negative effect of cardioactive drugs on the ^{45}Ca binding was not influenced by the presence of EGTA or ATP in washing solutions.

The lack of response to the cardiotonic drugs in the membrane fragments indicate either that the inotropic effect of these drugs is not primarily associated with the Ca^{++} movememt of membrane at rest as noted by other investigators (Grossman and Furchgott 1964a, 1964b, 1964c) or that the calcium transport system is rendered non-responsive by damage during the membrane preparation. Results obtained in this study of the effect of local anesthetics on Ca^{++} binding clearly show that local anesthetics inhibit calcium binding to the dried membrane fragments (see Table 2A and 2B). Whether the observed inhibition is of competitive nature is not demonstrated in this experiment. However, findings made by other investigators suggest that calcium ion and local anesthetics compete with each other for the same binding site in the membrane presumably residing at the phospholipid moieties (Feinstein 1964). Feinstein and Faimre(1969) found in non-dried skeletal muscle membrane that tetracaine (1 mM) inhibited Ca^{++} binding by 35 per cent. A similar degree of inhibition by tetracaine was observed in the present study of Ca^{++} binding to the membrane fragments dried on cover glasses.

Propranolol, a well-known beta receptor blocker which has also local anesthetic-like activity (Lucchesi and Iwami 1968), also inhibits calcium binding to the membrane fragments (see Table I). Since catecholamine was suggested to increase the calcium influx during the action potential(Reuter 1966), this inhibitory effect of proparnolol on Ca^{++} binding may be considered as influencing catecholamine-induced increase in Ca^{++} influx, thus resulting in, at least in part, adrenergic blocking action of propranolol. However, the concentrations of propranolol required to induce the inhibition are too high, far greater than those showing the adrenergic blocking action. In addition, NE does not influence the calcium binding or turnover rate in this present membrane preparation. Thus, it is likely that the inhibitory effect of propranolol on calcium binding is associated with its local anesthetic-like activity and not to its beta-blocking activity.

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