Biochemical Pharmacology, Vol. 18, pp 2025–2079. Pergamon Press. 1969. Printed in Great Britain

THE INFLUENCE OF AMOBARBITAL AND OUABAIN ON CALCIUM BINDING TO PHOSPHOLIPIDS ISOLATED FROM CARDIAC SARCOPLASMIC RETICULUM*

MICHAEL L. HESS, RICHARD F. LAIN and F. NORMAN BRIGGS†

Department of Physiology, University of Pittsburgh School of Medicine, Pittsburgh, Pa. 15213, U.S.A.

(*Received* 6 *December* 1968; *accepted* 21 *February* 1969)

Abstract—Calcium binding to phospholipid, prepared from isolated canine myocardial sarcoplasmic reticulum, was facilitated by amobarbital and unaltered by ouabain. Ouabain, however, did antagonize the amobarbital facilitation of calcium uptake. These results indicate that amobarbital reacts chemically with phospholipids and that ouabain antagonizes this reaction. The negative inotropic action of barbiturates may thus be explained by their interaction with phospholipids, perhaps those of the sarcoplasmic reticulum. The ouabain reversal of barbiturate depression of the myocardium could, in part, be explained by its ability to antagonize this barbiturate-phospholipid interaction.

ALTHOUGH it is well established that barbiturates depress cardiac contractility and that cardiac glycosides antagonize this action, neither the molecular site nor mechanism of these drug actions has been established. Briggs *et al.*[1] have suggested that the negative inotropic action of the barbiturates is due to depression of calcium uptake activity by the sarcoplasmic reticulum and that the cardiac glycosides antagonize the barbiturate-induced cardiac depression by reversing this inhibition. This proposal is based on the effects of these drugs on the calcium uptake activity of isolated fragments of the sarcoplasmic reticulum. The present report examines the possibility that phospholipids of the sarcoplasmic reticulum are a site of action of these drugs. This seemed plausible, since phospholipid, some of which is essential for calcium uptake,[1, 3] comprises approximately 35 per cent of these membranes. Furthermore, it has been observed that a number of drugs[4–7] influence the interaction of phospholipids with calcium. The most pertinent of these cases is the observation by Blaustein and Goldman[6] that barbiturates facilitate the binding of calcium to phospholipids.

MATERIALS AND METHODS

Myocardial fragmented sarcoplasmic reticulum was isolated by the method of Carsten,[8] as modified by Briggs *et al.*,[1] from hearts of mongrel dogs of either sex anesthetized with sodium pentobarbital (30 mg/kg of body weight) and oxygenated

* This investigation was supported by grants from the Public Health Service (HE 06782), the American Heart Association (66-554), and the Arthritis Foundation of Western Pennsylvania.

† Recipient of a Public Health Service Career Award (1K6-HE-5290).

with pure oxygen by positive pressure ventilation. The fraction of the muscle homogenate which has been designated the fragmented sarcoplasmic reticulum was obtained by discarding material sedimented at 12,500 *g* for 15 min and saving the pellet obtained after 90 min at 60,000 *g*. This pellet was washed once and its protein content determined by the method of Lowry *et al.*[9] The calcium uptake activity of this material was assayed as described by Gertz *et al.*[10] and all preparations were found to have calcium uptake rates greater than 0·2 μmole per min per mg protein. Before extraction of the phospholipid by the method of Masoro *et al.*,[11] the fragmented sarcoplasmic reticulum was collected into a pellet by centrifugation for 30 min at 198,000 *g*. The pellet, suspended in 100 ml chloroform–methanol (1:1), was placed in a Servall Omnimixer which was run at high speed for 1 min and then at low speed for 20 min. The resultant suspension was filtered through fine sintered glass. The precipitate was collected and placed in the Omnimixer and again extracted with 100 ml chloroform–methanol (1:1). The resultant 200 ml of filtrate was evaporated nearly to dryness in a rotary evaporator. To dissolve the phospholipid, $\frac{1}{3}$ ml chloroform was added to the rotary evaporator for each milligram of protein extracted. The dissolved phospholipid was transferred to a separatory funnel. This step was repeated twice. The methanol-soluble portion of the residue was then extracted with $\frac{1}{3}$ ml methanol per mg protein extracted and added to the separatory funnel. To the solution in the separatory funnel, 0·375 vol. of 0·15 M KCl was added, the mixture was shaken for 10 min, and the emulsion was permitted to break overnight in a cold room. A 1-ml aliquot of the chloroform phase was evaporated to dryness and digested for 20 min in 0·7 ml of 70% boiling perchloric acid. After cooling, inorganic phosphate was determined by the method of King.[12] The remainder of the chloroform phase was evaporated to dryness. The phospholipid was dissolved in a volume of chloroform–methanol (2:1) which would yield a final concentration of lipid phosphorous of 1·25 μmoles per ml.

The phospholipid-mediated partition of calcium into a phospholipid–chloroform phase was studied as described by Feinstein[4] and others.[5–7] One ml of a Ringer's solution, containing 116 μmoles NaCl, 3·2 μmoles KCl, 0·35 μmole $CaCl_2$, 10 μmoles Tris malate, pH 8·0 or 9·0 with 0·05 μc ^{45}Ca, was shaken for 10 min with 2 ml of a chloroform–methanol (2:1) solution containing 2·5 μmoles phospholipid. The partition was performed in a 4-ml glass tube closed at both ends with rubber stoppers. The emulsion was allowed to crack overnight in a cold room. Three phases were produced: a chloroform, a methanol–water, and a white, insoluble interface. Samples of the aqueous methanol and the chloroform phases were removed with the aid of a hypodermic needle which was inserted through the rubber stoppers. A 0·1-ml aliquot of the chloroform and the aqueous methanol phase was added to 15 ml of a liquid scintillation medium[13] and the intensity of the radioactivity was determined with a liquid scintillation spectrometer. Quenching due to chloroform was corrected with the use of an external radiation source calibrated against chloroform. Although in the control experiments, i.e. those containing no phospholipid, the transport of calcium never exceeded 2 per cent of that in the presence of phospholipid, this phospholipid independent transport was used to correct the phospholipid transport. The data were treated statistically by the method of analysis of variance, and the F test was used to determine if a difference existed between treatment groups. The *t*-test was applied ($P < 0·05$) for comparisons of the four treatment means, as described by Li.[14]

RESULTS

As shown in Fig. 1, each milligram* of phospholipid at pH 8·0 facilitated the loss of 0·135 μmole calcium from the aqueous phase and the gain of 0·110 μmole by the chloroform phase. The presence of an interphase in these studies made it necessary to examine the effect of phospholipid on both the loss of calcium from the aqueous phase and the gain by the chloroform phase. The amount of calcium at the interphase

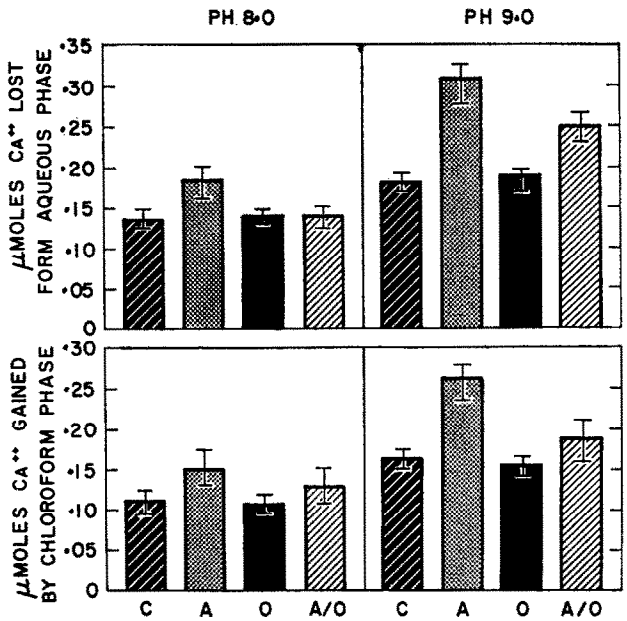

FIG. 1. Effect of amobarbital and ouabain on phospholipid-facilitated transport of calcium. These studies were carried out with phospholipids isolated from the sarcoplasmic reticulum of 8 canine hearts. The standard errors of the means are indicated by the vertical bars. The following abbreviations are used: C, phospholipid control; A, phospholipid plus 6 μmoles amobarbital; O, phospholipid plus 9 nmoles ouabain; A/O, phospholipid plus 6 μmoles amobarbital and 9 nmoles ouabain.

averaged 17 per cent of the calcium lost from the aqueous phase. At pH 9·0, the loss of calcium from the aqueous phase was 0·178 μmole while the gain by the chloroform phase was 0·160 μmole. Similar data have been reported by Feinstein,[4] Blaustein and Goldman,[6] and Nayler[5, 7] for phospholipids of diverse origin. When the phospholipid composition of sarcoplasmic reticulum[2, 15] and the calcium-binding characteristics of the individual phospholipids,[16] as shown in Table 1, are taken into account, it can be seen that the amount of calcium calculated to be bound at pH 8·0 (0·15 μmole) and 9·0 (0·23 μmole) closely approximates that observed in these experiments.

The addition of 6 μmoles amobarbital increased calcium uptake by the phospholipid (Fig. 1). At pH 8·0, the increase was 0·041 μmole per mg of phospholipid. This effect was statistically significant at the $P < 0·05$ level. Blaustein and Goldman[6] have

* The data are expressed per unit weight of phospholipid rather than per unit mole in order to facilitate comparison with other studies. A molecular weight of 800 was assumed for the phospholipid.

reported that thiopental (pH 8·0) increases calcium binding to phosphatidyl ethanolamine by 124 per cent and to phosphatidyl serine by 15 per cent. Applying these figures for calcium binding to phosphatidyl ethanolamine and phosphatidyl serine (Table 1), the increase in calcium binding would be expected to be 0·032 μmole per mg of phospholipid. Calcium binding to phosphatidyl inositol should not increase, since it is maximal at pH 8·0. Therefore, the remainder of the increase in calcium binding may take place with phosphatidyl choline. The barbiturate, at pH 9·0, increased

TABLE 1. CALCIUM BINDING ACTIVITY OF COMPONENT PHOSPHOLIPIDS OF THE SARCOPLASMIC RETICULUM*

	Reticulum		pH 8·0		pH 9·0	
Phospholipid	(% composition)	(μmoles PLP)	Ratio Ca/P	Bound Ca (μmoles)	Ratio Ca/P	Bound Ca (μmoles)
Phosphatidyl choline	69	0·86	0·02	0·03	0·02	0·02
Phosphatidyl ethanolamine	12	0·15	0·15	0·02	0·50	0·08
Phosphatidyl serine	6	0·07	0·78	0·05	1·00	0·07
Phosphatidyl inositol	9	0·11	0·50	0·06	0·50	0·06
Unidentified	4	0·05				
Total	100	1·24		0·15		0·23

* In order to make these data comparable to those of Fig. 1, an equivalent amount of phospholipid (1·25 μmoles phospholipid phosphorous, PLP) was used for these calculations. The phospholipid composition of the sarcoplasmic reticulum has been estimated from data provided by Martinosi[2] and Marinetti *et al.*[15] The affinity of the various phospholipids for calcium was taken from Joos and Carr.[16]

calcium uptake by 0·125 μmole. This increase in calcium binding is undoubtedly related to an increase in the ionization of the barbiturate which has a p*K*a of 7·94.[17] At pH 9·0 the calcium binding to phosphatidyl ethanolamine, phosphatidyl serine and phosphatidyl inositol is maximal; therefore, the barbiturate-induced increase in calcium binding probably takes place as a result of the barbiturate reaction with phosphatidyl choline. The increase in calcium binding induced by barbiturates is thought to be due to charge neutralization of the protonated amino group of the phospholipid.[18]

Ouabain, which has been shown to be without significant effect upon the ATP dependent uptake of calcium by the sarcoplasmic reticulum, has been shown to antagonize the barbiturate inhibition of calcium uptake.[1] A comparable result was obtained when the effect of ouabain on calcium uptake by phospholipids was examined. That is, although ouabain had no effect on calcium binding, it antagonized the barbiturate facilitation of calcium binding (Fig. 1). Calcium binding in the amobarbital-ouabain group at pH 9·0 was significantly ($P < 0·05$) different from that in the amobarbital group for both the loss of calcium from the aqueous phase and the gain by the chloroform phase. At pH 8·0 the amobarbital-ouabain group was significantly ($P < 0·05$) different from the amytal group for the loss from the aqueous phase, but the gain by the chloroform phase was not significantly different from that of either the amobarbital or the control group. The antagonism of amobarbital potentia-

tion by ouabain, measured by both the loss of calcium from the aqueous methanol phase and the gain by the chloroform phase, averaged 64 per cent and did not change with changing pH.

DISCUSSION

Our knowledge of the mechanism of calcium uptake by the sarcoplasmic reticulum does not permit the assignment of a specific role for phospholipid. That it has a role is suggested by the fact that when 75 per cent of the phospholipid is hydrolyzed by phospholipase C (EC 3.1.4.3) calcium uptake is severely depressed.[2, 3] Martinosi[2] has shown that this depression may be reversed by adding appropriate amounts of lysolecithin or lecithin to the enzyme-treated sarcoplasmic reticulum. Since all phospholipids do not share in the ability to restore uptake activity, there probably is molecular specificity to the action of phospholipids. These observations by Martinosi[2] suggest that phospholipid confers a conformation to membrane proteins which permits them to catalyze the hydrolysis of ATP and to transport calcium. Since the rate of calcium uptake by the sarcoplasmic reticulum is not inhibited by high concentrations of calcium,[19] there seems no reason to believe that the increase in calcium binding induced by amobarbital is the cause of the inhibition of calcium uptake, unless an increase in the affinity of phospholipid for calcium somehow interferes with calcium transport. It seems, therefore, more likely that the barbiturate-facilitated uptake of calcium should be viewed as evidence of an interaction between the phospholipid and the barbiturate. Blaustein and Goldman[6] and Blaustein[20] have indeed shown that phospholipids facilitate the uptake of barbiturate by phospholipids. It may be that the binding of barbiturates to phospholipids distorts their structure to the point that their ability to confer proper structure to the membrane proteins is lost. The reversal, by ouabain, of the negative inotropic action of barbiturates and of the inhibition of calcium uptake by cardiac sarcoplasmic reticulum may therefore be due to its ability to displace barbiturate bound to membrane phospholipid.

REFERENCES

1. F. N. Briggs, E. W. Gertz and M. L. Hess, *Biochem. Z.* **345**, 122 (1966).
2. A. Martinosi, *J. biol. Chem.* **243**, 61 (1968).
3. B. P. Yu, F. D. Martinis and E. J. Masoro, *J. Lipid Res.* **9**, 492 (1968).
4. M. B. Feinstein, *J. gen. Physiol.* **48**, 357 (1964).
5. W. G. Nayler, *Am. Heart J.* **71**, 363 (1966).
6. M. P. Blaustein and D. E. Goldman, *Science, N.Y.* **153**, 429 (1966).
7. W. G. Nayler, *J. Pharmac. exp. Ther.* **153**, 479 (1966).
8. M. E. Carsten, *Proc. Natn. Acad. Sci. U.S.A.* **52**, 1456 (1964).
9. O. H. Lowry, N. J. Rosenbrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
10. E. W. Gertz, M. L. Hess, R. F. Lain and F. N. Briggs, *Circulat. Res.* **20**, 477 (1967).
11. E. J. Masoro, L. B. Rowell and R. M. McDonald, *Biochim. biophys. Acta* **84**, 493 (1964).
12. E. J. King, *Biochem. J.* **26**, 292 (1932).
13. C. F. Gordon and A. L. Wolfe, *Analyt. Chem.* **32**, 574 (1961).
14. C. C. Li, *Introduction to Experimental Statistics*, chap. 16. McGraw-Hill, New York (1964).
15. G. V. Marinetti, J. Erbland and E. Kochen, *Fedn Proc.* **16**, 337 (1957).
16. R. W. Joos and C. W. Carr, *Proc. Soc. exp. Biol. Med.* **124**, 1268 (1967).
17. D. Kiichiro, A. Takaichi, H. Ryohei and R. Knoishi, *Chem. pharm. Bull., Tokyo* **15**, 1534 (1967).
18. M. B. Abramson, R. Katzman and H. P. Gregor, *J. biol. Chem.* **239**, 70 (1964).
19. A. Weber, R. Herz and I. Reiss, *Proc. R. Soc.* **160**, 489 (1964).
20. M. P. Blaustein, *Biochim. biophys. Acta* **135**, 653 (1967).