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# A CALCIUM-STIMULATED, OUABAIN-INHIBITED ATPase IN A MYOCARDIAL FRACTION ENRICHED WITH SARCOLEMMA

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## SUMMARY

An active intracellular to extracellular $Ca^{2+}$ efflux has been proposed in heart muscle. A myocardial sarcolemmal ATPase stimulated by an intracellular pCa and serving as a $Ca^{2+}$ pump has been postulated. A recently developed myocardial sarcolemmal preparation has now permitted a search for such an enzymatic activity. In these studies, an ATPase has been demonstrated in the sarcolemma which is activated by $Mg^{2+}$, stimulated as the $Ca^{2+}$ is raised to a pCa of 6, and is inhibited by ouabain. These findings suggest a mechanism by which $Ca^{2+}$ within the myocardium may be modulated and thus how the force of contraction may be altered by cardiac glycosides.

## INTRODUCTION

The presence of $(Na^+,K^+)$-stimulated ATPase in the surface membrane of cells and a role of this enzyme as a "pump" to maintain the $Na^+$ gradient across the cell membrane is well established[1]. A $Ca^{2+}$-stimulated ATPase and the coupled active efflux of $Ca^{2+}$ from the red blood cell have also been demonstrated[2]. A similar "pump" for $Ca^{2+}$ has been postulated in the sarcolemma of heart muscle[3,4]. The existence of such an ATP-dependent $Ca^{2+}$ "pump" involved in an active efflux of calcium from the myocardial cell is supported by the finding that the myocardial content of calcium is temperature-dependent[5]. A preparation of myocardial membrane enriched with sarcolemma was reported in 1970[6]. The ATPase activity of that preparation was activated by mmolar amounts of either $Mg^{2+}$ or $Ca^{2+}$, but only the former responded to added $Na^+$ *plus* $K^+$. This data has been confirmed by Sulakhe and Dhalla[7] but we feel that mmolar $Ca^{2+}$ activation in the absence of $Mg^{2+}$ is of questionable physiological relevance. Accordingly a $Mg^{2+}$-activated ATPase responsive to meaningful concentration of $Ca^{2+}$ (pCa 6) in our sarcolemma-enriched preparation has been sought. In this study such an ATPase activity was found. Further, the activity stimulated at a pCa 6 was inhibited by $\mu$molar amounts of ouabain.

These findings suggest a mechanism by which $Ca^{2+}$ within the myocardium may be modulated and how it may be altered by cardiac glycosides.

Abbreviation: EGTA, [ethylene bis(oxyethylenenitrilo)]-tetraacetic acid.

## METHODS

In 29 canine experiments, preparations of sarcolemma, mitochondria and microsomes were obtained as previously described[6]. ATPase activity was measured by the method of Stam and Honig[8] utilizing Elon for the assay of $P_i$ (ref. 9). The assay for the ATPase media contained 0.25 M sucrose, 5 mM $MgCl_2$, 4 mM $Na_2ATP$, 20 mM Tris maleate, pH 7.10, 0.5 mM [ethylene bis(oxyethylenenitrilo)]-tetra-acetic acid (EGTA), with or wihout added $CaCl_2$. For each preparation replicate sets of five were employed[10]. The reaction was run at 37 °C and water distilled in quartz glass was used throughout. ATP, Tris–maleate and ouabain octahydrate were from Sigma Chemical Co. Ultra pure, density gradient sucrose was from Schwartz/Mann and EGTA from Eastman Kodak Co. All other chemicals were American Chemical Society reagent grade. Calcium contamination was assayed with air–actylene flame of an atomic absorption spectrometer (Perkin–Elmer Model 303). The calcium contaminants of reagents were 0.5 mmole Ca/mole ATP and 0.04 mmole Ca/mole sucrose. ATP-dependent calcium uptake by mitochondria and microsomes extracted with 1 M NaI was determined using murexide and an Aminco Chance dual

TABLE I

SARCOLEMMAL $Mg^{2+}$-ACTIVATED, $Ca^{2+}$-STIMULATED ATPase

The $Q_{P_i}$ represents $\mu$moles $P_i$ appearing per min per mg protein. The control reaction media contained 0.25 M sucrose, 20 mM Tris maleate, pH 7.1, 5 mM $MgCl_2$, 4 mM $Na_2ATP$, and 0.5 mM EGTA. In addition, the pCa 6 media contained added $CaCl_2$, 0.44 mM. $n=22$.

| *pCa > 8.2** | *pCa 6.0** |
|---|---|
| 0.055 | 0.058 |
| 0.058 | 0.068 |
| 0.082 | 0.087 |
| 0.056 | 0.067 |
| 0.098 | 0.098 |
| 0.075 | 0.065 |
| 0.070 | 0.079 |
| 0.063 | 0.075 |
| 0.082 | 0.093 |
| 0.075 | 0.080 |
| 0.074 | 0.084 |
| 0.060 | 0.070 |
| 0.056 | 0.063 |
| 0.082 | 0.090 |
| 0.055 | 0.069 |
| 0.068 | 0.077 |
| 0.063 | 0.073 |
| 0.075 | 0.085 |
| 0.070 | 0.077 |
| 0.074 | 0.079 |
| 0.062 | 0.069 |
| 0.059 | 0.071 |
| $\bar{x}=0.068$ | $\bar{x}=0.076$ |

* $P<0.001$, paired $t=6.902$.

wavelength spectrophotometer by the method of Ohnishi and Ebashi[11]. The pCa was calculated according to Portzehl *et al.*[10]. The means of paired data were compared using a "double tailed" Student's *t* test[12].

## RESULTS

In Table I, the mean $Mg^{2+}$-activated ATPase activity of 22 cardiac sarcolemma preparations is shown at a pCa of 8.2 (control) and at a pCa of 6.0. The ATPase activity increased by 10% when the pCa was decreased to 6.

High levels of $Ca^{2+}$ (pCa 4–3) produced a significant depression of ATPase activity in the presence of $Mg^{2+}$ as previously reported[6]. In that prior study contamination of sarcolemmal preparations by mitochondria and reticular elements was demonstrated. However, no ATPase activity stimulated by $Ca^{2+}$ at a pCa of 6 was demonstrated in paired preparations of mitochondria and microsomes treated with 1 M NaI in experiments of this current study. Further, the 1 M NaI treatment was associated with loss of their ATP-dependent calcium uptake.

TABLE II

SARCOLEMMAL $Ca^{2+}$ STIMULATED ATPase *PLUS* OUABAIN

All media were the same as Table I except that $1 \cdot 10^{-6}$ M ouabain was added where noted. No ouabain effect was seen at pCa 8.2 but a significant decrease in the $Ca^{2+}$-stimulated ATPase was seen at pCa 6.0. The no-ouabain data of this table are included in the data of Table I. $n = 9$.

| *pCa > 8.2** | *+Ouabain** | *pCa 6.0*** | *+Ouabain*** |
|---|---|---|---|
| 0.082 | 0.081 | 0.090 | 0.085 |
| 0.055 | 0.062 | 0.069 | 0.063 |
| 0.068 | 0.068 | 0.077 | 0.068 |
| 0.063 | 0.061 | 0.073 | 0.066 |
| 0.075 | 0.075 | 0.085 | 0.079 |
| 0.070 | 0.074 | 0.077 | 0.072 |
| 0.074 | 0.076 | 0.079 | 0.078 |
| 0.062 | 0.058 | 0.069 | 0.064 |
| 0.059 | 0.063 | 0.071 | 0.065 |
| $\bar{x} = 0.068$ | 0.069 | 0.077 | 0.071 |

* $P < 0.4$, paired $t = 0.97$. Values are not significant
** $P < 0.001$, paired $t = 7.83$.

As shown in Table II, 1 $\mu$M ouabain was without effect at a pCa of 8.2 in 9 preparations. Further, in these 9 preparations, the sarcolemmal ATPase was stimulated 13% as the pCa was changed to 6.0. However, at a pCa of 6.0 1 $\mu$M ouabain depressed the ATPase $Q_{P_i}$ 0.006, or 66% of the net activity attributable to the increased $Ca^{2+}$. This depression due to ouabain was significant at the level of $P$ 0.001. 10 $\mu$M ouabain was without effect on the ATPase activated by 1.0 mM $CaCl_2$ in the absence of added $MgCl_2$.

As previously noted[6], $Na^+$ and $K^+$ stimulated the sarcolemmal ATPase in six paired preparations, and this stimulation was inhibited by $49 \pm 3\%$ by the addition

of $1 \cdot 10^{-6}$ M ouabain. As shown in Table III, no calcium stimulation could be seen in the presence of the added 120 mM $Na^+$, 12 mM $K^+$, an addition which itself led to substantial increments in ATPase activity.

TABLE III

SARCOLEMMAL $Ca^{2+}$-STIMULATED ATPase IN PRESENCE OF 120 mM NaCl *plus* 12 mM KCl

The reaction media was as in Table I except for the addition of 120 mM NaCl and 12 mM KCl. In the presence of $Na^+$ *plus* $K^+$, the stimulation by a pCa of 6 is no longer demonstrated. $n=7$.

| *pCa > 8.2** | *pCa 6.0** |
|---|---|
| 0.088 | 0.085 |
| 0.117 | 0.118 |
| 0.168 | 0.170 |
| 0.114 | 0.112 |
| 0.111 | 0.114 |
| 0.147 | 0.138 |
| 0.141 | 0.139 |
| $\bar{x}=0.127$ | 0.125 |

* $P < 0.047$, paired $t = 0.94$. Values are not significant.

DISCUSSION

In the present study a preparation enriched with myocardial sarcolemma was found to have a $Mg^{2+}$-activated ATPase demonstrable at a pCa of 8.2. This ATPase was stimulated by the addition of $\mu$molar amounts of $Ca^{2+}$ (pCa changed to 6). This stimulation by $\mu$molar $Ca^{2+}$ was inhibited 66% by ouabain, an inhibition not seen at a pCa of 8.2. This suggests that the depression seen with ouabain at a pCa of 6 is specific to Ca and is not attributable to a ouabain interaction with contaminating $Na^+$ and $K^+$. Such contaminants would also be present at a pCa of 8.2 where no ouabain effect was found.

This ATPase stimulated by $\mu$molar concentration of $Ca^{2+}$ may be meaningful in terms of supporting an active myocardial $Ca^{2+}$ efflux. However, the complete inhibition by interstitial levels of $Ca^{2+}$ and $Na^+$ suggests that it might be located on the intracellular sarcolemmal surface. The $Q_{P_i}$ of the $Ca^{2+}$ stimulation is less than that observed with $Na^+$ *plus* $K^+$ stimulation. However, it should be noted that the $Ca^{2+}$ stimulation involves $\mu$molar rather than mmolar ion concentrations. Whether this stimulation of ATPase activity in the preparation enriched in sarcolemma is coupled to $Ca^{2+}$ transport is not known, but the stimulating $Ca^{2+}$ level is consistent with that associated with intracellular activation. With this in mind, it is of interest that a possible lag in the $Na^+$ pump has been reported in association with the positive rate staircase and this lag has been held responsible for the observed increase in contractility through a secondary shift in intracellular $Ca^{2+}$ (ref. 13). A similar lag in a $Ca^{2+}$ pump might well ensue if the elevation of intracellular $Ca^{2+}$ following upon

increased rate of contraction leads to an increased sarcolemmal ATPase activity. The fact that $Ca^{2+}$ stimulation was not seen in the presence of added $Na^+$ and $K^+$ is consistent with a shared channel for the extrusion of $Na^+$ and $Ca^{2+}$ from the interior of the cell.

As noted elsewhere[6], this preparation of sarcolemma is contaminated by recognizable mitochondrial fragments and small vesicles possibly originating as sarcoplasmic reticulum. However, neither the 1-M NaI treated mitochondria nor microsomes demonstrated any ATPase responsiveness at a pCa of 6.

In summary, the data presented represents the first direct demonstration of a myocardial $Mg^{2+}$-activated, $Ca^{2+}$-stimulated ATPase that can be attributed to the sarcolemma and is responsive to a meaningfully low $Ca^{2+}$ level. Further, the inhibition of the $Ca^{2+}$-stimulated sarcolemmal ATPase by ouabain may reflect a mechanism of action for ouabain's positive inotropic effect. If the inhibition by ouabain of the $Ca^{2+}$-stimulated ATPase is related to its positive inotropic effect, then a net increase in total cell calcium should be present. A ouabain-induced net gain in total cell calcium has been reported in the perfused rabbit ventricular septum[14]. In this laboratory we have recently confirmed this net retention of calcium using threshold doses of ouabain on the blood perfused isolated dog heart (Stam, Jr, A. C., Serur, J., Urschel, C., Locksley, R. and Sonnenblick, E. H., unpublished). Thus we have provided further support for the hypothesis that ouabain's inotropic effect is at least in part due to inhibition of a sarcolemmal $Ca^{2+}$ pump.

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## REFERENCES

1 Skou, J. C. (1962) *Biochim. Biophys. Acta* 58, 314–325
2 Lee, K. S. and Shin, B. C. (1969) *J. Gen. Physiol.* 54, 713–729
3 Weber, A. M. (1966) in *Current Topics in Bioenergetics* (Sanadi, D. R., ed.), Vol. 1, pp. 203–254, Academic Press, New York
4 Sonnenblick, E. H. and Stam, Jr, A. C. (1969) *Annu. Rev. Physiol.* 31, 647–674
5 Lahrtz, H. G., Lüllmann, F. and Van Zwieten, P. (1967) *Biochim. Biophys. Acta* 135, 701–709
6 Stam, Jr, A. C., Weglicki, W. B., Feldman, D., Shelburn, J. C., and Sonnenblick, E. H. (1970) *J. Mol. Cell. Cardiol.* 1, 117–130
7 Sulakhe, P. V. and Dhalla, M. S. (1971) *Life Sci.* 10, 185–191
8 Stam, Jr, A. C. and Honig, C. R. (1962) *Biochim. Biophys. Acta* 60, 259–264
9 Harris, W. D. and Popat, P. (1954) *J. Am. Oil Chem. Soc.* 31, 124–127
10 Portzehl, H., Caldwell, P. C. and Ruegs, J. C. (1964) *Biochim. Biophys. Acta* 79, 581–591
11 Ohnishi, T. and Ebashi, S. (1963) *J. Biochem. Tokyo* 54, 506–511
12 Steel, R. G. and Torrie, J. H. (1960) *Principles and Procedures of Statistics*, pp. 67–87, McGraw Hill, New York
13 Langer, G. A. (1968) *Physiol. Rev.* 48, 708–757
14 Langer, G. A. and Serena, S. D. (1970) *J. Mol. Cell. Cardiol.* 1, 65–90.