the retina had occurred before rotation of the eye cup at stages 31, 32, and 35.

My results show that before larval stage 30, the ganglion cells do not yet have the information about the positions with which they must connect in the tectum, and the retina can be rotated without altering the normal pattern of retinotectal connections. Specification of retinal ganglion cells occurs first in the anteroposterior axis of the retina at stage 30. After this, the ganglion cells have the information which enables them to form their connections in the correct order in the rostrocaudal axis of the tectum. During the next 5 to 10 hours of development the ganglion cells become specified in the dorsoventral axis of the retina and are able to form the correct connections in the mediolateral axis of the tectum. Inversion of the retina after this stage of development results in total inversion of the retinotectal projection. Specification in both axes of the retina occurs before outgrowth of optic nerve fibers from the ganglion cells.

MARCUS JACOBSON
Department of Biological Sciences,
Purdue University,
Lafayette, Indiana 47907

References and Notes

1. L. S. Stone, *Proc. Soc. Exp. Biol.* **57**, 13 (1944); *Ann. N.Y. Acad. Sci.* **49**, 856 (1948); *J. Exp. Zool.* **145**, 85 (1960).
2. G. Székely, *Acta Biol. Acad. Sci. Hung.* **5**, 157 (1954); *Arch. Entwickl. Mech. Org.* **150**, 48 (1957).
3. R. M. Gaze, *Quart. J. Exp. Physiol.* **44**, 290 (1959); M. Jacobson, *ibid.* **47**, 170 (1962); R. M. Gaze, M. Jacobson, G. Székely, *J. Physiol. (London)* **165**, 484 (1963).
4. P. D. Nieuwkoop and J. Faber, *Normal Table of* Xenopus laevis (*Daudin*) (North-Holland, Amsterdam, 1956).
5. M. L. Wolbarsht, E. F. MacNichol, Jr., H. G. Wagner, *Science* **132**, 1309 (1960); C. Guld, *Med. Electron. Biol. Eng.* **2**, 317 (1964).
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Calcium-Induced Activation of Phosphorylase in Rat Hearts

Abstract. *Isolated perfused rat hearts exposed to increased calcium concentrations demonstrated a marked activation of phosphorylase, the maximum occurring about 15 seconds after the peak of the positive inotropic response. These observations provide an explanation for the failure of earlier investigations to demonstrate a significant calcium-induced increase in the active form of phosphorylase in the intact heart.*

The evidence that calcium is capable of inducing a significant activation of phosphorylase in the *intact* heart has been inconclusive. In the investigation described here, a marked activation of this enzyme has been demonstrated by studying the time course of the effects of calcium on phosphorylase activity in the isolated perfused rat heart.

Phosphorylase is an important enzyme, since it regulates the breakdown of glycogen to glucose-1-phosphate, thereby providing the heart with a substrate which can be catabolized to utilizable energy (*1*). This enzyme exists in two forms: phosphorylase *b* is the inactive form of the enzyme (it is active only in the presence of adequate amounts of adenosine-5′-monophosphate) and phosphorylase *a* is the active form. Phosphorylase is considered to be activated when the percent of phosphorylase *a* activity increases; this is computed in the following manner:

$$\frac{\text{phosphorylase } a \text{ activity} \times 100}{\text{phosphorylase } a + b \text{ activities}}$$

The activation of phosphorylase is a relatively complex biochemical process (Fig. 1) involving a number of enzymatic reactions (*2*).

Several investigators (*2*) have shown that calcium activates striated muscle phosphorylase *b* kinase in vitro by a mechanism that appears to be independent of that involving adenosine triphosphate (ATP) and magnesium and accelerated by adenosine-3′,5′-monophosphate (3′,5′-AMP). If such a process occurred in the *intact* heart it should lead to an enhanced conversion of phosphorylase *b* to phosphorylase *a*. However, Belford and Feinleib (*3*) observed that increasing the calcium concentration produced only a relatively small increase in the percent of phosphorylase *a* activity in isolated guinea pig atria. Similarly, Mayer and Moran (*4*) failed to demonstrate a significant activation of this enzyme in the open-chest dog preparation with doses of calcium that produced maximal increases in the contractile force of the heart.

It must be emphasized that in *all* of the above-mentioned experiments heart samples were taken at the moment the positive inotropic response reached its maximum. Hence, the failure of these experiments to demonstrate a significant activation of phosphorylase by calcium could be due to the fact that this activation process occurs sometime after the peak increase in contractile force. Such a delay in the maximum activation of cardiac phosphorylase following epinephrine administration has been recently demonstrated (*5*). We have therefore studied the time course of the effect of calcium on phosphorylase activity in the isolated perfused rat heart.

The methods used in the above experiments are briefly described below. Hearts obtained from female Wistar rats were perfused by the Langendorff technique with Tyrode's solution (37°C) at a flow rate of 5 ml/min. Five grams of diastolic tension was applied to each heart. All hearts were perfused for 8 minutes with control Tyrode's solution containing 1.8 mmole of calcium prior to exposing them to Tyrode's solution containing higher calcium concentrations. Subsequently, at a predetermined time interval, a portion of left ventricular tissue was frozen with a modified Wollenberger clamp (*6*) prechilled in liquid nitrogen. All samples were stored under liquid nitrogen until assayed. Phosphorylase *a* and total phosphorylase activities were assayed according to the procedure described earlier (*5*). Since no statistically significant change in total phosphorylase was observed during the course of any of the experiments, all data are presented in terms of the percent of phosphorylase *a* activity.

The data summarized in Table 1 demonstrate that when samples were taken at the peak of the inotropic response no significant activation of

Table 1. Effect of calcium on the percent of phosphorylase *a* activity, with means and standard errors, in isolated rat hearts.*

Calcium concentration (m*M*)	Phosphorylase *a* activity (% ± S.E.)
1.8†	11.1 ± 0.8
3.6	10.7 ± 0.7
7.2	13.7 ± 1.3
14.4	10.3 ± 0.5

* Heart samples were taken at the peak of the inotropic response. See text for details. † Control samples were taken after 8 minutes of perfusion.

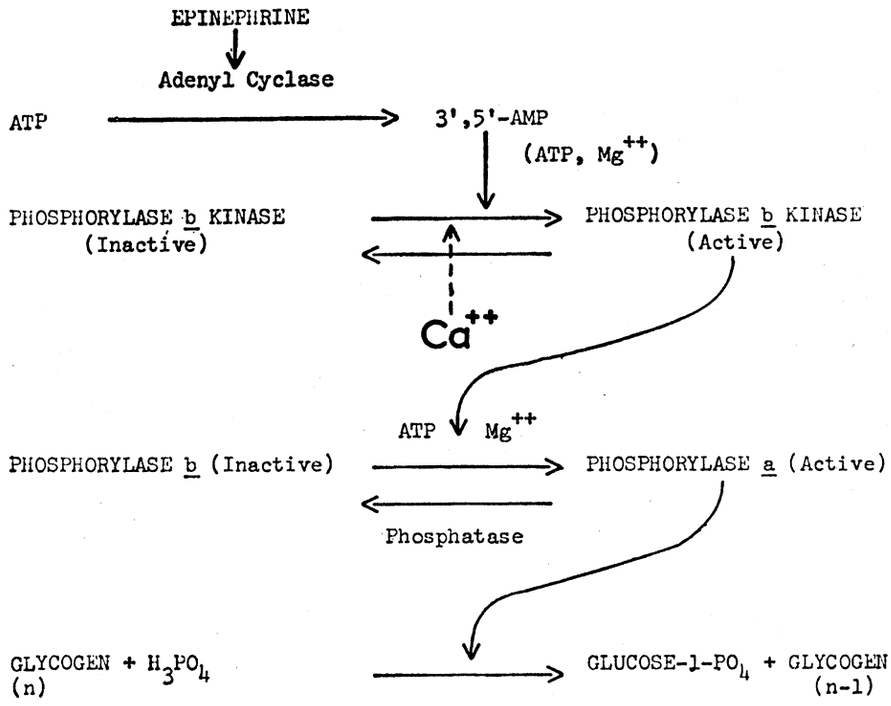

Fig. 1. Striated muscle phosphorylase system.

phosphorylase occurred in hearts exposed to Tyrode's solution containing two to eight times the control calcium (1.8 m*M*) concentration. In contrast, when samples were removed at various times after the peak of the inotropic response a marked increase in the percent of phosphorylase *a* activity was obtained. Figure 2 illustrates the time course of calcium-induced activation of phosphorylase in rat hearts exposed to 3.6 mmole of calcium. The data demonstrate that phosphorylase *a* activity increased from a control value of about 7 percent to a maximum of 50 percent ($P < .01$) within 30 seconds after the administration of Tyrode's solution containing 3.6 mmole of calcium. Since the maximum activation of phosphorylase did not occur until approximately 15 seconds after the peak of the positive inotropic response, it is evident that the increased conversion of inactive to active phosphorylase elicited by calcium lags behind the course of the positive inotropic response.

We also have studied the time course of the effects of calcium on cardiac phosphorylase in the open-chest dog preparation (*7*). In ten such experiments the average control value of phosphorylase *a* activity was about 4 percent. After the intravenous administration of $CaCl_2$ (40 mg/kg) (*8*) a sevenfold increase ($P < .01$) in the percent of phosphorylase *a* occurred approximately 12 seconds after the peak of the positive inotropic response, indicating that calcium has the ability to markedly activate cardiac phosphorylase *in situ*.

The results of these time-course studies clearly establish the ability of calcium to accelerate the conversion of inactive to active phosphorylase in the *intact* heart. In addition, the data provide an explanation for the failure of earlier investigations to demonstrate a significant activation of this enzyme by calcium.

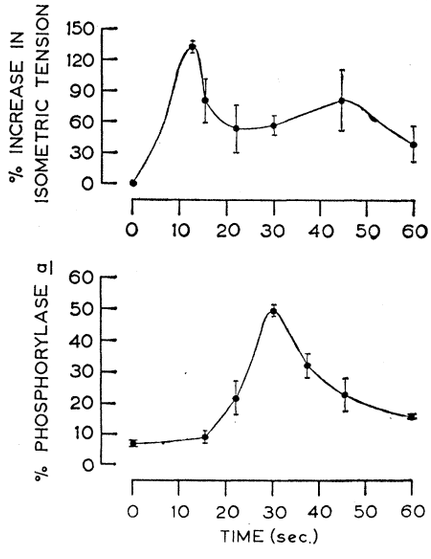

Fig. 2. Effect of calcium on isometric tension and phosphorylase *a* activity of isolated rat hearts. At time 0, the Tyrode's solution perfusing the heart was changed for one containing 3.6 mmole of calcium. Each point is the mean value obtained from four or more hearts. I-shaped bars represent standard errors.

It is doubtful whether the calcium-induced activation of phosphorylase is mediated by the release of endogenous catecholamines, since marked activation of this enzyme still occurred in hearts from reserpinized rats when they were exposed to increased calcium concentrations. The dosage schedule of reserpine (*9*) used in these experiments has been shown by others (*10*) to virtually deplete rat hearts of their catecholamine stores. A direct activation by calcium of phosphorylase *b* kinase presumably mediates the increased conversion of phosphorylase *b* to phosphorylase *a*. However, further evidence is required to establish the validity of such a mechanism.

ABRAM J. D. FRIESEN
GLENNA ALLEN
JOSEPH R. E. VALADARES
Department of Pharmacology,
University of Ottawa,
Ottawa, Ontario, Canada

References and Notes

1. E. G. Krebs and E. H. Fischer, *Vitamins Hormones* **22**, 399 (1964).
2. E. G. Krebs, D. J. Graves, E. H. Fischer, *J. Biol. Chem.* **234**, 2867 (1959); W. L. Meyer, E. H. Fischer, E. G. Krebs, *Biochemistry* **3**, 1033 (1964); K. E. Hammermeister, A. A. Yunis, E. G. Krebs, *J. Biol. Chem.* **240**, 986 (1965); G. I. Drummond, L. Duncan, A. J. D. Friesen, *ibid.* **240**, 2778 (1965); G. I. Drummond and L. Duncan, *ibid.* **241**, 3097 (1966).
3. J. Belford and M. R. Feinleib, *J. Pharmacol. Exp. Therap.* **127**, 257 (1959).
4. S. E. Mayer and N. C. Moran, *ibid.* **129**, 271 (1960).
5. J. R. E. Valadares and A. J. D. Friesen, *Life Sci.* **5**, 521 (1966).
6. A. Wollenberger, O. Ristau, G. Schoffa, *Arch. Ges. Physiol.* **270**, 399 (1960).
7. Open-chest dog heart studies were conducted according to the procedure of S. E. Mayer, M. de V. Cotten and N. C. Moran, *J. Pharmacol. Exp. Therap.* **139**, 275 (1963). Blunt forceps prechilled in liquid nitrogen were used to remove samples of right ventricular tissue.
8. Appropriate volumes of a 20 percent solution of $CaCl_2$ were given via the femoral vein. Control heart samples were taken after the injection of equal volumes of a NaCl solution of the same tonicity as the $CaCl_2$ solution.
9. Reserpine (0.5 mg/kg) was administered intraperitoneally once a day for 3 days. Hearts were then excised on the 4th day.
10. B. Bhagat, N. Saini, W. L. West, W. M. Booker, *Arch. Int. Pharmacodyn.* **155**, 126 (1965).
11. Supported by grants from the Medical Research Council of Canada. A.J.D.F. is a postdoctoral fellow of the Canadian Heart Foundation. We thank Dr. G. M. Ling for constructive criticism of the manuscript.

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