Protection and Reactivation of Cardioglobulin-A by High Energy Phosphate Compounds

By Edward J. Leonard, M.D., and Stephen Hajdu, M.D.

A plasma protein system of mammalian origin which increases the contractile force of isolated frog heart has recently been described. It is composed of three globulins, designated cardioglobulin A, B, and C, all of which are necessary for the activity of the system. The cardioglobulin activity of normal human plasma falls within a characteristic range, deviations from which have been found in certain types of cardiovascular disease.

The assay of cardioglobulin is performed on excised frog hearts mounted on cannulae. An attempt was made to eliminate the technically difficult cannulation step by using strips instead of whole ventricle, but this approach was unsuccessful because cardioglobulin solutions were inactivated in the presence of heart strips. It was thought that inactivation of cardioglobulin might be due to release of material from the cut surface of the heart strips. Further investigation showed that cardioglobulin-A was inactivated by tissue homogenates, not only of heart but also of other organs.

A study of the conditions under which this inactivation occurs or can be prevented supports the idea that biologically active cardioglobulin-A contains a phosphate bond, which is utilized normally during the action of the whole cardioglobulin system on the heart, but may be hydrolyzed under other conditions by various tissue enzymes. The evidence related to these conclusions is presented in this paper.

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Methods

PREPARATION AND ASSAY OF CARDIOGLOBULIN FRACTIONS

Cardioglobulin-B was used in these studies for "sensitizing" frog hearts, i.e., exposing frog hearts to a solution containing a standard amount of cardioglobulin-B. This material becomes bound to the heart, and after washing with Ringer-Conway solution the tissue can then be used for the assay of cardioglobulins A and C. The preparation, storage and assay of cardioglobulin-B was essentially as previously described, except that the sodium sulfate precipitation step was eliminated, and glycerol was dialyzed directly into human serum. The preparation of cardioglobulins A and C from rat plasma is similar in principle to that previously described for human serum, in that fractionation of the globulins by salting out yields cardioglobulin-C at low salt concentration and cardioglobulin-A at high salt concentration. A saturated solution of ammonium sulfate, neutralized to pH 7.0 with ammonium hydroxide, was added directly to rat plasma in an amount sufficient to make the final solution 30% saturated. The cardioglobulin-C containing precipitate was removed by centrifugation, and cardioglobulin-A was obtained by increasing the ammonium sulfate in the supernatant to 60% saturation. The salt was removed by elution of the protein on G-25 Sephadex. Sodium ascorbate and either ATP or CP were maintained in a concentration of 1 mg/ml throughout the preparative procedure.

The assay of rat cardioglobulins A and C is similar to that described for the fractions from human serum. The addition of cardioglobulins A and C to a sensitized heart (a heart to which cardioglobulin-B has been added previously) causes contracture, the extent of which depends on the amount of cardioglobulins A and C in the assay sample. The degree of contracture is graded on a seven-point scale and the cardioglobulin activity is expressed in units/ml of plasma.

TISSUE HOMOGENATES

One to two gram amounts of tissue from various sources were suspended in ice cold Ringer-Conway solution and homogenized in a glass homogenizer. The starting material for homogenates of glycerinated muscle was prepared according to the method of Szent-Györgyi and stored at -20°C. A sufficient number of fibers for a day's use was teased away from the stored sample, washed with cold distilled water, and then homogenized. The homogenates contained 10 to 35 mg tissue per ml of suspension. The action of homogenates on the cardioglobulin activity of rat plasma was determined in a reaction mixture of the following composition: 0.5 ml of rat plasma, 0.2 to 0.75 ml homogenate, the total volume being brought to 2.0 ml with Ringer-Conway solution. The volume of homogenate chosen in any given experiment was an amount sufficient to inactivate the rat plasma cardioglobulin in a 5- or 10-minute equilibration at 26°C.

ANIMALS

*Rana pipiens* for cardioglobulin assay were obtained and treated as previously described. Female Sprague-Dawley rats were used in these studies. For experiments with phosphate-labelled plasma proteins, P32 obtained from Oak Ridge as phosphoric acid and neutralized with sodium hydroxide to pH 7.0 was added to the drinking water in a concentration of approximately 0.7 mc/100 ml for a period of two to four days before sacrifice of the animal.

SEPHADEX COLUMN TECHNIQUES FOR P32 EXPERIMENTS

In order to obtain P32 labelled plasma proteins free of small phosphate-containing molecules, rat plasma was eluted on G-25 or G-50 Sephadex columns, the eluate being collected in serial fractions. The protein peak appears in the first portion of the eluate, followed by the nonprotein material. Either G-25 or G-50 Sephadex can be used, separation being somewhat better on G-50. The columns were prepared in the usual way, column dimensions were 1 x 20 cm, sample volumes were not more than 2 ml, and flow was sufficiently fast at a pressure of 20 cm of water so that the protein peak could be eluted within five to ten minutes. Separation of protein from nonprotein P32 was virtually complete on one passage through the column, since passage of the collected protein peak through Sephadex a second time resulted in the appearance of more than 99% of the counts in the protein peak, the mean value for the nonprotein counts being only 0.7% of the total. This value of 0.7% thus represented a base line amount of nonprotein phosphate obtained on the second column run, and was subtracted from each nonprotein count in the various experimental series.

CHEMICALS AND CHEMICAL METHODS

Nucleotides, creatine, creatine phosphate, and creatine kinase were obtained from commercial sources. In addition, a sample of creatine kinase was kindly supplied by Dr. Lafayette Noda, Dartmouth College, Hanover, New Hampshire. Protein samples for phosphorus determination were ashed by heating in the presence of 10% magnesium nitrate in alcohol (Ruth Kielley, personal communication), and phosphorus was determined by the method of Chen et al. Protein concentration was estimated by absorption at 280 mg/ml. Radioactivity of phosphate-labelled fractions was determined by transferring aqueous samples directly into Bray's solution and counting in a well-type scintillation counter.

Results

1. DESTRUCTION OF CARDIOGLOBULIN-A BY TISSUE HOMOGENATES

Fresh homogenates from a wide variety of tissues were found to destroy cardioglobulin activity of rat plasma. Cardioglobulin could be inactivated by homogenates of heart and skeletal muscle of frog, and heart, skeletal muscle, spleen, kidney, and liver of rat. Homogenates prepared from glycerinated rabbit or rat psoas were also effective. The glycerinated material had the advantage that a large amount could be stored in glycerol for a period of weeks before homogenization, and thus provided a source of material with uniform cardioglobulin-destroying activity.

The homogenate destroys cardioglobulin-A, not cardioglobulin-C. This was shown by inactivating rat plasma (containing cardioglobulins A and C) under the conditions described in Methods. The inactivated system could be restored by addition of cardioglobulin-A, but not by cardioglobulin-C. The destruction by homogenate proceeds rapidly under the conditions outlined. Although no effect is seen without incubation, cardioglobulin-A loss is evident at 45 seconds and is virtually complete in five minutes.

The addition of certain high energy phosphate compounds to the reaction mixture, in a final concentration of 1 to 2 mg/ml, protects cardioglobulin-A against the action of the homogenate. In each experiment a control was run simultaneously with the high energy phosphate compound being tested. At the end of the reaction period the mixtures were...
TABLE 1

<table>
<thead>
<tr>
<th>Agent tested</th>
<th>Number of paired experiments</th>
<th>Units of cardioglobulin without agent</th>
<th>Units of cardioglobulin with agent tested</th>
<th>Mean</th>
<th>$\Delta \bar{x}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CP, 1 to 2 mg/ml</td>
<td>5</td>
<td>1.9</td>
<td>0.1</td>
<td>1.8</td>
<td>0.3</td>
</tr>
<tr>
<td>ATP, 2 mg/ml</td>
<td>8</td>
<td>1.6</td>
<td>0.1</td>
<td>1.5</td>
<td>0.4</td>
</tr>
<tr>
<td>ADP, 2 mg/ml</td>
<td>3</td>
<td>2.7</td>
<td>0.2</td>
<td>2.5</td>
<td>0.3</td>
</tr>
</tbody>
</table>

* Paired experiments, with and without agent tested. Each reaction mixture contained five units of cardioglobulin-A at start. Muscle homogenates were prepared as described in Methods. Samples assayed at end of equilibration with homogenate.

† Number of units of cardioglobulin remaining in mixture at end of equilibration. Mean values with and without agent tested. Two of the values with ATP were more than three units, but upper limit was not assayed and values were placed at three for purposes of calculating mean of the group.

$\Delta$ units of cardioglobulin remaining in phosphate sample at end of equilibration minus units of cardioglobulin remaining in paired control. All mean differences significant by $t$-test, $P < 0.01$.

§ $S_{\bar{x}}$: standard error of mean difference.

assayed, the activity of each sample being expressed in cardioglobulin units per ml. The difference between control and high-energy phosphate treated samples for each experimental pair was then tabulated. Table 1 shows that CP, ATP and ADP all caused significant retention of cardioglobulin activity. On the other hand, no protection against homogenate action was obtained by addition of comparable concentrations of adenylic acid, adenosine, UTP or creatine.

After cardioglobulin-A was inactivated by incubation with homogenate, an attempt was made to restore biological activity by a subsequent ten-minute equilibration with added CP. Despite the continued presence of the cardioglobulin-destroying homogenate, reactivation occurred in three out of five experiments (table 2, I-A). The addition of creatine kinase to the medium containing CP did not enhance the action of the CP.

Reactivation of the homogenate-treated cardioglobulin was also accomplished in another way. The reaction mixture was added to the frog heart, and after a five-minute equilibration, during which there was no sign of cardioglobulin activity, CP was added directly to the solution in the heart cannula. This resulted in a rapidly developing contracture characteristic of the cardioglobulin reaction in seven out of ten experiments (table 2, I-B).

TABLE 2

<table>
<thead>
<tr>
<th>Reactivation of Cardioglobulin by CP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inactivation</td>
</tr>
<tr>
<td>--------------</td>
</tr>
<tr>
<td>I. Tissue homogenates *</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>II. Dilution †</td>
</tr>
<tr>
<td>III. During action on frog heart †</td>
</tr>
</tbody>
</table>

* See Methods for composition of reaction mixture.
† See Results for description of inactivation by dilution.
‡ See figure 2.
2. DESTRUCTION BY A MECHANISM INTRINSIC TO PLASMA

Although cardioglobulin-A is stable in whole plasma for short periods of time at room temperature, slight modification of the plasma frequently results in rapid loss of activity. The simplest alteration causing cardioglobulin-A loss consists of threefold dilution of plasma followed by 10 to 20 minutes of shaking on a Yankee rotator at 26°C. This reaction is temperature dependent, no loss being observed if diluted samples are shaken for 20 minutes at 4°C. Inactivation of undiluted plasma can be caused by two to three hours of dialysis against Ringer-Conway solution at room temperature. Addition of cardioglobulin-A to plasma inactivated by either method restores the activity. The results suggest that inactivation of cardioglobulin-A may be catalyzed by a plasma enzyme, the activity of which is inhibited in whole plasma by a molecule that can be removed by dialysis or can be diminished in effect by dilution.

Destruction of cardioglobulin-A by these methods is inconstant. For example, loss of activity was observed regularly in the autumn of 1961 and 1962, but during the winter the same method caused little or no cardioglobulin-A destruction. It is not known whether this difference is seasonal or a fortuitous result due to another variable. Day-to-day variation in response to dialysis has also been observed.

As in the case of the tissue homogenate experiments, destruction can be prevented or diminished by certain high energy phosphate compounds. The addition of barium to the reaction mixture also prevents destruction. The results of the experiments on diluted plasmas are summarized in table 3. Protection was virtually complete with CP and barium, partial with ADP, and not evident with ATP. The ATP result is surprising and has not been explained. Both ATP and CP (one experiment with each) have been shown to prevent loss of cardioglobulin-A activity on dialysis.

After inactivation of cardioglobulin-A by dilution, reactivation can be accomplished by addition of CP (2 to 8 mg/ml) and incubation at 32 to 37°C for 10 minutes. This was carried out successfully in four out of five experiments (table 2, II). Reactivation proceeds partially or not at all at 4°C.

3. INACTIVATION OF CARDIOGLOBULIN-A DURING ACTION OF THE COMPLETE CARDIOGLOBULIN SYSTEM

The results summarized in the previous two sections suggested the possibility that the observed inactivation of cardioglobulin-A could be due to enzymatic hydrolysis of a high energy phosphate compound bound to the protein. Cardioglobulin-A activity is also lost when the whole cardioglobulin system acts on the isolated frog heart. The effect of cardioglobulin on the isolated heart does not last indefinitely, but after a number of min-

<table>
<thead>
<tr>
<th>Agent tested</th>
<th>Number of paired experiments*</th>
<th>Units of cardioglobulin with agent †</th>
<th>Units of cardioglobulin without agent ‡</th>
<th>Mean Δ‡</th>
<th>$S_2$§</th>
</tr>
</thead>
<tbody>
<tr>
<td>CP, 4 mg/ml</td>
<td>5</td>
<td>3.0</td>
<td>0.1</td>
<td>2.9</td>
<td>0.1</td>
</tr>
<tr>
<td>ATP, 4 to 6 mg/ml</td>
<td>5</td>
<td>0.6</td>
<td>0.4</td>
<td>0.2</td>
<td>0.5</td>
</tr>
<tr>
<td>ADP, 4 to 5 mg/ml</td>
<td>5</td>
<td>1.7</td>
<td>0.0</td>
<td>1.7</td>
<td>0.2</td>
</tr>
<tr>
<td>Ba++, 0.1 μM/ml</td>
<td>3</td>
<td>3.0</td>
<td>0.0</td>
<td>3.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

* Paired experiments, with and without agent tested. Each reaction mixture contained three units of cardioglobulin-A at start. Samples assayed at end of equilibration.
† Number of units of cardioglobulin remaining in mixture at end of equilibration. Mean values with and without agent tested.
‡ Δ: units of cardioglobulin remaining in sample containing agent being tested minus units of cardioglobulin remaining in paired control. All mean differences, except for ATP group, significant by t-test, $P \leq 0.001$.
§ $S_2$: standard error of mean difference.

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Action of cardioglobulin on frog heart. Continuous tracing of twitch tension. Height of black column is proportional to tension developed. Individual twitches not seen because of slow speed of chart paper. Heart was sensitized with cardioglobulin-B. At arrow a solution was added which contained cardioglobulin A and C. After an initial rise in tension, contracture occurs (fall in tension, rise in base line). Although solution was not changed, the heart recovered from the contracture, as indicated by restoration of twitch tension and declining base line. Contracture can be induced again in such a heart by addition of fresh cardioglobulin-A.

utes gradually disappears (fig. 1). This appears to be due to loss of cardioglobulin-A activity, since addition of fresh cardioglobulin-A (without cardioglobulins B and C) restores the action of the system. It is also possible to reactivate the cardioglobulin solution in the cannula by the addition of a large amount of creatine phosphate (which has no effect on the heart in the absence of cardioglobulin). This can be done either by adding 10 mg of creatine phosphate directly to the 2 ml of cannula solution, or by removing the cannula contents and dissolving an amount of creatine phosphate sufficient to make a final concentration of 20 mg/ml (table 2, III). A typical result is shown in figure 2. The question, then, is whether loss of cardioglobulin-A, which occurs during the action of the system on the frog heart, is associated with loss of protein-bound phosphate.

The ideal approach to this problem would be to add P32 labelled cardioglobulin-A to the reaction mixture and look for release of protein bound P32. However, the actual experiments are far from ideal, mainly because it is impossible at the present time to obtain pure cardioglobulin-A. The essential steps in the experiment are as follows: (1) administration of P32 to rats for several days in order to label the plasma proteins; (2) collection
Release of Protein-bound P\textsuperscript{32}

<table>
<thead>
<tr>
<th>Experiment*</th>
<th>Number of experiments</th>
<th>Counts released (%) of total</th>
<th>(S_E)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Shaking control</td>
<td>8</td>
<td>0.4</td>
<td>.15</td>
</tr>
<tr>
<td>II. Sensitized hearts</td>
<td>16</td>
<td>2.2</td>
<td>.47</td>
</tr>
<tr>
<td>III. Nonsensitized hearts</td>
<td>9</td>
<td>0.4</td>
<td>.17</td>
</tr>
</tbody>
</table>

* Rat plasma P\textsuperscript{32} labelled protein, shaken 1 hour at 26°C or added to frog hearts as described in text. Then separated into protein and nonprotein fractions by gel filtration and each fraction counted.

\(S_E\): standard error of mean. Difference between experiment groups II and III significant by \(t\)-test, \(P = 0.01\).

The results are presented in table 4, which shows the extent of phosphate release from rat plasma protein under various experimental conditions. The "Shaking control" experiments (I) were designed to determine how stable the protein-bound phosphate was after the first Sephadex run (step (2) of the outline in the previous paragraph). The plasma proteins were separated from nonprotein material by gel filtration on Sephadex, shaken on a Yankee rotator for 60 minutes at 26°C, and then rerun on Sephadex for counting of protein and nonprotein fractions. A small release of radioactivity from the protein occurred during the hour of shaking, the mean nonprotein count being 0.4% of the total. In the "Sensitized hearts" group (II), rat plasma protein collected from one column run was added to a sensitized heart. Since cardioglobulin-B was bound to the frog heart and the rat plasma protein fraction provided cardioglobulins A and C, the inotropic cardioglobulin reaction occurred. The solution was transferred through a succession of hearts (three to six total) until the cardioglobulin response no longer occurred. This took a total of 20 to 30 minutes, following which the solution was rerun on Sephadex for separation into protein and nonprotein fractions. The nonprotein count was 2.2% of the total, more than five times that of the "Shaking control." The "Nonsensitized hearts" group (III) was designed to determine whether phosphate was released on exposure of protein to hearts in the absence of the cardioglobulin reaction. Sephadex-treated plasma was run through a succession of four hearts that had not been sensitized by the addition of cardioglobulin-B, five minutes in each heart for a total of 20 minutes. In the absence of the prior addition of cardioglobulin-B, the cardioglobulin reaction in these hearts was minimal or absent. A very small amount of phosphate was released under these conditions, comparable to that in the "Shaking control" experiments. The difference in the percentage of phosphate counts released between sensitized and nonsensitized heart groups is 1.8%. It can therefore be stated that in these experiments the occurrence of the cardioglobulin reaction was associated with a statistically significant release of protein bound phosphate.

**Discussion**

Protein-bound phosphate release is much greater in sensitized hearts in which the cardioglobulin reaction occurs and in which cardioglobulin-A activity is gradually depleted than in nonsensitized hearts in which these activities do not occur (table 4, II and III). These findings are consistent with the idea that the depletion of cardioglobulin-A in the
heart during the cardioglobulin reaction is due to phosphate release from cardioglobulin-A.

The essential features of the cardioglobulin-A inactivation experiments (Results, sections 1 and 2) are that under the conditions noted the presence of CP, ATP or ADP generally prevents loss of activity and generally the addition of CP to an inactivated cardioglobulin-A has restored activity. It would thus appear that both tissue homogenates and plasma contain an enzyme system which causes hydrolysis of a bound phosphate of cardioglobulin-A that is required for biological activity. The protection of activity by ATP or ADP could be explained by assuming that the enzyme systems are of low specificity, cardioglobulin-A-P, ATP or ADP serving as substrates. Since CP can reactivate cardioglobulin-A, the action of CP on the other hand (both in protection and reactivation experiments) could be that of a phosphate donor.

If ATP or ADP can serve as substrates for the enzyme, and if CP can restore cardioglobulin-A activity after the action of the enzyme, it is possible that the phosphate group is bound to cardioglobulin-A in a high energy linkage. On the other hand it is conceivable that the reactivation of cardioglobulin-A by CP involves the formation of a low energy bond. Phosphate transfer from CP to glycerol or glucose in the presence of intestinal phosphatase has been shown, though it should be noted that the reaction mixture included inorganic phosphate and polyalcohol concentrations in the high range of 1.5 to 2 molar. Since there is no direct evidence on how ATP protects or CP reactivates, the nature of the cardioglobulin-A-P bond remains open to conjecture.

Evidence presented in a previous paper led to the hypothesis that the overall action of the three proteins of the cardioglobulin system involves the release or transport of cardioglobulin-C calcium. Cardioglobulin-A may contain a high energy phosphate, the energy of which is utilized during the action of the cardioglobulin system.

Summary

The biological activity of cardioglobulin-A can be destroyed enzymatically by a short equilibration with homogenates prepared from a wide variety of tissues. Blood plasma alone also contains a cardioglobulin-A inactivating system which is stimulated by the removal or dilution of an unidentified small molecule normally present in the plasma. Inactivation of cardioglobulin-A can be prevented by the addition of certain high energy phosphate compounds, notably ATP, ADP or CP. Furthermore, the biological activity of inactivated cardioglobulin-A can frequently be restored by addition of CP.

When the whole cardioglobulin system acts on the frog heart, cardioglobulin-A activity is gradually depleted. This is associated with a loss of protein-bound phosphorus from a component of the mixture. Activity can be restored by addition of CP.

It has been proposed that the action of the cardioglobulin system involves the release of bound cardioglobulin-C calcium. A possible role of cardioglobulin-A may be to provide, through a P linkage, the energy for the release of this calcium.

References


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