Effect of Cardiotonic Lactones on Calcium Metabolism of Dog Cardiac Microsomes

By Mark L. Entman, M.D., Joseph W. Cook, Jr., M.D., and Rubin Bressler, M.D.

ABSTRACT

The effects of several lactones were studied in a microsomal fraction of dog myocardium thought to be sarcoplasmic reticulum. The lactones increased the steady-state accumulation and turnover of calcium only in the presence of ATP, and augmented the calcium-stimulated ATPase activity. When the effective concentrations of the lactones were exceeded, there were no further alterations in calcium accumulation or turnover. A correlation between the capacity of these lactones to increase calcium accumulation and turnover and their relative cardiotonic activity, as reported in the literature, was noted. The potency of the lactones in relation to calcium metabolism in the microsomes is influenced by ring saturation, position of the double bond, and presence of a steroid ring system to the lactone moiety.

ADDITIONAL KEY WORDS lactone structure steric configuration
cardiotonic activity calcium-stimulated ATPase microsomal fraction
lactone ring saturation sarcoplasmic reticulum

We recently showed that ouabain increased the accumulation and turnover of calcium in a microsomal fraction of dog myocardium thought to represent sarcoplasmic reticulum (1). These effects required the presence of ATP and were associated with an increased “calcium-stimulated ATPase.” The data suggested that the cardiotonic activity of ouabain might be due to its increasing the quantity of calcium available for release (upon membrane depolarization) by the sarcoplasmic reticulum, in close proximity to the junctions of the actin and myosin filaments in the A band and at the A-I junction so that cross bridges can be formed.

The requirement of a lactone ring for cardiotonic activity in digitalis agents has led to the investigation of other lactones for cardiotonic activity. Several simple lactones possess such activity when tested on the isolated hypocalcemic frog heart (2-4). In this study, a number of these lactones (Fig. 1) that have previously been evaluated for cardiotonic activity (2-5) were examined for their effect on calcium transport. We found that the concentrations of these lactones reported to possess cardiotonic activity also increase steady-state accumulation and turnover of calcium. The effects of these agents on calcium metabolism required ATP and were associated with an augmentation of calcium-stimulated ATPase activity.

Methods

Immediately following pentobarbital anesthesia, cardiac microsomes were isolated and purified from hearts of mongrel dogs by the procedure of Katz and Repke (6), using a 20% to 35% sucrose gradient as the final purification step. Alpha tocopherol (0.2 mM) was added to all isolation media. The purified microsomal preparation was stored at 4°C and used within 3 days. Cellulose vessels and polypropylene syringes were used in all procedures to avoid calcium binding. Protein determinations were done by a modification of the biuret method (7).
Calcium accumulation was assayed by incubating 0.5 to 1.0 mg of microsomal protein in 5 ml of basic reaction mixture. The basic reaction mixture contained 0.12 M KCl, 5.0 m M Mg ATP, 10 m M histidine buffer (pH 7.0), and $2.5 \times 10^{-5} \text{M} \ CaCl_2$ (25,000 counts/min/μmole). Incubations were carried out in a reaction mixture free of sodium. The reactions proceeded at 25°C for 5 minutes, sufficient time to attain equilibrium (1, 6, 8). The incubation mixtures were extruded through a Swinny adapter containing a Millipore filter (0.45 μm pore diameter) and aliquots of the filtrate counted in triton X-100: toluene (1:2 v/v) containing 4 g/liter 2,5 diphenyloxazole and 0.1 g/liter 1,4 bis [2(5 phenyloxazolyl)] benzene in a liquid scintillation spectrometer.

Calcium turnover at equilibrium was measured by incubation of the microsomes with nonradioactive CaCl₂ (2.5 × 10⁻⁵ M) for 5 minutes at 25°C. At the end of this period 0.1 ml of ⁴⁵CaCl₂, 2.5 × 10⁻⁵ M (3.12 × 10⁶ counts/min), was added to the 5-ml incubation mixture so that the resultant mixture did not appreciably differ in calcium concentration from the original reaction mixture (containing 125 μmole of Ca²⁺ in 5 ml, whereas the reaction with ⁴⁵CaCl₂ added contained 127.5 μmole of Ca²⁺ in 5.1 ml). Filtered samples were obtained at zero time, 0.5, 1.0, and 2.0 minutes after the addition of ⁴⁵CaCl₂. This method measured calcium turnover at equilibrium. Values given represent the ⁴⁵Ca²⁺ bound to the microsomes in exchange for nonradioactive Ca²⁺.

ATPase activity was assayed by measuring Mg ATP hydrolysis to inorganic phosphate. Incubations were carried out in the absence and presence of 10⁻⁵ M CaCl₂. The incubation samples were extruded through a Swinny adapter containing a Millipore filter (0.45 μ). Inorganic phosphate assays were carried out on the filtrate by the procedure of Post and Sen (9). Calcium-stimulated ATPase activity was calculated by subtracting the phosphate values in the absence of calcium from those in the presence of calcium.

Mitochondrial contamination of the microsomal preparations was assessed by electron microscopy and by assay of glutamic dehydrogenase activity. No intact mitochondria were visible in osmium-tetroxide-fixed preparations. Glutamic dehydrogenase activity (10) of the preparation was less than 1% of that of a preparation of dog heart mitochondria with similar protein concentration.

Endogenous calcium was measured by ashing the microsomes in a nickel crucible and resuspending the sediment in a solution of 1% lanthanum in 5% HCl. Calcium was determined by atomic absorption spectrometry (Perkin-Elmer). The total endogenous calcium of the microsomes was 4 to 5 μmole/mg protein.

Ouabain (Nutritional Biochemicals Corporation) was ascertained to be pure by thin-layer chromatography (11) and its characteristic infrared absorption spectrum. Dihydro-ouabain was prepared from ouabain by catalytic hydrogenation using platinum. The reduction was confirmed by the disappearance of the double bond peak on infrared spectroscopy. Alpha and beta angelica lactone and gamma butyrolactone were ascertained to be pure by gas-liquid chromatography and infrared spectroscopy. All reagents were assayed by atomic absorption and found to have negligible levels of calcium.

Experiments were analyzed using the paired t-test for the data from a single microsomal preparation.
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### TABLE 1

**Effect of Lactones on Calcium Accumulation by Cardiac Microsomes**

<table>
<thead>
<tr>
<th>Agent</th>
<th>Minimal effective concn</th>
<th>Ca(^{2+}) binding(^a) (umoles/mg protein)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>13.1 ± 3.2</td>
<td></td>
</tr>
<tr>
<td>Ouabain</td>
<td>10(^{-6})M</td>
<td>17.0 ± 4.1</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>Dihydro-ouabain</td>
<td>10(^{-5})M</td>
<td>17.7 ± 6.2</td>
<td>&lt;.05</td>
</tr>
<tr>
<td>Alpha angelica lactone</td>
<td>10(^{-3})M</td>
<td>16.0 ± 1.7</td>
<td>&lt;.05</td>
</tr>
<tr>
<td>Beta angelica lactone</td>
<td>10(^{-2})M</td>
<td>20.1 ± 3.2</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>Gamma butyrolactone</td>
<td>10(^{-1})M</td>
<td>12.9 ± 3.1</td>
<td>&gt;.50</td>
</tr>
</tbody>
</table>

The reaction mixtures contained 0.2 mg/ml of cardiac microsomes, 10 mM histidine buffer (pH 7.0), 0.12M KCl, 5 mM Mg ATP, 2.5 \(\times\) 10\(^{-5}\)M \(^{45}\)CaCl\(_2\) (3.12 \(\times\) 10\(^6\) counts/min), and the indicated amounts of lactones. Final incubation volume was 5 ml. Reactions proceeded at 25°C for 5 minutes.

\(^a\)Values are means ± SD; n = 21.

### Results

**Effect of Lactones on Calcium Accumulation by Cardiac Microsomes.**—The data in Table 1 show that all of the lactones tested, except gamma butyrolactone, increased the binding of calcium to cardiac microsomes. The minimal effective concentration of each agent necessary to augment calcium binding is shown in Table 1, as is the resulting increase in microsomal calcium binding. Although there were great differences in the minimal effective concentrations of the lactones tested, the degree of augmentation achieved with the various lactones was not significantly different. Further increase in concentration of the lactones above the effective concentration did not result in additional calcium accumulation by the microsomal preparation. In the absence

**FIGURE 2**

*Effect of lactones on calcium turnover at equilibrium. Incubations were carried out as described for the calcium accumulation studies of Table 1, except for the use of non-radioactive calcium. After 5 minutes of incubation at 25°C, 0.1 ml of \(^{45}\)CaCl\(_2\), 2.5 \(\times\) 10\(^{-5}\)M (3.12 \(\times\) 10\(^6\) counts/min) was added to the reaction mixture. Samples were filtered at the times indicated to ascertain the exchange of \(^{45}\)Ca\(^{2+}\) for unlabeled bound Ca\(^{2+}\) at equilibrium.*

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of ATP, these agents exerted no effect on calcium accumulation.

**Effect of Lactones on Calcium Turnover of Cardiac Microsomes.**—We have previously shown that microsomal calcium is rapidly exchangeable with calcium in the incubating media, and that the ouabain-stimulated increase in microsomal calcium is also exchangeable with the media (1). Ouabain thus increased the calcium turnover of the microsomes. The effect of lactones on calcium turnover in the microsomes is shown in Figure 2. The lactones tested, except gamma butyrolactone, at minimal effective concentrations of the same magnitude as the steady-state accumulation studies of Table 1, increased the

![Figure 3](image)

**FIGURE 3**
Effect of varying concentrations of dihydro-ouabain on calcium turnover at equilibrium. Incubations were carried out as described in Figure 2 except for the use of several concentrations of dihydro-ouabain instead of several different lactones.

**TABLE 1**

<table>
<thead>
<tr>
<th>Agent</th>
<th>Basic ATPase</th>
<th>Calcium-stimulated ATPase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8.4</td>
<td>2.1</td>
</tr>
<tr>
<td>Ouabain (10^{-5}M)</td>
<td>4.6</td>
<td>3.2</td>
</tr>
<tr>
<td>Dihydro-ouabain (10^{-6}M)</td>
<td>4.8</td>
<td>3.3</td>
</tr>
<tr>
<td>Alpha angelica lactone (10^{-3}M)</td>
<td>5.0</td>
<td>3.65</td>
</tr>
<tr>
<td>Beta angelica lactone (10^{-2}M)</td>
<td>5.2</td>
<td>2.0</td>
</tr>
<tr>
<td>Gamma butyrolactone (10^{-1}M)</td>
<td>7.9</td>
<td>1.7</td>
</tr>
</tbody>
</table>

The reaction mixtures contained 0.1 mg/ml of cardiac microsomes, 4 mM Mg ATP, 8 mM histidine buffer pH 7.1, 0.12 M KCl, the indicated amounts of lactones and 10^{-4}M CaCl2 where indicated. Final incubation volumes were 5 ml. Reactions were carried out at 25°C for 30 minutes. (n = 2)

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total calcium turnover. Figure 3 shows that a further increase in the concentration of dihydro-ouabain does not increase the total calcium turnover. This phenomenon is common to all of the effective lactones and is similar to the nonlinear response described in the steady-state accumulation studies.

Effect of Lactones on ATPase Activity of Cardiac Microsomes.—The data in Table 2 show that ouabain and a number of other lactones inhibited "basic" ATPase activity (ATPase activity in the presence of potassium and magnesium but in the absence of calcium). These agents, however, stimulated ATPase activity in the presence of calcium. The inhibition of basic ATPase activity by ouabain has previously been shown for red blood cells (12) and cardiac microsomes (1, 13).

Discussion

Our data show ouabain-like augmentation of calcium accumulation and turnover in cardiac microsomes by lactones (1) known to possess cardiotonic activity in isolated heart preparations (2-5). As has been previously discussed (1), the inability to measure initial rates by these methods prevents further characterization of this effect except that it requires ATP and increases the amount of calcium in a pool(s) that is rapidly exchangeable with the external media and is associated with an increased calcium-stimulated ATPase activity.

Simple lactones with beta-gamma unsaturation were eight times as potent as cardiotonic agents in the hypocalcemic frog heart (2) and ten times as potent in stimulating calcium transport as the alpha-beta unsaturated isomers, although the alpha-beta unsaturated configuration is the one found in active digitals glycosides. Saturation of the lactone ring eliminated cardiotonic effects (2) and augmentation of calcium transport in the simple lactones, but saturation of the lactone ring of ouabain decreased its capacity to stimulate calcium accumulation and turnover in cardiac microsomes and diminished its cardiotonic potency (5) in the isolated heart by only tenfold. The capacity of alpha-beta unsaturated lactones to form polar resonance forms (12), the effect of the steroid ring system and saturation of the lactone ring, all influence steric and solubility properties of the lactones and their action on membrane systems. This suggests that these physical properties relate to their biologic activity.

Table 3, based on information from the literature and our own data, shows a positive correlation between the capacity of the lactones to augment calcium uptake and turnover by cardiac microsomes and their ability to stimulate myocardial contraction (2, 5). The effect of the nonsteroidal lactones has been observed with the isolated hypocalcemic frog heart (2-4); similar cardiotonic activity could not be demonstrated in the heart of the intact cat (4) because relatively low doses caused toxicity. We postulate that the limited membrane solubility and permeability of these compounds, in contrast to steroidal lactones, required such high dosage for effective concentration to reach the sarcotubular calcium transport system that arrhythmias or central nervous system toxicity resulted from their effects on the external membrane. (Like ouabain, they also inhibit basic ATPase.) In contrast, the frog lacks a developed sarcotubular system and depends on sarcolemmal calcium transport for contraction. This surface site might allow the agents easier access to the site that augments calcium transport. It is probable that a correlation between relative concentration necessary for cardiotonic activity and for augmentation of calcium transport

<table>
<thead>
<tr>
<th>Agent</th>
<th>Relative cardiotoxicity required for calcium transport effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ouabain</td>
<td>1/10</td>
</tr>
<tr>
<td>Dihydro-ouabain</td>
<td>1/100</td>
</tr>
<tr>
<td>Alpha angulica lactone</td>
<td>1/10,000</td>
</tr>
<tr>
<td>Beta angulica lactone</td>
<td>1/4,770</td>
</tr>
<tr>
<td>Gamma butyrolactone</td>
<td>No effect</td>
</tr>
</tbody>
</table>

Cardiotonic activity data taken from the literature (2, 5).
requires that the agents have access to the site of calcium transport as it does in the isolated in-vitro system reported here.

This report, therefore, is not intended to suggest new therapeutic agents. It is intended to demonstrate the positive correlation between microsomal calcium transport and cardiotonic activity and to delineate the molecular alterations that may markedly affect the potency of these lactones in exerting their pharmacologic action.

References


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