Intracellular Calcium and Myocardial Contractility

II. EFFECTS OF POSTEXTRASYSTOLIC POTENTIATION IN THE ISOLATED RABBIT HEART

By Josef Suko, M.D., Yasuyuki Ueba, and Charles A. Chidsey, M.D.

ABSTRACT

Postextrasystolic potentiation was studied in the isolated perfused rabbit heart to determine its effect on intracellular calcium, sodium, and potassium concentrations, $^{45}$Ca uptake, and calcium distribution in mitochondria and microsomes. Paired stimulation at 80/min augmented myocardial contractility with a gradually developing increase in total intracellular calcium and sodium. A greater $^{45}$Ca uptake was also observed and this was proportionate to the increase of intracellular calcium so that no change in exchangeable calcium occurred. A small but statistically insignificant increment was observed in mitochondrial and microsomal calcium. An increase of the rate of single stimuli to 160/min produced a minimal change in myocardial contractility compared to paired stimulation at 80/min, no change in total intracellular calcium, and a greater calcium uptake so that there was a significant increment in exchangeable calcium. Although these experiments do not identify the cellular processes responsible for the positive inotropic response to paired stimulation, they suggest that an alteration of myocardial calcium metabolism is involved. Furthermore, it is apparent that this alteration is distinct from that occurring during a comparable increase in the rate of delivery of single stimuli to the heart.

ADDITIONAL KEY WORDS

single stimulation, paired stimulation, myocardial calcium, sodium, and potassium, $^{45}$Ca uptake and exchange, myocardial contractility

Postextrasystolic potentiation constitutes a profound inotropic intervention characterized by an increase in both rate of development of tension and in maximal developed tension (1-4). The cellular basis for this response to the delivery of a paired stimulus pattern remains largely unknown. Although studies have suggested that there is an augmented potassium loss from the heart into the coronary blood flow (5, 6), no change in the resting membrane potential has been observed under these circumstances (1), and it is unlikely that this loss is of sufficient magnitude to reduce intracellular potassium substantially. Release of norepinephrine stored in cardiac adrenergic nerves does not seem to be involved, since denervation, reserpine pretreatment, and beta-receptor blockade have all been ineffective in reducing the response to paired stimulation (7, 8). Calcium plays a central role in the excitation contraction process of muscle (9) and alterations of this cation may be determinants in the regulation of the contractile state of cardiac muscle (10). Since little attention had been directed previously to the study of myocardial calcium metabolism in the positive inotropic response to paired stimulation, we undertook such a study using the isolated perfused rabbit heart.
Methods

Albino rabbits weighing from 1.1 to 1.6 kg were anesthetized intravenously with 37.5 mg/kg of sodium pentobarbital and the hearts were removed rapidly and perfused in a Langendorff apparatus. The perfusion medium used as standard nutrient solution was prepared to contain in mmoles/liter: NaCl 91, NaHCO3 20, KCl 3, KH2PO4 1, MgSO4•7H2O 1, Na-glutamate 5, Na-pyruvate 5, Na2-fumarate 5, CaCl2•5H2O, glucose 2 g/liter, and insulin 5 IU/št. The medium was gassed continuously with a mixture of 95% O2 and 5% CO2 and the temperature was maintained at 37°C. The intrinsic heart rate was reduced to less than 65/min by dissecting off both atria. A fine fishhook electrode was attached to the left ventricle and an indifferent electrode to the aorta. This electrode attachment provided for both stimulus and recording of the electrogram. The apex of the heart was fixed to a Statham force transducer and recording of the active tension during spontaneous beating averaged 23.6 ± .07 g and was unchanged during 40 ± 2 (SE) min during spontaneous contractions and increased to the driven frequency of 80/min during stimulation. Active tension during spontaneous beating averaged 23.6 ± .07 g and was unchanged during 80 seconds, blotted dry, and weighed. Both ventricles were perfused through a tissue sieve, and two aliquots of pressed ventricle were homogenized in 20 volumes of 4M perchloric acid containing 23Na-lanthanum chloride (LaCl3) using a Virtis tissue homogenizer. An aliquot of pressed tissue was homogenized in 20 volumes of 25% sucrose for 10 seconds, and mitochondria and microsomes were isolated by differential centrifugation with mitochondria prepared between 1,000 and 12,000 × g for 10 minutes and microsomes between 12,000 and 35,000 × g for 10 minutes. The mitochondrial and microsomal protein was resuspended in deionized water and extracted in 4M perchloric acid containing 24Ca, for at least 1 hour. Calcium, sodium, and potassium were assayed from the perfusion medium and the supernatant of the tissue extracts and extracts of mitochondrial and microsomal fractions using atomic absorption spectrophotometry. 44Ca was measured by adding 1 mg of carrier CaCl2 (1 ml) to 1 ml of perfusion medium or 1 ml of supernatant fluid of the tissue homogenate and assayed in 10 ml of counting solution (11) in a liquid scintillation spectrometer. All values were expressed as counts per minute and were corrected for quenching estimated by channels ratio method. Inulin spaces were measured with 3H-inulin in single as well as paired stimulated hearts. The inulin space did not differ in the two groups and averaged 43.2 ± 7% after 30 minutes of perfusion with 3H-inulin.

The concentrations of calcium, sodium, and potassium in the perfusion media were determined in each experiment, and the average of these determinations was, respectively, 36, 140, and 4.98 ME values virtually identical with these average values in the perfusion medium and an extracellular space value of 43%. Statistical analysis were carried out by the t-test determined for nonpaired samples.

Results

The changes observed in the function of the isolated rabbit heart after removal of the atria were minimal upon institution of electrical stimulation (Table 1). The heart rates averaged 40 ± 2 (ac) during spontaneous contractions and increased to the driven frequency of 80/min during stimulation. Active tension during spontaneous beating averaged 23.6 ± .07 g and was unchanged during 20 seconds, blotted dry, and weighed. Both ventricles were perfused through a tissue sieve, and two aliquots of pressed ventricle were homogenized in 20 volumes of 4M perchloric acid containing 23Na-lanthanum chloride (LaCl3) using a Virtis tissue homogenizer. An aliquot of pressed tissue was homogenized in 20 volumes of 25% sucrose for 10 seconds, and mitochondria and microsomes were isolated by differential centrifugation with mitochondria prepared between 1,000 and 12,000 × g for 10 minutes and microsomes between 12,000 and 35,000 × g for 10 minutes. The mitochondrial and microsomal protein was resuspended in deionized water and extracted in 4M perchloric acid containing 24Ca, for at least 1 hour. Calcium, sodium, and potassium were assayed from the perfusion medium and the supernatant of the tissue extracts and extracts of mitochondrial and microsomal fractions using atomic absorption spectrophotometry. 44Ca was measured by adding 1 mg of carrier CaCl2 (1 ml) to 1 ml of perfusion medium or 1 ml of supernatant fluid of the tissue homogenate and assayed in 10 ml of counting solution (11) in a liquid scintillation spectrometer. All values were expressed as counts per minute and were corrected for quenching estimated by channels ratio method. Inulin spaces were measured with 3H-inulin in single as well as paired stimulated hearts. The inulin space did not differ in the two groups and averaged 43.2 ± 7% after 30 minutes of perfusion with 3H-inulin.

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**TABLE**

<table>
<thead>
<tr>
<th>No.</th>
<th>Time (mm)</th>
<th>Heart rate</th>
<th>Spont. contr. (beats/min)</th>
<th>Active tension</th>
<th>Maximal response</th>
<th>Spont. contr. (g)</th>
<th>Maximal response contr. (g)</th>
<th>Rate of tension development</th>
<th>Spont. contr. (g/sec)</th>
<th>Maximal response contr. (g/sec)</th>
<th>Rate of tension development</th>
<th>Spont. contr. (g/m)</th>
<th>Maximal response contr. (g/m)</th>
<th>Rate of tension development</th>
<th>Spont. contr. (cm/min)</th>
<th>Maximal response contr. (cm/min)</th>
<th>Rate of tension development</th>
</tr>
</thead>
<tbody>
<tr>
<td>SS-80</td>
<td>6</td>
<td>15</td>
<td>63.6 ± 5.3</td>
<td>21.9 ± 1.7</td>
<td>21.1 ± 1.3</td>
<td>4.5 ± 0.2</td>
<td>102 ± 11</td>
<td>159 ± 11</td>
<td>0.1 ± 0.2</td>
<td>26.5 ± 2.1</td>
<td>24.7 ± 2.5</td>
<td>24 ± 2.1</td>
<td>26.5 ± 2.1</td>
<td>24.7 ± 2.5</td>
<td>24 ± 2.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SS-80</td>
<td>4</td>
<td>5</td>
<td>65.0 ± 9.5</td>
<td>22.3 ± 1.2</td>
<td>21.1 ± 1.3</td>
<td>5.0 ± 0.3</td>
<td>150 ± 11</td>
<td>143 ± 26</td>
<td>4.8 ± 0.6</td>
<td>28.3 ± 1.2</td>
<td>26.7 ± 1.4</td>
<td>26 ± 1.0</td>
<td>28.3 ± 1.2</td>
<td>26.7 ± 1.4</td>
<td>26 ± 1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SS-160</td>
<td>4</td>
<td>15</td>
<td>39.7 ± 6.5</td>
<td>21.5 ± 3.5</td>
<td>22.3 ± 3.0</td>
<td>8.5 ± 0.3</td>
<td>155 ± 11</td>
<td>159 ± 22</td>
<td>3.8 ± 4.1</td>
<td>38.3 ± 2.0</td>
<td>24.0 ± 2.0</td>
<td>23.3 ± 1.3</td>
<td>38.3 ± 2.0</td>
<td>24.0 ± 2.0</td>
<td>23.3 ± 1.3</td>
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</tr>
</tbody>
</table>

Mean values ± sd. Values for the single stimulus-induced contraction at 80/min (SS-80) are given only for 15 minutes of stimulation since values at 1, 3, 5, and 10 minutes are essentially identical; values for stimulation at 160/min (SS-160) were obtained only at 15 minutes; PS-80 = paired stimulation at 80/min; Spont. contr. = spontaneous contraction; Simult. contr. = stimulus-induced contraction; Control = first period of stimulus-induced contraction; Expt. = second period of stimulus-induced contraction; No. = number of animals.
TABLE 1

Effect of Single and Paired Stimulation on Ca and ^Ca Tissue Concentration and Relative Specific Activity

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>No.</th>
<th>Ca (μmole/g heart)</th>
<th>Ca* (μmole/ml ICF)</th>
<th>^Ca (10^6 cpm/g heart)</th>
<th>^Ca* (10^6 cpm/ml ICF)</th>
<th>Fraction of exchangeable Ca</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4</td>
<td>0.98 ± 0.06</td>
<td>1.23 ± 0.07</td>
<td>8.24 ± 0.07</td>
<td>7.01 ± 0.12</td>
<td>0.50 ± 0.05</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>0.98 ± 0.06</td>
<td>1.30 ± 0.09</td>
<td>10.37 ± 0.27</td>
<td>10.10 ± 0.50</td>
<td>0.43 ± 0.04</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>0.97 ± 0.04</td>
<td>1.37 ± 0.06</td>
<td>10.91 ± 0.23</td>
<td>9.99 ± 0.35</td>
<td>0.44 ± 0.02</td>
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<tr>
<td>15</td>
<td>6</td>
<td>1.00 ± 0.02</td>
<td>1.24 ± 0.02</td>
<td>11.03 ± 0.74</td>
<td>11.23 ± 0.22</td>
<td>0.52 ± 0.04</td>
</tr>
<tr>
<td>30</td>
<td>5</td>
<td>1.04 ± 0.03</td>
<td>1.39 ± 0.05</td>
<td>12.15 ± 0.36</td>
<td>13.74 ± 0.92</td>
<td>0.55 ± 0.02</td>
</tr>
</tbody>
</table>

Mean values ± se. SS-80 and PS-80 = single and paired stimulation at 80/min; SS-160 = single stimulation at 160/min; Ca = tissue calcium; Ca* = intracellular calcium; ICF = intracellular fluid.

No tendency for either a further increase at 15 minutes or further decline following that time could be observed. Single stimulation at 160/min produced a smaller increment in rate of tension development (average 38%). During the stimulus-induced contractions, resting tension showed a small reduction from the initial value of 16.4 ± 0.9 g during spontaneous contraction. The reduction occurred in hearts in which contraction was induced by either single or paired stimulation and at 15 minutes averaged 13.9 ± 0.9 g and 12.9 ± 0.9 g, respectively. Coronary flow was relatively constant during these experiments and did not change consistently during the positive inotropic response induced by paired stimulation and was only minimally increased by stimulation at 160/min. The tracing of a typical experiment is shown in Figure 1. Active tension and the electrogram are represented in the nonstimulated, spontaneously beating heart during stimulation with a single impulse, as the stimulus pattern was changed from single to paired stimuli, and after 15 minutes of paired stimulation. It is evident that an increase in contractile force and rate of development of tension occurs immediately upon establishing this pattern of stimulus; the response is maintained for 15 minutes of stimulation in the preparation.

The calcium concentration of the control hearts, which were stimulated during the experimental period with single stimuli, appeared to be unchanged during this 30-minute period of stimulation (Table 2). The total tissue calcium concentrations and the calculated intracellular calcium averaged respectively, 100 ± 0.01 μmole/g of heart and 1.32 ± 0.02 μmole/ml of intracellular fluid. Comparable values were observed in hearts stimulated with single impulses at 160/min. However, there was an increase in calcium concentration in those hearts in which contractions were induced by paired stimulation. This change in concentration occurred in both total tissue calcium and intracellular calcium. The increases of these values developed progressively over a period of 15 minutes of double stimulation to levels of 1.21 ± 0.04 μmole/g and 1.70 ± 0.07 μmole/ml, respectively, values significantly greater than those observed in the control hearts (P < 0.01). During continued contraction induced by paired stimulation the calcium declined so that total tissue and intracellular calcium concentrations, although still elevated, were
not significantly different from control values at 30 minutes.

Corresponding to the increase in calcium concentration in the hearts driven with paired stimulation there was also a greater uptake of ⁴⁵Ca during perfusion of these hearts with media containing this radioisotope. The total tissue and intracellular ⁴⁵Ca concentrations were both significantly greater than the control values at 5 and 15 minutes, averaging, respectively at 15 minutes: 22.52 ± 0.22 vs. 11.33 ± 0.14 cpm/g of heart and 15.06 ± 0.40 vs. 12.33 ± 0.22 cpm/ml of intracellular fluid (P<.01). These data indicated that the rate of uptake of ⁴⁵Ca was augmented significantly after 5 minutes of double stimulation, suggesting that the entry of calcium had been increased in these hearts. This can be appreciated visually from a graphic presentation of these data (Fig. 2).

However, the fraction of exchangeable calcium in these hearts, calculated by dividing the specific activity of calcium in the heart by that in the media, was unchanged in the paired stimulated hearts. In single and paired stimulated hearts the exchangeable fraction rose with time to a relative plateau value at 15 to 30 minutes. In contrast, hearts stimulated at 160/min with single impulses demonstrated a marked increment in exchangeable calcium at 15 minutes, indicating that the increased uptake of ⁴⁵Ca in these hearts was accountable through a greater exchange of calcium.

The distribution of calcium within the myocardial cell was examined by comparing the contents of calcium in mitochondria and microsomes. An increase in mitochondrial calcium was observed in the paired stimulated hearts at 15 minutes, but this was not significantly greater than the values in the control hearts, averaging, respectively, 20.8 ± 1.7 and 17.8 ± 0.7 μmole/g of mitochondrial protein. Similarly, there was a small increase without statistical significance in

<table>
<thead>
<tr>
<th>TABLE 3</th>
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<tbody>
<tr>
<td>Effects of Single and Paired Stimulation on Intracellular Sodium and Potassium Concentration</td>
</tr>
<tr>
<td>Condition</td>
</tr>
<tr>
<td>----------</td>
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<tr>
<td>[ ]</td>
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<tr>
<td>SS-80</td>
</tr>
<tr>
<td>PS-80</td>
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<tr>
<td>SS-160</td>
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<td>[ ]</td>
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<tr>
<td>Intracellular Potassium</td>
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<td>SS-80</td>
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<tr>
<td>PS-80</td>
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<tr>
<td>SS-160</td>
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<td>[ ]</td>
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<tr>
<td>Mean values = μmol/ml of intracellular fluid; SS-80 and PS-80 = single and paired stimulation at 80/min; SS-160 = single stimulation at 160/min.</td>
</tr>
<tr>
<td>*P &lt; .01 compared to SS-80.</td>
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</tbody>
</table>

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microsomal calcium, averaging, respectively, 19.2 ± 0.9 and 17.5 ± 1.7 μmole/g of microsomal protein.

The calculated intracellular sodium concentration showed no change in the control hearts during 30 minutes of single stimulation (Table 3). However, there was a greater intracellular sodium concentration during paired stimulation at 15 minutes which averaged 38.4 ± 1.2 compared to 31.0 ± 1.8 μmole/ml of intracellular fluid (P < 0.1). With continued paired stimulation to 30 minutes, the intracellular sodium concentration decreased to control levels, a finding comparable to that observed with calcium concentrations. Calculated intracellular potassium concentration was unchanged in both groups during the entire stimulation period, averaging 79.9 ± 2.1 and 80.9 ± 2.7 μmole/ml of intracellular fluid after 15 minutes of single and paired stimulation, respectively. No change in sodium or potassium was observed in the hearts stimulated at 160/min with single impulses.

Discussion

These observations do not define the cellular mechanism responsible for the positive inotropic response to paired stimulation, but they do indicate that an alteration of myocardial calcium metabolism may be involved in this response. Our findings are in contrast to those recently reported by Hasak and associates (12) in which no change in total calcium or calcium exchange was observed in guinea pig atria. However, these studies utilized higher extracellular calcium and lower temperature, both of which are known to increase intracellular calcium (13). Calcium exchange is less affected by changes in rate of stimulation in high extracellular calcium (14, 15), and it is likely that small differences of total intracellular calcium would be obscured under circumstances in which a higher extracellular calcium was present. The relation of the increased total intracellular calcium to the positive inotropic response is uncertain. Since this occurred after the inotropic response had been established, it seems unlikely that there was a direct causal relation between these two. Rather, the progressive calcium accumulation within the cell may reflect the altered myocardial metabolism of calcium which is responsible for the postextrasystolic potentiation. Also, it seems unlikely that the increase of calcium, and of sodium as well, resulted from a relative deficiency of oxygenation of these hearts during the augmented contractile state produced by paired stimulation. The absence of a change in calcium or sodium during the increase of single stimulation to 160/min would indicate that this is an unlikely explanation.

The finding that 45Ca uptake is augmented by paired stimulation suggests that the progressive increase in calcium concentration of the heart may be the direct consequence of an altered rate of entry. However, doubling the rate of stimulation with single impulses also produced a greater uptake of 45Ca, a finding which is in accord with several earlier studies (14-17). Under these conditions, no change in calcium or sodium concentration was observed, and the fraction of exchangeable calcium was increased. It is also notable that the change in contractility associated with this increment in frequency of stimulation was not comparable to that achieved during paired stimulation. The fraction of exchangeable calcium in the experiments with paired stimulation cannot be interpreted readily since 45Ca uptake was measured in a nonsteady state during increases of intracellular calcium.

An explanation for the increased calcium concentration produced by paired stimulation must involve a consideration of the temporal relation of the two membrane depolarizations induced by this mode of stimulation and the potential effect of this relation on the free intracellular calcium concentration within the muscle cell. Activation of the contractile state and initiation of relaxation are the consequence of changes in free intracellular calcium resulting from altered calcium permeability or affinity of the plasma membrane (18, 19) and perhaps the sarcoplasmic reticulum (5). The contraction pattern induced by two paired stimuli, in which essentially no relaxation can occur,
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be seen, suggests that the free intracellular calcium is elevated for a greater time. In addition, it is possible that a higher free calcium within the cell may result from the temporal coupling of two depolarizations, each of which results in a higher calcium permeability of the membrane. Thus, if the free intracellular calcium concentration were higher and maintained for a longer period of time, an increased uptake of calcium might occur in binding sites not normally utilized to sequester calcium. Uptake into these binding sites may decrease the calcium available for removal from the cell and cause a progressive calcium accumulation within the cell.

The hypothesis which we have proposed, that there is a more prolonged and greater free intracellular calcium concentration during paired stimulation, could provide the basis for the inotropic response of the heart. Considering that during relaxation, this calcium may be bound to sites with different affinities for this cation than those of the normal sites, it is possible that the increase of free intracellular calcium following subsequent depolarizations may be enhanced. Wood and associates have suggested that the inotropic effects of prolonged subthreshold depolarizing current may be due to an increased calcium entry since they observed a prolongation of the plateau phase of the action potential with this stimulus (20). This suggestion was based on the observation that calcium permeability is enhanced during this phase of the action potential (18, 19). However, no such alteration in the action potential duration has been demonstrated during paired stimulation (1), and it is unlikely that the hypothesis proposed by Wood and associates alone can explain the increased contractility produced by such a stimulus. Another possible mechanism whereby increased free calcium could be effected during paired stimulation would involve an alteration of the distribution of calcium within intracellular binding sites so that greater quantities might be available for release during depolarization, but there is no experimental evidence to substantiate this mechanism. Finally, the role of sodium as a determinant of the free calcium concentration must be considered. It is possible that increases of free calcium could result simply from effects of increased intracellular sodium as suggested by Langer (21). However, the increases of sodium we observed were only significant after prolonged stimulation, and it is unlikely that these could explain the contractile response. Thus the present results do not offer an explanation for the cellular mechanisms responsible for increased contractility during postextrasystolic potentiation, but the observed increase in calcium concentration suggests that alteration in the intracellular disposition of this ion may be involved in the response.

Acknowledgment

The authors wish to recognize the excellent technical assistance of Ruth Eussner and Dorothy Walker.

References

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Circ Res. 1970;27:227-234
doi: 10.1161/01.RES.27.2.227

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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