Ventricular Pressure-Heart Rate Product before Induction of Ischemia as a Determinant of the Reperfusion-Induced Accumulation of Calcium within Myocardium

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Abstract—Using the product of heart rate (HR) and left ventricular developed pressure (LVP) as a measure of the total mechanical energy required for contraction (TMEFC), experiments were performed with isolated perfused heart preparations of the guinea pig to establish the importance of TMEFC before induction of ischemia for induction of calcium accumulation within ischemic reperfused myocardium. Two calcium antagonists, diltiazem and nicardipine, and prazosin produced a dose-related suppression of the calcium accumulation when given prior to induction of ischemia (except for the highest dose of nicardipine), while they were ineffective when given only during the period of reperfusion, and the suppression was found to be closely correlated with the decrease in HR×LVP before induction of ischemia. The failure of the highest dose of nicardipine to suppress calcium accumulation (while producing a dose-related decrease in HR×LVP) was not associated with an increase in cyclic AMP. These findings are compatible with the idea that TMEFC before induction of ischemia is a prime determinant of calcium accumulation within the ischemic reperfused myocardium.

More than 10 years ago, Jennings and his collaborators (1, 2) demonstrated that whereas the calcium content of the myocardium was unaffected by prolonged periods of ischemia, a substantial accumulation of calcium occurred during the periods of reperfusion after prolonged ischemia of longer than 30 min and that the time-course of this accumulation corresponded to the time course of onset of irreversible injury. It is often assumed that the massive and abrupt increase in calcium influx that occurs during post-ischemic reperfusion is due to the entry of calcium through the voltage-activated slow channels. Yet there is little evidence to support such a hypothesis. It is true that slow channel blockers such as verapamil, nifedipine and diltiazem have been shown to suppress the reperfusion-induced accumulation of myocardial calcium (3–7), but the suppression was observed in these experiments only when doses of the substances producing a definite inhibition of myocardial mechanical function were given prior to the induction of the global ischemia or during the period of low-flow ischemia. In the studies by Nayler et al. (5), calcium antagonists were given to the animals for 4 to 5 days before the experiments. Thus, the most plausible explanation at present for the prevention of calcium accumulation is to ascribe it to the depressant effects of these compounds on the myocardial mechanical function exerted prior to induction of ischemia and a resultant diminution of energy expenditures early during ischemia. Higgins and Blackburn (7) recently demonstrated that doses of calcium antagonists not producing a decrease in cardiac output given before induction of ischemic insult could produce a suppression of accumulation of calcium within the myocardium following ischemia and reperfusion. However, as has long been recognized (8), cardiac output and work are poor indicators of the rate of energy expenditure.
In view of the circumstances an attempt was made in the present study to establish the importance of the total mechanical energy required for contraction (9-11) before induction of ischemia as a prime determinant of reperfusion-induced accumulation of calcium. To bring in changes in reperfusion-induced accumulation of calcium, various concentrations of two types of calcium antagonists of diverse chemical structure and prazosin were used. Prazosin was included in the present study, for the substance was recently demonstrated to reduce the reperfusion-induced accumulation of calcium, when given prior to induction of ischemia (12, 13).

Materials and Methods

Experiments were performed in the isolated perfused heart preparation of the guinea-pig (Langendorff's method). Male guinea-pigs weighing around 300-400 g were lightly anesthetized with ether. Immediately after opening the thorax, the hearts were excised and transferred to ice-chilled modified Krebs-Ringer bicarbonate solution to induce rapid cessation of the heart beat. The adherent connective tissue was removed and the ascending aorta cannulated. Retrograde perfusion with a modified Krebs-Ringer bicarbonate solution from a reservoir 75 cm above the heart was begun immediately. The perfusion fluid contained NaCl (127.2 mM), KC1 (4.7 mM), CaCl2 (2.5 mM), KH2PO4 (1.2 mM) and NaHCO3 (24.9 mM). It was oxygenated with 95% O2 + 5% CO2 gas by means of an oxygenating device as described by Neely et al. (14) to ensure P02 values higher than 600 mmHg and kept at a temperature of 38°C. Sodium pyruvate (2 mM) and glucose (5.5 mM) were added to the perfusion fluid as substrates.

The latex balloon was introduced into the left ventricular cavity from the left atrium through the mitral valve. The left ventricular pressure was measured by a pressure transducer (Statham P50) connected to the balloon with a saline filled polyethylene tubing.

Heart rate (HR) was counted with a cardiotachometer triggered by pressure pulses of the left ventricular pressure (LVP). As a measure of the total mechanical energy required for contraction (9-11), the product of HR x LVP was calculated.

After a 40 min equilibration period, the global ischemia was induced by cross-clamping of the aortic inflow line, after which the hearts were reperfused for 40 min. The period of reperfusion was set at 40 min, for previous experiments conducted in this laboratory demonstrated that for the myocardial mechanical function to attain a steady level after a relatively long period of ischemia required for induction of the reperfusion-linked accumulation of calcium, it was necessary to reperfuse the heart for 40 min. Calcium antagonists were infused into the aortic inflow line at a speed of 0.1 ml/min with a syringe pump (Harvard 940) for 10 min prior to induction of global ischemia or for the entire period after resumption of perfusion. Prazosin was infused for 15 min prior to induction of ischemia and for 7 min after resumption of the perfusion.

In the preliminary section of this study, myocardial "tissue calcium" was measured without any previous complicated treatment of the preparation. The hearts cut off the perfusion cannula at the end of the reperfusion period were blotted with filter papers to remove surface fluid, dried overnight at a temperature of 80°C and weighed. After extraction from 10 mg of dried tissue with the method developed by Sparrow and Johnstone (15), calcium was determined with an atomic absorption spectrometer (Hitachi 180-30). The calcium that is measured under these conditions is referred to as "tissue calcium" in this paper.

In the main part of this study, the hearts were quickly perfused with 10 ml of ice-cold calcium-free solution containing 0.35 M sucrose and 5 mM histidine (pH 7.4) to minimize the contamination by extracellular calcium (16). The flushing solution was prepared using Dowex 50-W cation exchange resin to remove contaminant calcium. After washout, the hearts were blotted and then dried at 100°C to constant weight. Calcium was extracted and measured as described above. The calcium which is measured under these conditions is referred to as "cell calcium".
For determination of cyclic AMP, the hearts were frozen with a Wollenberger tongue precooled in liquid nitrogen. The frozen tissue was pulverized in a percussion mortar at liquid nitrogen temperature and homogenized with a Polytron (PT-20) in 0.1 N HCl (at maximal speed, 25 sec x2). The homogenate was heated at 100°C for 3 min and centrifuged (3,000 r.p.m. 15 min), and the cyclic AMP in the supernatant was determined with a radioimmunoassay kit (Yamasa Shoyu, Noda).

Drugs used were nicardipine (Bayer), diltiazem (Tanabe) and prazosin (Taito-Pfizer).

Results are presented as the mean±S.E.M. of n experiments. The significance of difference was determined using the unpaired Student’s t-test or the Aspin-Welch test, whichever appropriate, with P<0.05 as the limit of significance.

Results

1) Preliminary experiments on myocardial “tissue calcium”

In order to determine the length of global ischemia necessary for induction of accumulation of calcium within the myocardium upon reperfusion, the isolated perfused guinea pig hearts were subjected to global ischemia of various lengths and reperfused.

Figure 1 depicts the relation between the duration of ischemia and the amount of “tissue calcium” accumulated after reperfusion of 40 min. As is evident from this figure, the accumulation of calcium within the myocardium after reperfusion did not occur with ischemia of shorter than 20 min, while the reperfusion after ischemia of 40 min was associated with a significant accumulation of calcium within the myocardium. Thus, in the following experiments, the length of ischemia was set at 40 min. On reperfusion accumulation of calcium progressed gradually attaining a steady level beyond 20 min.

2) Experiments on myocardial “cell calcium”

In the following experiments, myocardial “cell calcium” was measured instead of “tissue calcium”.

Effects of calcium antagonists and prazosin on the accumulation of myocardial “cell calcium”: As shown in Fig. 2, myocardial “cell calcium” concentration of the isolated perfused guinea pig heart (perfused for 120 min with normal oxygenated perfusate) was 7.43±0.56 μmole/g dry weight (n=6). Ischemia for 40 min did not result in a change in this concentration, the value being 7.28±0.27 (n=6), while 40 min reperfusion after 40 min ischemia resulted in a significant increase in “cell calcium” to 12.18±0.84 (n=10). Prazosin as well as diltiazem suppressed this increase in calcium in a dose-dependent manner (Fig. 2). Nicardipine suppressed the calcium accumulation at lower doses, but the inhibitory effect was not observed at a higher dose (Fig. 2). In contrast, all three compounds failed to produce such effects when given during the period of reperfusion (Table 1).

Effects of calcium antagonists and prazosin on the myocardial mechanical function and oxygen demand: Figure 3 depicts the percent changes produced by the two calcium antagonists and prazosin in the product of heart rate (HR) and left ventricular developed pressure (LVP). As will be discussed later in the “Discussion” section, HR×LVP is a reliable predictor of the
Fig. 2. Effects of prazosin, diltiazem and nicardipine on the accumulation of "cell calcium" in the ischemic reperfused myocardium. Values (mean±S.E.M.) represent 6-10 hearts per group. Time of ischemia: 40 min. Time of reperfusion: 40 min. Normal: perfused for 120 min with normal oxygenated Krebs-Ringer bicarbonate solution. Ischemia: at the end of ischemia of 40 min. Control: reperfusion for 40 min after ischemia of 40 min without treatment of drugs. All the other data: reperfusion for 40 min after ischemia of 40 min. Diltiazem and nicardipine were infused for 10 min prior to induction of ischemia. Prazosin was infused for 15 min prior to induction of ischemia and for 7 min after resumption of perfusion. *P<0.05, **P<0.01 vs. control. †P<0.05, †P<0.01 vs. normal.

| Ca²⁺ μmol/g dry tissue | Ca²⁺ μmol/g dry tissue |
|------------------------|------------------------|
| **Control**            | 10.83±1.11             | **Control**            | 11.23±0.45             |
| **Prazosin**           |                        | **Nicardipine**        |                        |
| 3x10⁻⁸ M               | 10.20±0.85             | 10⁻⁶ M                 | 11.32±0.87             |
| 3x10⁻⁷ M               | 10.54±0.57             | 10⁻⁷ M                 | 11.01±0.74             |
| 3x10⁻⁶ M               | 10.71±0.38             | 10⁻⁸ M                 | 11.07±0.57             |
| **Diltiazem**          |                        |                        |                        |
| 10⁻⁶ M                 | 10.82±1.07             |                        |                        |
Fig. 3. Effects of diltiazem, nicardipine and prazosin infused before induction of ischemia on the product of heart rate (HR) × left ventricular developed pressure (LVP) calculated as a measure of the total mechanical energy required for contraction and expressed as percent of the value just before administration of the drugs. Each point represents the mean ± S.E.M. (n=5–6).

Relation between the changes in HR × LVP just prior to induction of ischemia and the accumulation of myocardial cell calcium after reperfusion of 40 min: As shown in Fig. 4, a close relationship was observed between the mean levels of HR × LVP just prior to induction of ischemia and the mean concentrations of "cell calcium" at the end of 40 minutes' reperfusion after 40 minutes' global ischemia, except for the data obtained with the highest concentration of nicardipine, indicating that the suppression of calcium accumulation produced by these compounds was mainly due to the decrease in the total mechanical energy required for contraction attributable in these cases to the depression of the left ventricular developed pressure.

Effects of calcium antagonists on the tissue cyclic AMP content: In order to find out the reason why the calcium accumulation after
the highest concentration of nicardipine was not correlated with HR × LVP, the effects of this substance on the myocardial tissue cyclic AMP content were examined, for it was demonstrated that calcium antagonists of the dihydropyridine type were capable of inhibiting cyclic nucleotide phosphodiesterase (17, 18). In Table 2 are listed the cyclic AMP content of the myocardium reperfused for 40 min after ischemia of 40 min, and the effects of nicardipine and diltiazem. Nicardipine as well as diltiazem did not produce any effect on the cyclic AMP content of the myocardium.

Discussion

In the present study it was demonstrated that calcium antagonists, when administered prior to induction of ischemia, could produce a dose-related inhibition of the accumulation of calcium within the myocardium subjected to reperfusion after relatively long period of ischemia of 40 min, while they were ineffective when administered simultaneously with the resumption of the perfusion. Protective effects of calcium antagonists administered prior to induction of ischemia against the accumulation of calcium upon reperfusion of the ischemic heart were repeatedly reported and were generally ascribed to the depression of the myocardial mechanical function (for references see “Introduction” section). However, Higgins and Blackburn (7) recently demonstrated that doses of calcium antagonists not producing a decrease in cardiac output given before induction of ischemic insult could produce a suppression of accumulation of calcium within the myocardium following ischemia and reperfusion. Therefore, in the present study, we attempted to define a factor(s) related to the mechanical function that determines the ischemia-reperfusion induced accumulation of calcium. A close correlation was found between the amount of calcium accumulated and the heart rate (HR) × left ventricular developed pressure (LVP) just prior to induction of ischemia. In an isovolumically
beating or a normally ejecting canine left ventricular preparation, Suga and his collaborators (9–11) have demonstrated that the area in the pressure-volume (PV) diagram which is circumscribed by the end-systolic PV relation line, the end-diastolic PV relation curve and the systolic segment of the PV loop trajectory, an area they designated as the systolic pressure-volume area (PVA), represents the total mechanical energy that the contracting ventricle requires to increase its wall's elastic state from the end-diastolic compliant level to the end-systolic stiff level and consists of the external mechanical work and the end-systolic potential energy. In isovolumic contractions in which the external mechanical work is zero, only the end-systolic potential energy term remains. In other words, PVA becomes proportional to systolic pressure. Thus, HR × LVP represents the total mechanical energy required for contraction per unit time under this condition. Therefore, the close correlation found in the present study between the amount of calcium accumulated upon reperfusion and HR × LVP before induction of ischemia indicates that the observed inhibition of the calcium accumulation is mainly ascribable to the reduction of the total mechanical energy required for contraction resulting from the inhibition of the myocardial mechanical function produced by calcium antagonists.

With the highest dose of nicardipine, the inhibition of the calcium accumulation was no longer observed, despite the stronger inhibition of the myocardial mechanical function and a larger decrease in HR × LVP. An inhibition of phosphodiesterase was reported with calcium antagonists of the dihydropyridine type (17–20), and an increase in the slow inward current was observed by Iijima et al. (21) by intracellularly applying nifedipine and nicardipine. Therefore, the amount of cyclic AMP within the myocardium was measured in order to see whether the failure of the highest dose of nicardipine to induce a suppression of the calcium accumulation was due to the accumulation of cyclic AMP and resultant increase in the slow inward current. No accumulation of cyclic AMP was found. Thus, the mechanism of reversal of action of nicardipine on the accumulation of calcium under these conditions remained obscure. Presumably the compound functioned as a calcium agonist during reperfusion, when a small amount still remained in the myocardium. Thomas et al. (22) reported on the mixed effects of calcium-antagonists of the dihydropyridine type; in the isolated perfused guinea pig heart, these compounds produced a small but definite positive inotropic effect at low concentrations, indicating a calcium agonistic action.

The attenuation of the calcium accumulation produced by prazosin agreed with the results obtained by Sharma et al. (13) and Nayler et al. (12). However, like those of Nayler et al., our results differed from those of Sharma et al. in that no protective effects were found when prazosin was given coincidently with reperfusion. As Nayler et al. stated, this discrepancy may have resulted from the differences in the experimental model and the experimental techniques. Nayler et al. found an enhancement of the reperfusion-induced gain in calcium with high doses of prazosin, which they ascribed to the release of catecholamine through inhibition of the presynaptic α-receptor and resultant activation of β-receptors. In our experiments, no such an enhancement was observed. According to Roach et al. (23), prazosin was equieffective as phentolamine in antagonizing the attenuation by clonidine of the tachycardia evoked by electrical stimulation of the cardiac sympathetic fibers in the rat, the species Nayler et al. used, while it failed to effectively inhibit the clonidine-induced attenuation in the dog and the cat. Such a species difference may explain the lack of an enhancement in our experiments, although we have at present no data as regards the presynaptic α-receptor of the guinea pig.

In the present study, prazosin produced negative inotropic and chronotropic effects. Nayler et al. (12) also found the depression of the developed tension (with no change in heart rate) after prazosin, albeit the effects were transient. As a mechanism of the negative inotropic effect, blockade of the myocardial α-adrenoceptor subserving the positive inotropic effect may be excluded. Although Karliner et al. (24) reported on the specific binding of 3H-prazosin to the guinea
pig myocardial membranes ($B_{\text{max}}$ of 58 fmole/mg protein and $K_b$ of 0.58 nM), and the existence of $\alpha$-adrenoceptors subserving the positive inotropic effects was demonstrated by Skomedal and Osnes (25) in the guinea pig papillary muscles with phenylephrine in the presence of propranolol, it was shown by Shibata et al. (26) that $\alpha_1$-adrenoceptors were of minor functional importance in the guinea pig ventricle. Furthermore, the concentrations of prazosin that produced a definite inhibition of the myocardial mechanical function in the present study were much higher than those necessary to produce a definite blockade of $\alpha_1$-receptor. They are almost equal to those necessary to produce definite stabilizing effects, i.e., $2 \times 10^{-6}$ M (27).

Anyway, the protective effects of prazosin against the reperfusion-induced gain in calcium was closely related with the decrease in the total mechanical energy required for contraction just prior to induction of ischemia. This finding combined with those obtained with calcium antagonists stress the importance of the total mechanical energy required for contraction at the induction of ischemia as a determinant factor in the accumulation of calcium after ischemia and reperfusion. Higgins and Blackburn (7) reported on the attenuation of calcium accumulation induced by doses of calcium antagonists producing no change in the cardiac output under normally perfused conditions. However, as has long been recognized (8), cardiac output is not a good indicator of the rate of energy expenditure. Moreover, as no data were given in Higgins and Blackburn’s paper on the changes in the parameters more closely related to energy expenditure, we could not compare their results with ours.

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