Role of protein phosphatases in hypoxic preconditioning

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Several studies (18, 25, 32) have demonstrated that protection of the myocardium against ischemia-reperfusion injury caused by a brief preceding ischemia (ischemic preconditioning) is due to the release and accumulation of endogenous mediators, such as adenosine or noradrenaline, during ischemic preconditioning and a subsequent activation of protein kinase C (PKC). There are, however, data (20, 23, 27) indicating that protection by ischemic preconditioning can also be achieved independent of PKC activation. The mechanism of this PKC-independent protection could not be explained. Brief periods of hypoxia, i.e., hypoxic preconditioning (HP), which do not allow accumulation of ischemic mediators, can also provide protection against ischemia-reperfusion injury (17, 19, 30, 32). Previous studies (14, 26) of isolated hearts demonstrated that protection induced by HP does not involve the activation of adenosine receptors or G proteins, suggesting a protective signaling different from well-known mechanisms of ischemic preconditioning. A protective role of PKC in HP was suggested for cultured embryonic cardiac myocytes (6, 29), but this was not demonstrated in adult cardiac myocytes or the adult whole heart. In general, the mechanisms of HP-induced protection are still not fully understood. For the present study, we hypothesized that HP leads to protection due to a PKC-independent mechanism. We (1) tested whether protection induced by HP is PKC independent and (3) which other type of signaling is responsible for HP-induced protection. Motivated by reports (2, 3) that inhibitors of these protein phosphatases (PP) can imitate ischemic preconditioning in some experimental models, we focused our study on the role of serine thereonine PP1 and PP2A.

We used the experimental model of isolated Langendorff-perfused rat hearts exposed to 60 min of global ischemia and 60 min of reperfusion. To test for implication of PKC we applied the PKC inhibitor bisindolylmaleimide (BIM), and to test for implication of PP1/PP2A we applied the PP inhibitors cantharidin or okadaic acid. To analyze the mechanism of HP protection on the cellular level, experiments were performed on isolated adult ventricular cardiomyocytes exposed to 60-min simulated ischemia (hypoxia at extracellular pH 6.4) and 20-min reoxygenation. Effects of HP on homeostasis of cations (Ca²⁺, Na⁺, and H⁺) and cell length were analyzed. We used cantharidin or okadaic acid to differentiate between the roles of PP1 and PP2A (9, 10). Cantharidin has an IC₅₀ for PP1 of 10⁻⁶ M, for PP2A of 10⁻⁷ M. To inhibit both isoforms we used 20 or 5 μM. Okadaic acid has an IC₅₀ for PP1 of 10⁻⁷ M, for PP2A of 10⁻⁹ M. To inhibit only PP2A we used 5 nM.

MATERIALS AND METHODS

The investigation conforms to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, Revised 1996).

Experiments on isolated perfused hearts. Hearts from adult male Wistar rats (250–300 g) were mounted on a Langendorff system in a temperature-controlled chamber (37°C) and perfused for a 20-min stabilization period at constant flow of 10 ml/min with solution A containing (in mmol/l) 120.0 NaCl, 3.5 KCl, 1.2 KH₂PO₄, 1.2 MgSO₄, 2.0 CaCl₂, 22.0 NaHCO₃ and 11.0 glucose, pH 7.35, at 37°C. The perfusate was saturated with 95% O₂-5% CO₂. These conditions are called normoxic standard perfusion. Left ventricular pressure was measured using a pressure transducer connected to a transducer gain converter (Häm-O-Mat 3050, Siemens, Erlangen, Germany). Left ventricular pressure was expressed as absolute values and as percent of baseline values. After 60 min of normoxic standard perfusion, hearts were exposed to 60 min of hypoxic standard perfusion, followed by 20 min of reoxygenation. Heart rate and left ventricular pressure were measured at 1.0-s intervals. Data are presented as means ± SE. The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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monitored with the use of a water-filled latex balloon connected to a pressure transducer. A second pressure transducer was used to monitor pressure in the aorta, i.e., the coronary perfusion pressure.

For ischemia-reperfusion controls, hearts were subjected for another 20 min to normoxic standard perfusion. This was followed by 60 min of global ischemia (37°C) and 60 min of normoxic standard perfusion (reperfusion). In the HP group, hearts were first subjected to 10 min of perfusion (10 ml/min) with glucose-free hypoxic solution A saturated with 95% N₂-5% CO₂, followed by 10 min of normoxic standard perfusion (HP). Thereafter, ischemia-reperfusion was performed as described earlier. Drugs (BIM, 1 μmol/l; cantharidin, 20 or 5 μmol/l; okadaic acid, 5 nmol/l) were applied alone or in combination during 20-min preischemic normoxia or 20-min preischemic HP protocol.

**Experiments on isolated cardiomyocytes.** Ventricular heart muscle cells were isolated from adult male Wistar rats (250–300 g) as previously described (21) and were used in experiments 5 to 10 h after being plated on glass coverslips. To measure cytosolic Ca²⁺, Na⁺, or H⁺ concentrations, cardiomyocytes were loaded in medium 199 at 35°C for 30 min with acetoxymethyl esters of 2.5 μmol/l fura 2, 5.0 μmol/l sodium-binding benzofuran isophthalate (SBFI), or 15 μmol/l 2’,7’-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF), respectively. After being loaded, cells were washed twice with medium 199, followed by incubation for 30 min to allow hydrolysis of the acetoxymethyl esters within the cell. The fluorescence from dye-loaded cells was 30–40 times higher than background fluorescence from unloaded cells. Dye compartmentation was assessed by using digitonin (14) and did not exceed 10% for fura 2, 15% for SBFI, and 12% for BCECF. Fluorescence ratios of fura 2, SBFI, and BCECF were calibrated with 5 μmol ionomycin (14), 6 μmol/l gramicidin D (8), and 10 μg/ml nigericin (13), respectively. Simultaneously to the fluorescence measurement, the microscopic image of the cells was recorded with a video camera. The extent of cell hypercontracture was expressed as a percentage, relating the cell shortening to cell length before anoxia.

Cardiomyocytes were superfused at a flow rate of 0.6 ml/min with solution B containing (in mmol/l) 140.0 NaCl, 2.6 KCl, 1.2 KH₂PO₄, 1.2 MgSO₄, 1.0 CaCl₂, 5.0 glucose, and 25.0 HEPES (pH 7.4, at 37°C). Normoxic medium was equilibrated with air. The medium was made anoxic by autoclaving as previously described (1). The anoxic medium was glucose-free and equilibrated with 100% N₂.

Under all protocols, cells were exposed to 60-min anoxia at pH 6.4 and 20-min reoxygenation at pH 7.4. To exclude PKC-dependent effects, all experiments were performed in the presence of 1 μmol/l BIM. For anoxia-reoxygenation controls, cells were superfused for 20 min with normoxic medium (pH 7.4). HP was induced by 10-min superfusion of cells with anoxic medium (pH 6.4), followed by 10 min of reoxygenation before sustained anoxia. PP inhibitors were applied during the initial 20 min of normoxic superfusion or 20-min HP protocol and during sustained anoxia.

**Statistics.** Data are given as means ± SE. For each experimental protocol, 20–40 individual cells were used, with not >6 cells from the same cell isolate. Comparisons between groups were performed by one- or two-way analysis of variance, followed by the Student-Newman-Keuls test where appropriate. Statistical significance was accepted when P < 0.05.

**RESULTS**

**Effect of HP in isolated hearts.** To produce HP, hearts were perfused for 10 min with hypoxic medium, followed by reoxygenation for another 10 min (20-min HP protocol) before 60 min of global ischemia. At the end of the hypoxic period, left ventricular developed pressure (LVdevP) was reduced to 39 ± 5% (n = 15, P < 0.05) of its normoxic control value, but contractile performance rapidly recovered thereafter. HP did not alter hemodynamic parameters immediately before 60 min of ischemia (not shown). In contrast, HP significantly improved postischemic recovery of left ventricular function. After 60 min of reperfusion, LVdevP was 40 ± 7 mmHg in HP-treated hearts (n = 9) versus 16 ± 5 mmHg in control hearts (n = 15, P < 0.05) (Fig. 1A). The rate-pressure product showed similar differences (Table 1). This was associated with reduced left ventricular end-diastolic pressure (LVEDP), i.e., contractile dysfunction in HP-treated hearts. After 60 min of reperfusion, LVEDP was 39 ± 6 mmHg after HP (n = 9) versus 59 ± 6 mmHg in non-HP controls (n = 15, P < 0.05) (Fig. 1B).

It was analyzed whether the protective effect of HP could be affected by PKC inhibition. For this purpose, PKC blockade with 1 μmol/l BIM was applied during the 20-min normoxic reperfusion or the 20-min HP protocol before ischemia. In either case, it had no
significant effect on the functional recovery of hearts during reperfusion (Fig. 1). We tested previously whether this concentration of BIM could abolish a PKC-dependent preconditioning effect. By following previous experiments (14), we exposed the cardiomyocytes to the PKC activator 1,2-dioctanoyl glycerol (DOG; 20 μmol/l) under normoxic conditions instead of the anoxic conditions in the HP protocol. DOG-treated cells showed less reoxygenation-induced hypercontracture than control cells not treated with DOG (11.8 ± 2.0% vs. 27.9 ± 2.1%; n = 29, P < 0.05). The copresence of BIM (1 μmol/l) during the DOG-preconditioning protocol abolished this protection of cells (hypercontracture: 28.4 ± 1.8% vs. 27.1 ± 3.0%; n = 34, not significant).

To analyze the role of PP in the protective effect of HP, hearts were treated during the 20-min preconditioning period (either normoxic perfusion or HP protocol) with 20 μmol/l cantharidin. This concentration is one order of magnitude above the IC50 of PP1 and two orders above the IC50 of PP2A, i.e., it blocks both isoforms. Because this represents a high-pharmacological concentration with the possibility of side effects, we also exposed the cardiomyocytes to the PKC activator 1,2-dioctanoyl glycerol (DOG; 20 μmol/l) under normoxic conditions instead of the anoxic conditions in the HP protocol. DOG-treated cells showed less reoxygenation-induced hypercontracture than control cells not treated with DOG (11.8 ± 2.0% vs. 27.9 ± 2.1%; n = 29, P < 0.05). The copresence of BIM (1 μmol/l) during the DOG-preconditioning protocol abolished this protection of cells (hypercontracture: 28.4 ± 1.8% vs. 27.1 ± 3.0%; n = 34, not significant).

Cantharidin treatments did not alter the transient depression of contractile performance during the HP period. On reperfusion, the elevation of aortic pressure was still apparent under treatment with 20 μmol/l cantharidin, but LVdevP was no longer higher but lower than in the ischemic-reperfused controls (Fig. 2A). This lowering of LVdevP was also apparent at 5 μmol/l (7.7 ± 2.2 vs. 17.3 ± 5.1 mmHg at 60-min reperfusion, n = 5, P < 0.05). More importantly, the enhanced contractile recovery in HP-treated hearts was abolished after cantharidin pretreatment. The development of contracture, i.e., LVEDP, in reperfused control hearts was not affected by pretreatment with cantharidin (Fig. 2B). The reduction of contracture seen after HP, however, was abolished in presence of cantharidin 20 or 5 μmol/l (with or without 5 μmol/l: 48.8 ± 4.7 vs. 38.6 ± 5.7 mmHg at 60-min reperfusion, n = 5, P < 0.005). The rate-pressure product of the postischemic hearts could not be determined because the cardiac excursions were too small to allow undisturbed monitoring of the beating frequency (Table 1).

To further analyze the role of PPs, treatment with 5 nmol/l okadaic acid was combined with the HP proto-

Table 1. Developed pressure, heart rate, aortic pressure, and rate pressure product in ischemic-reperfused hearts

<table>
<thead>
<tr>
<th></th>
<th>LVdevP, mmHg</th>
<th>Heart Rate, beats/min</th>
<th>LVdevP × Heart Rate/1,000, mmHg/min</th>
<th>Aortic Pressure, mmHg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Preischemia</td>
<td>60-min</td>
<td>postischemia</td>
</tr>
<tr>
<td>Control HP</td>
<td>15</td>
<td>71.0 ± 4.0</td>
<td>165.5</td>
<td>4.7</td>
</tr>
<tr>
<td>HP</td>
<td>9</td>
<td>67.4 ± 6.8</td>
<td>40.2</td>
<td>6.8</td>
</tr>
<tr>
<td>BIM</td>
<td>5</td>
<td>69.7 ± 5.9</td>
<td>13.0</td>
<td>3.1</td>
</tr>
<tr>
<td>HP + BIM</td>
<td>5</td>
<td>68.0 ± 8.5</td>
<td>42.8</td>
<td>6.1</td>
</tr>
<tr>
<td>Cantharidin</td>
<td>6</td>
<td>82.6 ± 4.9</td>
<td>7.0</td>
<td>1.8</td>
</tr>
<tr>
<td>HP + cantharidin</td>
<td>5</td>
<td>84.0 ± 5.0</td>
<td>5.0</td>
<td>1.5</td>
</tr>
<tr>
<td>OA</td>
<td>5</td>
<td>66.0 ± 6.7</td>
<td>20.0</td>
<td>7.0</td>
</tr>
<tr>
<td>HP + OA</td>
<td>5</td>
<td>74.0 ± 6.0</td>
<td>58.0</td>
<td>5.0</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of hearts. LVdevP, left ventricular developed pressure; HP, hypoxic preconditioning; BIM, bisindolylmaleimide; OA, aortic pressure; ND, not determined. Data represent values taken during the last minute before ischemia and values at 60 min of reperfusion. *P < 0.05 vs. control; †P < 0.05 vs. HP.
col. At this concentration, okadaic acid has been shown to selectively inhibit PP2A but not PP1 (9, 10). In contrast to cantharidin, okadaic acid led to improved functional recovery of the reperfused HP-treated hearts; LVdevP and the rate-pressure product were significantly increased (Fig. 3A, Table 1). No significant changes were seen for LVEDP (Fig. 3B). In normoxic controls, treatment with 5 mmol/l okadaic acid had no effect. The transient contractile depression during the HP period and the rapid return to control values were the same as in HP protocols without okadaic acid. Aortic pressure was not increased in the presence of okadaic acid. In summary, the experiments showed that the HP protocol provides protection in a PKC-independent but PP-dependent manner.

Effects of HP in isolated cardiomyocytes. To analyze the cellular mechanism of HP-induced PKC-independent protection, cytosolic ion homeostasis (Ca\(^{2+}\), Na\(^{+}\), pH) and cell length were investigated in isolated cardiomyocytes exposed to simulated ischemia (anoxia at pH 6.4). For this purpose, all experiments with isolated cardiomyocytes were performed in the presence of 1 mmol/l BIM. In the control group, a 60-min superfusion of cardiomyocytes with anoxic glucose-free medium at pH 6.4 (simulated ischemia) caused an accumulation of Ca\(^{2+}\) in the cytosol as indicated by an increase in the fura 2 ratio (Fig. 4). Calibration of the fura 2 ratio showed that cytosolic intracellular Ca\(^{2+}\) (Cai) concentration was 2.14 ± 0.09 mmol/l (n = 18) at the end of anoxia. The HP protocol was simulated by preexposure of the cells to 10 min of these anoxic conditions, followed by 10 min of normoxia. HP significantly reduced Cai overload at the end of sustained anoxia (Cai: 0.75 ± 0.06 mmol/l, n = 37, P < 0.05 vs. control). Treatment with 20 mmol/l cantharidin or 5 mmol/l okadaic acid, 20 min before and 60 min during anoxia. Treatment with cantharidin completely abolished the protective effect of HP against Cai overload, but treatment with okadaic acid further enhanced the reduction of Cai overload by HP (Cai: 0.55 ± 0.05 mmol/l, n = 18, P < 0.05 vs. HP). Treatment of non-HP control cells with cantharidin or okadaic acid had no effects on Cai overload during sustained anoxia.

Accumulation of cytosolic intracellular Na\(^{+}\) (Na\(_i\)) can play an important role for Cai overload through the activation of the reverse mode of the Na\(^{+}\)/Ca\(^{2+}\) exchanger in anoxic or ischemic myocardial cells (16). To analyze whether an alteration in the Na\(_i\) accumulation is the cause of the HP effect on Cai overload, Na\(_i\) concentration was monitored by the SBFI fluorescence ratio. Under control conditions, the SBFI ratio rose during 60 min of anoxia from 1 to 1.45 (Fig. 5), indicating an increase in Na\(_i\) concentration from 5.0 ± 0.7 to 8.5 ± 6 mmol/l (n = 38). HP significantly reduced the rise of the SBFI ratio. This corresponds with a reduction of Na\(_i\) overload under this condition to 41 ± 6 mmol/l, n = 39, P < 0.05 vs. control. Treatment with 20

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Fig. 3. A: LVdevP before 60 min of global ischemia (C) and during reperfusion. B: LVEDP before 60 min of global ischemia and during reperfusion. The following protocols are shown: control, ● (n = 15); control + okadaic acid, ■ (n = 5); HP, ○ (n = 9); HP + okadaic acid, □ (n = 5). Data are means ± SE. *P < 0.05 vs. control; #P < 0.05 vs. HP.

Fig. 4. Fura 2 ratio (arbitrary units) after 60-min anoxia in control (solid bars) and HP-treated (open bars) cardiomyocytes. Under all protocols, treatment with 1 μmol/l BIM was applied. The following experimental protocols are shown: control (n = 18); control + cantharidin (n = 17); control + okadaic acid (n = 25); HP (n = 37); HP + cantharidin (n = 27); HP + okadaic acid (n = 18). The initial value of fura 2 ratio was equal to 0.47. Data are means ± SE. *P < 0.05 vs. control; #P < 0.05 vs. HP.
μmol/l cantharidin abolished this reduction of Na\textsubscript{i} overload at the end of anaoxia (Na\textsubscript{i}: 80 ± 7 mmol/l, n = 27, P > 0.05 vs. control). In contrast to cantharidin, treatment with 5 nmol/l okadaic acid enhanced the HP-induced protection against Na\textsubscript{i} overload. Treatment of non-HP control cells with cantharidin or okadaic acid had no effects on Na\textsubscript{i} overload during sustained anoxia.

In the present study, hypoxia was combined with low extracellular pH (6.4) to simulate ischemic acidosis. Under these conditions, cytosolic pH (pH\textsubscript{i}) was reduced from 7.15 ± 0.02 to 6.47 ± 0.02 (n = 42) in control cells during 60 min of anoxic incubation. HP alone (6.49 ± 0.03, n = 29) or in combination with cantharidin (6.45 ± 0.04, n = 21) or okadaic acid (6.50 ± 0.03, n = 19) did not affect pH\textsubscript{i} at the end of anoxia. During reoxygenation, pH\textsubscript{i} recovered to the preanoxic level with similar rapidity under all experimental conditions.

It has been shown previously (15) that the degree of cytosolic Ca\textsuperscript{2+} overload is an important determinant for the reoxygenation-induced hypercontracture of isolated cardiomyocytes. Similarly to this effect on Ca\textsuperscript{2+} overload, HP produced a significant reduction of reoxygenation-induced hypercontracture. Hypercontracture was 10.1 ± 1.1% (n = 37) vs. 27.4 ± 1.8% (n = 18, P < 0.05) in ischemic-reoxygenated control cells. The protection by HP could be abolished by treating the cells with cantharidin (25.5 ± 1.5%, n = 27) but not with okadaic acid (8.5 ± 1.1%, n = 18, P < 0.05 vs. control). Treatment of ischemic-reoxygenated control cells with cantharidin or okadaic acid had no effect on hypercontracture during reoxygenation.

**DISCUSSION**

There were several findings of this study. First, in isolated ischemic-reperfused hearts, HP caused a significant improvement of functional recovery and a reduction of contracture development during reperfusion. This effect was not altered by PKC inhibition. Second, the protective effect could be abolished by the presence of cantharidin, an inhibitor of PP1 and PP2A. The result of experiments with a low dose of okadaic acid suggests that PP1 plays a beneficial role in HP-mediated protection. Third, in isolated cardiomyocytes HP provided PKC-independent protection against Na\textsubscript{i} and Ca\textsubscript{2+} overload during simulated ischemia and against hypercontracture during reoxygenation. This protection had the same responsiveness to PP inhibitors as HP protection in whole hearts.

Ischemic preconditioning represents a powerful procedure to protect ischemic-reperfused myocardium in most investigated animal species as well as in humans. It is now clear that numerous triggers and mediators can elicit this protective mechanism. It is still not clear whether all forms of protection elicited by an ischemic-preconditioning protocol have a common final end point (effector) within the protective signaling. As mentioned above, in some models ischemic preconditioning provides protection independent of PKC and exchanging preconditioning ischemia for brief hypoxic perfusion can also provide protection. We found in a rat model that hypoxic preconditioning provides protection independent of PKC inhibition. To our knowledge others have not investigated this aspect of HP in a rat model. In mouse neonatal cell cultures, HP protection was found to be PKC dependent (6), but this may be due to species or developmental differences. In rabbit cardiomyocytes and hearts, Armstrong et al. (3) reported that application of a PP2A inhibitor provides protection in a PKC-independent manner. Therefore, we tested the role of PP1/PP2A using appropriate inhibitors. Inhibition of PP1/PP2A with a dose of cantharidin (20 μmol/l) well above either of the respective IC\textsubscript{50}, abolished HP protection. Inhibition of PP2A with a dose of okadaic acid two orders below the IC\textsubscript{50} of PP1 but above that of PP2A augmented the protective effects of HP on LVdevP and the rate-pressure product in reperfused hearts. These observations were made in hearts as well as in cardiomyocytes. They indicate that PP1 activation represents a mediator of HP protection in this rat model, whereas PP2A activation is adverse to this beneficial effect. In this latter aspect, our results resemble the findings of Armstrong et al. (2, 3). In contrast to their observations, however, we did not see a protective effect of PP2A inhibition alone. The difference may be related to differences in model ischemia (dense cell pellet vs. cells superfused with anoxic acidic medium) or in species (rabbit or pig vs. rat).

A word of caution seems appropriate: as in all studies that are based on the use of pharmacological inhibitors, our experiments and those described by Armstrong’s group (2, 3) could be affected by unknown side effects of these inhibitors. This consideration may ap-
ply particularly to the use of cantharidin at a concentration (20 μmol/l) well above the IC50 of PP1 and PP2A. Therefore, we also performed experiments with 5 μmol/l. Under normoxic conditions, 20 μmol/l cantharidin also affected contractile performance and coronary resistance, as previously described (12). These hemodynamic effects, however, are not accountable for the HP-antagonistic effects of cantharidin. First, because hearts were perfused at a constant flow rate, an increase in coronary resistance did not infringe on coronary flow. Second, the increase in contractility observed before ischemia cannot explain the reduction of contractile recovery in the reperfused hearts because these effects are opposite. Third, these preschismic hemodynamic effects were not seen with 5 μmol/l cantharidin. Fourth, the finding that cantharidin interferes with HP protection also in isolated cells provides another argument that its effect on HP protection is not due to its hemodynamic side effects. Okadaic acid (5 nmol/l) had no such side effect on normoxic hemodynamics.

To analyze the downstream cellular mechanisms of PKC-independent HP protection in isolated hearts, a model of isolated cardiomyocytes was used. Cells were exposed to simulated ischemic conditions (anoxia, extracellular pH 6.4) and subsequent reoxygenation. This model was characterized before in detail (14–16). The alteration of ion homeostasis, especially Ca2+ overload, during ischemia was shown to play an important role in the development of ischemic injury of hearts (26). The effect of HP on Ca2+ homeostasis in isolated cardiomyocytes during simulated ischemia was analyzed under conditions excluding the role of PKC, i.e., in the presence of the PKC inhibitor BIM. HP led to a significant reduction of anoxic Ca2+ overload. The mechanism of this HP-induced protection in isolated cardiomyocytes seems similar to that found in whole hearts. Treatment with cantharidin completely abolished protection in either model, whereas treatment with okadaic acid partly augmented it. Taken together, these data indicate that attenuation of Ca2+ overload in cardiomyocytes represents the cellular basis of the HP-induced improvement of postischemic heart function.

An important consequence of Ca2+ overload is the development of hypercontracture in reoxygenated cardiomyocytes (15). Similar to Ca2+ overload, hypercontracture was reduced by HP in a PP-sensitive manner. In whole hearts, hypercontracture of cardiomyocytes seems to be the basis for the observed rise of LVEDP during reperfusion (4). Indeed, in the present study, the effects of HP with or without cantharidin or okadaic acid treatment were similar in respect to LVEDP in whole hearts and hypercontracture in cardiomyocytes.

Koop and Piper (13) showed that the main cause for Ca2+ accumulation in metabolically inhibited cardiomyocytes consists in the influx of extracellular Ca2+ through the reverse mode of the Na+/Ca2+ exchanger. The rise of Na+ is one of the driving forces for the activation of the reverse mode of Na+/Ca2+ exchanger in cardiomyocytes (5, 28). Consistent with this causal sequence is the finding that the changes in Na+ overload are perfectly matched with changes in Ca2+ overload under corresponding experimental conditions. These data indicate that the mechanism leading to Na+ overload is indeed the primary target of HP-induced action. The mechanism of ischemic Na+ overload is not yet fully understood. It has been speculated that the noninactivating Na+ current (also called sustained or persistent Na+ current) is responsible for Na+ overload under ischemic conditions (7, 11). Recent studies (24) demonstrated that the Na+ channel represents a target molecule for PP1. It may, therefore, be suggested that the observed changes in Na+ overload in HP-treated cardiomyocytes under phosphatase inhibition are due to a change in the phosphorylation of Na+ channels.

It is noteworthy that the phosphatase-dependent hypoxic preconditioning effect is found in the same experimental model of isolated cardiomyocytes from the adult rat as a previously described PKC-dependent mechanism of protection (15). It shows that these cells contain multiple pathways that may confer signaling of ischemic preconditioning.

In conclusion, the results of this study indicate that HP provides protection of isolated hearts and isolated cardiomyocytes against ischemic injury by a mechanism endogenously antagonized by PP2A activation and supported by activation of PP1. It may, therefore, be suggested that PP are targets for new strategies to protect the ischemic-reperfused heart. Because hypoxia-reoxygenation transition is also part of the protocol of ischemic preconditioning, it may be supposed that the described mechanism of protection is contained within the complex protective mechanism of ischemic preconditioning. It is possibly responsible for the finding in some biological models that ischemic preconditioning can provide myocardial protection independent of PKC activation (20, 21, 23, 27).

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REFERENCES


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