Biochemical mechanism(s) of stunning in conscious dogs

HARTMUT LÜSS,1 ANDREAS MEISSNER,2 NORBERT ROLF,2 HUGO VAN AKEN,2 PETER BOKNIK,1 UWE KIRCHHEFER,1 JÖRG KNAPP,1 STEPHANIE LÄER,3 BETTINA LINCK,1 IVA LÜSS,1 FRANK U. MÜLLER,1 JOACHIM NEUMANN,1 AND WILHELM SCHMITZ1

1Institut für Pharmakologie und Toxikologie; 2Klinik und Poliklinik für Anästhesiologie und operative Intensivmedizin, Universität Münster, D-48149 Münster; and 3Institut für Pharmakologie, Universität-Krankenhaus Eppendorf, D-2024 Hamburg, Germany.

Received 20 August 1999; accepted in final form 4 January 2000

Biochemical mechanism(s) of stunning in conscious dogs. Am J Physiol Heart Circ Physiol 279: H176–H184, 2000.—The mechanism(s) underlying contractile dysfunction in cardiac stunning is not completely understood. The expression and/or the phosphorylation state of cardiac Ca2+ homeostasis-regulating proteins might be altered in stunning. We tested this hypothesis in a well-characterized model of stunning. Conscious dogs were chronically instrumented, and the left anterior descending artery (LAD) was occluded for 50 min. Thereafter, reperfusion of the LAD was initiated. Tissues from reperfused LAD (stunned) and Ramus circumflexus (control) areas were obtained when left ventricular regional wall thickening fraction had recovered by 50%. Northern and Western blotting revealed no differences in the expression of the following genes: phospholamban, calsequestrin, sarco(endo)plasmic reticulum Ca2+-ATPase 2a, and the inhibitory subunit of troponin I (TnI). However, the phosphorylation state of phospholamban and TnI remained depressed for more than 3 h (22). Others (32, 44) demonstrated that stunning in the open-chest dog can lead to biochemical alterations not related to ischemia itself, supporting the view that ischemia in conscious animals may be physiologically more relevant. Therefore, we performed the present study in conscious dogs.

From a variety of models, several hypotheses have been set forward to explain stunning (for review see Refs. 5, 9, 20). Although stunned myocardium is characterized by a number of metabolic alterations, there is no evidence that an impairment of oxidative phosphorylation, energy transport, or energy utilization are the cause for stunning (for review see Ref. 9). Moreover, it is unlikely that vascular abnormalities contribute to stunning, because coronary occlusion for 5–10 min leads to stunning despite an unaltered vascular function (for review see Ref. 9). Several biochemical and pharmacological studies implied the formation of free radicals as a possible mechanism (3, 4, 40). The duration and severity of ischemia determine in part the magnitude of free radical generation. The source of the radicals (at least in humans) is unclear (for review see Ref. 9). Generation of nitric oxide may also be involved (18). These reactive compounds might impair the function of regulatory proteins like the contractile proteins or the proteins in the sarcoplasmic reticulum (SR) directly. The formation of free radicals may explain why ischemia-reperfusion can induce the expression of chaperone proteins like heat shock proteins (28). Activity of the renin-angiotensin system is increased during ischemia (12). Addition of angiotensin-converting enzyme inhibitors reduces stunning (10). Therefore, angiotensin-converting enzyme by degrading bradykinin may contribute to stunning (10).

In addition, altered cAMP levels or an altered inositol 1,4,5-trisphosphate [Ins(1,4,5)P3] pathway may cause myocardial dysfunction (37, 42). Altered Ca2+ handling might also be involved. Ca2+ uptake into the SR, which is brought about by the sarco(endo)plasmic reticulum Ca2+ pump (SERCA), was impaired in glo-
bally ischemic hearts (29). In isolated perfused hearts from small laboratory animals, \( \text{Ca}^{2+} \) overload occurred at the beginning of reperfusion after global ischemia before \( \text{Ca}^{2+} \) returned to normal levels even though stunning persisted (30). This has led to the conclusion that a defect distal to cytosolic \( \text{Ca}^{2+} \) levels exists in stunning. There is evidence for a decreased \( \text{Ca}^{2+} \) sensitivity of the myofilaments (17). However, others failed to detect a decrease in \( \text{Ca}^{2+} \) sensitivity or a decrease in the maximum \( \text{Ca}^{2+} \)-activated force (21, 23). This was explained by different protocols, different parameters to assess myocardial function, and species differences (9).

The transient \( \text{Ca}^{2+} \) overload at the beginning of reperfusion can lead to biochemical alterations of myocardial proteins: \( \text{Ca}^{2+} \) can activate proteases (calpain I) and may thereby lead to the proteolysis of various cardiac proteins (46), notably of the inhibitory subunit of troponin I (TnI) (14). Because the phosphorylation of TnI hastens relaxation (11, 47), it is conceivable that proteolysis of TnI impairs relaxation, which is a hallmark of stunning.

However, in the conscious dog model, which bears physiological relevance, no biochemical data on the putative mechanism(s) of stunning are currently available. Hence, in the present study we started to test whether alterations in the expression or posttranslational modifications of \( \text{Ca}^{2+} \) regulatory proteins occur in regional stunning, which may contribute to stunning.

MATERIALS AND METHODS

Chronic instrumentation and monitoring. The experimental protocol was approved by the local animal welfare committee. Mongrel dogs (either sex, weight 20–26 kg) were injected intramuscularly with 15 mg piritramide and 5 mg/kg ketamine. Animals were anesthetized intravenously with 10 mg/kg propofol and 0.01 mg/kg fentanyl. After tracheal intubation anesthesia was maintained with enflurane in a mixture of oxygen in air. Cefamandole (30 mg/kg) was given as a prophylactic perioperative antibiotic. Details of the instrumentation have been described earlier (39, 41). Briefly, a left thoracotomy in the fifth intercostal space was performed under aseptic conditions. Eighteen-gauge catheters (Tygon, Serpi-Erpe, Wilsele, Belgium) were inserted into the descending aorta and the left atrium for measurement of pressures and withdrawal of blood. A pressure microtransducer (Janssen Pharmaceutica, Beerse, Belgium) was inserted into the left ventricle through an apical stab wound for measurement of left ventricular pressure (LVP) and rate of increment of LVP (LV dp/dt) (48). Pulsed Doppler blood flow velocity probes (20 MHz, Baylor College of Medicine, Houston, TX) were fitted around the left anterior descending (LAD) and the left circumflex (Ramus circumflexus, RCX) coronary arteries. For measurement of regional myocardial wall thickening fraction (WTF), 10-MHz pulsed Doppler crystals (Baylor College of Medicine) were placed on the myocardium of the LAD- and RCX-perfused areas. Proximal to the Doppler flow probe, a pneumatic occluder (Dimed, Belgium) was positioned around the LAD for induction of reversible ischemic episodes in the LAD-perfused myocardium. After the thorax was closed, all leads were tunneled subcutaneously and exited the body between the scapulae. After instrumentation, the animals were trained daily to get accustomed to the experimental environment and to lie quietly in a cage when connected to the data acquisition system. Aortic and left atrial pressures were measured by disposable pressure transducers (PVB Medizintechnik, Kirchseeon, Germany). Pressures, flow velocities, and wall thickening signals were processed by a six-channel pulsed Doppler system (Baylor College of Medicine). WTF was measured in the LAD and RCX regions as follows: The start of systole was taken as the onset of the development of LVP, and the end of cardiac systole was taken as coinciding with the maximum rate of decline of LVP. Systolic wall thickening was defined as the maximum systolic increase in wall thickness from its end-diastolic baseline and expressed as percent systolic thickening fraction according to the formula: \( \text{WTF} = 100 \times S/R \), where S is systolic excursion of the myocardial wall and R is the range-gated sample volume depth in millimeters. Systolic excursion was determined by integrating the velocity of myocardial layers passing through a range-gate sample volume, which was positioned at a depth less than the end-diastolic thickness of the left ventricular wall. This procedure has been validated previously (48). The left ventricular micromanometer was calibrated to the pressures measured in the aorta and left atrium. The LVP signal was electronically differentiated (Gould, Cleveland, OH). All signals were recorded on an eight-channel thermal writing polygraph (Gould). Experiments were only performed after the animals had completely recovered from the instrumentation and when normal blood gas values and hemodynamic variables were obtained. The experiments were performed 7 days after instrumentation. Animals were not adapted to ischemia by several occlusions of the LAD because we wanted to exclude preconditioning (8). After measurement of baseline values, LAD ischemia was induced for 10 min. No drugs were given during ischemia. During reperfusion, WTF was followed until 50% recovery occurred compared with baseline values. We studied 50% of WTF, because we hypothesized that biochemical alterations should be still detectable at this point. We did not choose a constant time after reperfusion for biochemical measurements, because we wanted to compare biochemical alterations at a similar extent of decrease in regional contractile function in all animals. When this 50% recovery level was reached, animals were anesthetized with 300 mg propofol, 0.5 mg fentanyl, and 15 mg midazolam. The trachea was intubated, and the dogs were ventilated with 100% oxygen. A parasternal thoracotomy was performed, the heart was excised, and samples from the LAD- and RCX-perfused territories were frozen in liquid nitrogen.

Obviously we cannot exclude that anesthesia alters biochemical parameters in stunning. It is even conceivable that the alterations would be different in postischemic tissue compared with nonischemic tissue. However, tissue harvesting was not possible without anesthesia.

Analysis of mRNA and Northern blotting. Total RNA was extracted from the frozen samples from LAD- and RCX-perfused areas as previously described (15). Samples were homogenized using a microdismembrator (B. Braun Melsungen, Melsungen, Germany) in 1 ml of TriStar-Reagent (AGS, Heidelberg, Germany) containing guanidinium thiocyanate and phenol. Total RNA was extracted in the following way: to 800 \( \mu \)l of homogenate 200 \( \mu \)l of chloroform were added, and the resulting two phases were separated by centrifugation. The RNA present in the top aqueous phase was precipitated by 10.220.33.3 on November 9, 2017 http://ajpheart.physiology.org/ Downloaded from
The plasmid (pGEM) with cDNA inserts for dog phospholamban has been described previously (1). This insert was isolated by digestion with EcoRI and purified from 1.5% agarose gels. The size was about 610 bp for the dog phospholamban probe. Other cDNA probes were constructed by RT-PCR. First-strand cDNA was reverse transcribed from 1 μl of total dog ventricle RNA in 10 μl of 50 mM Tris·HCl (pH 8.3), 40 mM KCl, 6.0 mM MgCl₂, 1.0 mM each dNTP (Pharmacia, Uppsala, Sweden), 5.0 μl of dithiothreitol, 50 μg/ml BSA, 10 units of human placental RNAase inhibitor (AGS), and 90 units of TrueScript reverse transcriptase (AGS) at 41°C for 60 min. Primers (Table 1) for SERCA were designed based on the published dog cDNA sequence (1). Probes for cardiac calsequestrin and cardiac inhibitory subunit of TnI were generated by cross species RT-PCR (Table 1). All PCR reactions were carried out in a total volume of 50 μl containing 20 mM Tris·HCl (pH 8.55 at 25°C), 16 mM (NH₄)₂SO₄, 200 μM each dNTP, 1.5–2.0 mM MgCl₂, and 1.5 units Taq DNA polymerase (AGS). Each reaction was subjected to 35 cycles of denaturation (1 min at 94°C), annealing (2 min), and extension (2 min, 72°C). All PCR reactions were performed in a thermal cycler (model TR3 CM220, Omigene, Ebersberg, Germany). Sizes of PCR products were compared with DNA size markers (MBI Fermentas, Vilnius, Latvia). MgCl₂ titration curves were performed with each primers to optimize amplification specificity. Single bands of the expected size were obtained. PCR products were visualized on 2% agarose gels, cut out, purified by dialysis, and used as probes in Northern blots. To confirm the identity of PCR products, cycle sequencing using AmpliTaq-FS DNA polymerase (Applied Biosystems, Weiterstadt, Germany) and an ABI PRISM-310 automated sequencer (Applied Biosystems) was performed.

For Northern blots, total RNAs (20 μg) were separated on 1% denaturing agarose gels and transferred to nylon membranes (Amersham Buchler, Braunschweig, Germany) by capillary transfer in 20% saline sodium citrate (SSC). After prehybridization in a solution containing 50% deionized formamide, 5× Denhardt solution, 0.9 M NaCl, 60 mM NaH₂PO₄, 6.0 mM EDTA, 0.2 mg/ml tRNA from yeast, and 0.1% SDS at pH 7.4, membranes were hybridized overnight at 42°C in the same buffer containing the probes, which were labeled with [α-³²P]dCTP (NEN DuPont, Bad Homburg, Germany) by random priming (Megaprime-kit, Amersham Buchler). Hybridized membranes were washed at a stringency of 0.2× SSC and 0.1% SDS at 60°C, exposed to Kodak screens, visualized in a PhosphorImager, and quantified by the ImageQuant software version 3.3 (Molecular Dynamics, Krefeld, Germany). To normalize the amount of RNA bound to membranes, all blots were also hybridized for 18S ribosomal RNA as described previously (15).

Quantitative immunoblotting. Frozen myocardium from LAD and RCX areas was homogenized at 4°C three times for 30 s each with a Polytron PT-10 (Kinematica, Luzerne, Switzerland) in 300 μl of 10 mM NaHCO₃ and 100 μl of 20% SDS. Mixtures were kept at 25°C for 30 min before centrifugation to remove debris. Thereafter, supernatants (called homogenates) were kept at −20°C until further analysis. Homogenate protein samples (20 μg) were loaded per lane. These amounts were in the linear range for each protein (Fig. 1). Gels were run using 10% polyacrylamide separating gels. After gel electrophoresis, separated proteins were electrophoretically transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) (15). Nitrocellulose sheets were incubated with antibody A1 raised against phospholamban (UBI, Lake Placid, NY), antibody 2A7-A1 against SERCA, an affinity-purified antibody to calsequestrin, and antibody 2F6.6.51 to the inhibitory subunit of TnI. These antibodies have been characterized previously (2, 36). Proteins binding antibodies were visualized using 125I-labeled anti-mouse IgG (ICN Biomedicals, Eschwege, Germany) for phospholamban, 125I-labeled protein A (ICN Biomedicals) for SERCA, the inhibitory subunit of TnI and calsequestrin. Radioactive bands were visualized in a PhosphorImager as described above.

Determination of cAMP content. cAMP levels were determined in control (RCX) and ischemic (LAD) areas from a canine left ventricle by the Biotrak cAMP125I assay system from Amersham (Amersham Buchler) according to the instructions of the manufacturer.

Determination of [Ins(1,4,5)P₃] and [Ins(1,4,5)P₃]ₐ. [Ins(1,4,5)P₃] was determined in control (RCX) and ischemic (LAD) areas from a canine left ventricle as described previously (31). To each sample 500 μl of a freshly mixed solution containing 8% trichloroacetic acid, 1 μmol EDTA per 20 mg tissue wet wt, and 1 μmol NaF per 20 mg wet wt were added. Frozen samples were homogenized in a microdismembrator (B.

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**Table 1. PCR primers used to generate probes for Northern blot analysis**

<table>
<thead>
<tr>
<th>Gene</th>
<th>5' Primer</th>
<th>3' Primer</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dog SERCA</td>
<td>CCTCCGGTGGGCAATTTGATCGG</td>
<td>CCTCCATGAACTGGTCTATAGG</td>
<td>7–723</td>
</tr>
<tr>
<td>cCSQ</td>
<td>ATAGGGTGTGGATTTGCAAG</td>
<td>TCCAGTCCTCAAGCTTGAGG</td>
<td>407–1218</td>
</tr>
<tr>
<td>TnI</td>
<td>GGGGGGATTAGGACGGCAT</td>
<td>GTGGGGGCTTTAATAATGGGGC</td>
<td>108–538</td>
</tr>
</tbody>
</table>

SERCA, sarco(endo)plasmic reticulum Ca²⁺-ATPase 2a; cCSQ, consensus calsequestrin; TnI, the inhibitory subunit of troponin.
Hemodynamic data in conscious dogs before, during, and after ischemia.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Ischemia</th>
<th>10 min</th>
<th>Excision</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAP, mmHg</td>
<td>134.1 ± 2.8</td>
<td>118.2 ± 3.4*</td>
<td>132.4 ± 5.8</td>
<td>135.7 ± 8.8</td>
</tr>
<tr>
<td>DAP, mmHg</td>
<td>84.6 ± 3.0</td>
<td>75.8 ± 4.1</td>
<td>85.6 ± 6.2</td>
<td>85.3 ± 7.4</td>
</tr>
<tr>
<td>MAP, mmHg</td>
<td>106.2 ± 2.7</td>
<td>94.1 ± 3.4*</td>
<td>107.8 ± 5.6</td>
<td>107.7 ± 6.2</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>88 ± 4.3</td>
<td>97.3 ± 5.2</td>
<td>91.3 ± 6.6</td>
<td>104.4 ± 6.3</td>
</tr>
<tr>
<td>dP/dt(_{\text{max}}), mmHg/s</td>
<td>3085.3 ± 177.6</td>
<td>2328.8 ± 150.2*</td>
<td>2651.4 ± 161.3</td>
<td>2896.7 ± 168.9</td>
</tr>
<tr>
<td>dP/dt(_{\text{min}}), mmHg/s</td>
<td>2789.8 ± 129.2</td>
<td>1988.3 ± 176.8*</td>
<td>2455.6 ± 180.3</td>
<td>2518.8 ± 130.2</td>
</tr>
<tr>
<td>Flow, kHz</td>
<td>5.1 ± 0.6</td>
<td>5.3 ± 0.7</td>
<td>6.4 ± 1.9</td>
<td></td>
</tr>
</tbody>
</table>

Data are presented as means ± SE; n = 9 dogs. Excision, at time of excision; 10 min indicates 10 min after start of reperfusion; SAP, systolic arterial blood pressure; DAP, diastolic arterial blood pressure; MAP, mean arterial blood pressure; HR, heart rate; dP/dt, maximum and minimum rate of left ventricular pressure rise and decline; Flow, blood flow velocity in left anterior descending coronary arteries. *P < 0.05 vs. control.
vs. 0.68 ± 0.13 arbitrary units, n = 9), calsequestrin mRNA (0.73 ± 0.12 vs. 0.77 ± 0.09 arbitrary units, n = 9), and TnI mRNA (1.44 ± 0.20 vs. 1.58 ± 0.12 arbitrary units, n = 9) in LAD and RCX areas, respectively. It was conceivable that mRNA levels show regional variation. Hence, we also measured mRNA for the genes of interest in LAD and RCX areas from sham-operated animals. No differences were noted (data not shown).

Quantification of protein levels for SERCA, phospholamban, calsequestrin, and the inhibitory subunit of TnI. Quantitative immunoblotting was used to determine the expression of SERCA, phospholamban, calsequestrin, and the inhibitory subunit of TnI. Initial experiments revealed that the detection method was linear between 10 and 40 μg of protein of ventricular homogenate (Fig. 1). Thus 20 μg of protein were used for quantitative immunoblotting. SERCA protein expression was unchanged in LAD versus RCX areas (1.95 ± 0.17 vs. 2.12 ± 0.20 PhosphorImager units, respectively, n = 9). Likewise, the expression of phospholamban, calsequestrin, and the inhibitory subunit of TnI was unchanged (6.62 ± 0.37 vs. 7.72 ± 0.72 PhosphorImager units for phospholamban, 1.70 ± 0.11 vs. 1.64 ± 0.10 PhosphorImager units for calsequestrin, 7.62 ± 0.45 vs. 8.02 ± 0.49 PhosphorImager units for the inhibitory subunit of TnI, n = 9). In addition, it was conceivable that these proteins are altered between LAD and RCX areas in sham-operated animals. This was however not the case. Values amounted to 7.42 ± 0.43 vs. 8.01 ± 0.70 units for phospholamban, 1.74 ± 0.10 vs. 1.79 ± 0.13 units for calsequestrin, and 8.23 ± 0.41 vs. 7.90 ± 0.43 units for the inhibitory subunit of TnI (n = 9) in LAD and RCX areas, respectively.

CAMP and [Ins(1,4,5)P₃]. We compared cAMP and [Ins(1,4,5)P₃] levels in the same RCX and LAD tissues from which also Western and Northern blotting was done. Ten minutes of occlusion and reperfusion decreased cAMP levels by 28% (from 15.9 ± 1.6 pmol/mg protein in control area to 11.5 ± 1.1 pmol/mg protein in the reperfused area, n = 9, P < 0.05, Fig. 3A). However, the level of Ins(1,4,5)P₃ was unchanged following reperfusion compared with control (1.4 ± 0.3 vs. 1.1 ± 0.1 pmol/mg protein, n = 9, Fig. 3B).

Assay of phosphorylated form of the inhibitory subunit of TnI. With a specific antibody, the level of the phosphorylated form of the inhibitory subunit of TnI in ischemic area (LAD) compared with control area (RCX) was determined. In the ischemic area the inhibitory subunit of TnI was less phosphorylated than in the control area (6.3 ± 1.1 vs. 12.5 ± 2.0 PhosphorImager units, n = 8, P < 0.05, Fig. 4). In contrast, the total amount of TnI (phosphorylated and unphosphorylated form) was unchanged. The level of phosphorylated TnI was not significantly different in LAD and RCX areas in sham-operated animals (12.8 ± 2.1 vs. 11.8 ± 1.9 PhosphorImager units, n = 8, P > 0.05).

In vitro phosphorylation of phospholamban and the inhibitory subunit of TnI. To study the CAMP-induced phosphorylation state of proteins in the dog myocardium, the back-phosphorylation technique was employed, using an excess of the catalytic subunit of the CAMP-dependent protein kinase and [γ-³²P]ATP. The amount of phosphate incorporated into phospholamban and the inhibitory subunit of TnI in control and ischemic area are shown in Fig. 5. After 10 min of occlusion and reperfusion, the amount of phosphate incorporated into phospholamban increased to 188 ±

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**Fig. 2.** Autoradiograms of Northern blot analysis for sarco(endo)plasmic reticulum Ca²⁺-ATPase 2a (SERCA), phospholamban (PLB), calsequestrin (CSQ), and the inhibitory subunit of troponin (TnI) in ramus circumflexus (RCX) and left anterior descending artery (LAD) areas from conscious dogs. Total RNA was separated on agarose gels, blotted onto nylon membranes, and then hybridized with specific ³²P-labeled cDNA probes as described in MATERIALS AND METHODS. The location of 18S and 28S ribosomal RNAs is indicated on the right.
33% of the corresponding RCX value ($n = 6$, $P < 0.05$) and into the inhibitory subunit of TnI to 218 ± 49% of RCX ($n = 6$, $P < 0.05$), indicating a lower phosphorylation state of these proteins in vivo.

**DISCUSSION**

The main new finding of the present study is that decreased levels of cAMP and subsequently a reduced phosphorylation state of cardiac regulatory proteins accompanied myocardial stunning in conscious mammals. These changes may or may not be germane to the pathophysiology of stunning.

In this study we used a well-established model of stunning in conscious dogs where the regional contractile dysfunction after 10 min of LAD occlusion was completely reversible (40). For better comparison we measured biochemical parameters when the WTF had recovered to 50% of the preischemic value. However, it can be questioned in what way the present data add new information to previous work by us and others: it is important to discriminate between models of stunning in open-chest and closed-chest animal models. Probably, the situation in closed-chest preparations approximates more closely many clinical situations than do open-chest models. For instance, free radical formation is much smaller in closed-chest preparations than in open-chest preparations (32). Isolated saline-perfused rodent hearts are even more likely to be dissimilar from stunning in humans. Hence, it is important to study the mechanism of stunning in closed-chest animals. We are not aware that similar biochemical studies like those reported here have been performed in any model of regional stunning in conscious animals.

We studied the expression of phospholamban, SERCA, calsequestrin, and TnI based on previous work from others and our group (13, 27, 35, 43). β-Adrenergic stimulation of the heart leads via stimulation of cAMP production and subsequent activation of the cAMP-dependent protein kinase to phosphorylation of phospholamban and TnI (11, 33). Phospholamban phosphorylation is probably relevant because this relieves the tonic inhibition of SERCA by phospholamban. More specifically, unphosphorylated phospholamban decreases the affinity of SERCA for $Ca^{2+}$, whereas phosphorylation of phospholamban by the cAMP-dependent protein kinase has the opposite effect (7). Knock out of the phospholamban gene elevates basal contractility of the mouse heart, accelerates time to peak tension, and hastens relaxation (34). Moreover, after ablation of the phospholamban gene, the heart is less sensitive to the positive inotropic and the relaxant

![Fig. 3](http://ajpheart.physiology.org/)

**Fig. 3.** Levels of cAMP (A) and [Ins(1,4,5)P$_3$] (B) in RCX and LAD areas. cAMP and [Ins(1,4,5)P$_3$] levels were assayed as described in MATERIALS AND METHODS. Each bar represents the mean ± SE; $n = 9$, $^*P < 0.05$ vs. RCX.

**Fig. 4.** Western blot analysis and quantification of the phosphorylated form of the inhibitory subunit of TnI (phospho-TnI) in RCX and LAD areas. Twenty micrograms of ventricular homogenate were separated by SDS-PAGE. After transfer of proteins to nitrocellulose membranes, blots were cut and probed with the antibody 1E11.3 specific for phospho-TnI. Each bar represents the mean ± SE; $n = 8$. **★ p < 0.05 vs. RCX**
effects of β-adrenoceptor stimulation (34). Overexpression of phospholamban on the other hand reduces force of contraction and impairs relaxation (24), and the effect of β-adrenergic stimulation is accentuated. One can ask whether phosphorylation of phospholamban can affect Ca\(^{2+}\) transients. First, phosphorylation of phospholamban in canine membrane preparations enhances the Ca\(^{2+}\) uptake from the cytosol to the SR, whereas dephosphorylation reduces the Ca\(^{2+}\) uptake (26). Second, β-adrenergic stimulation leads to phosphorylation of phospholamban in the intact heart (33). Third, disruption of the phospholamban gene, which removes phospholamban as a protein and thus abolishes any phosphorylation of phospholamban, enhances the amplitude and shortens the duration of Ca\(^{2+}\) transients (34). These evidences strongly indicate that phosphorylation of phospholamban can affect Ca\(^{2+}\) transients in the heart (for review see Ref. 38).

The rate of transcription of the phospholamban gene was stimulated by repetitive stunning in open-chest pigs (13, 27). These data prompted us to test whether phospholamban is altered in our model. In the present work we did not find any change of phospholamban at either mRNA or protein level. It is possible that this is due to the mode by which stunning was initiated or caused by species differences. However, even in repetitive stunning in open-chest pigs, phospholamban mRNA was also not significantly altered, and protein levels of phospholamban were not studied (13). Consistent with our present data is a previous report by our group where we also failed to observe changes in phospholamban expression in a pig model of low-flow hibernation and subsequent stunning (35). In the present work we also measured the expression of calsequestrin because it is the main Ca\(^{2+}\) binding protein of the SR. In stunned open-chest pigs, both the transcriptional rate of the calsequestrin gene and its expression at the mRNA level were higher than in control pigs, but protein levels of calsequestrin were not reported (13). However, we failed to note any change in calsequestrin expression (protein or mRNA level). Again, this could be due to species differences or different protocols. We used namely a single occlusion in conscious dogs, whereas others (13) used open-chest, multiple occlusion in pigs. Because phospholamban acts via interaction with SERCA, it was important to study the expression of this protein. Overexpression of SERCA in the heart of transgenic mice enhanced relaxation consistent with previous in vitro and in vivo findings, indicating the importance of SERCA levels for relaxation of the heart (19). Interestingly, in the pig stunning model mentioned above, increased transcription and increased expression of the SERCA gene at the mRNA level were noted though protein data were not provided (13). However, in the present work no change of SERCA expression at the mRNA level (which should be an early indicator of changes) or at the protein level (which is the functionally relevant form) was noted. It is quite conceivable that we measured too early in reperfusion to find any change in protein levels after transcription and translation of a gene. However, changes due to proteolysis should be measurable under our experimental conditions. Therefore, we studied whether or not protein levels are changed in our model. Global stunning in isolated rodent hearts leads to proteolysis of TnI (14). In the more physiological model of regional stunning that we have used, no expressional changes were observed. Like ourselves Thomas et al. (43) recently failed to detect alterations of TnI protein expression during regional ischemia in pigs. They explained these divergences by the different degrees in ischemia in the various models and by species differences (43). We chose to measure at 50% restitution of regional contractile function for the following reason: At a later time, for instance, where regional function had normalized, sufficient time for transcription and translation will have passed, and we may find changes in protein levels. However, these changes would be too late to cause stunning. This information may also be useful but only to understand compensatory mechanisms to ischemia after stunning has subsided.

Acute ischemia can reduce cAMP content in dog hearts, but data on cAMP in stunned dog hearts have not yet been published (45). More recently, a reduction of cAMP content during prolonged global ischemia was reported in the rat heart (42). The steady-state level of cAMP can decline when the activity of adenyl cyclase(s) is decreased or the activity of phosphodiesterase(s) is enhanced. At present we cannot discriminate between these mechanisms. In rat hearts, global ischemia reduced the activity of the adenyl cyclase conceivably by protein kinase C-induced phosphorylation of the enzyme (42). Moreover, the transient Ca\(^{2+}\) overload at the beginning of reperfusion can lead to biochemical alterations of myocardial proteins by activa-
tion of proteases. Hence, proteolytic cleavage of proteins like adenylyl cyclases may take place. Whatever its cause, the decline in cAMP is probably not unspecific because the level of Ins(1,4,5)P$_3$ was not altered.

The reduced level of cAMP may lead to less activity of the cAMP-dependent protein kinase and thus may be functionally important. This conclusion is supported by three lines of evidence. First, the phosphorylation of TnI was reduced as directly visible by a phosphorylation state-specific antibody. Second, in vitro phosphorylation, which is complementary to the in vivo phosphorylation state, indicates that the phosphorylation state of TnI in the intact animal is reduced by stunning. Third, in vitro phosphorylation also indicates that the in vivo phosphorylation state of phospholamban is reduced in stunning. These qualitatively similar alterations in the phosphorylation state of phospholamban and TnI are plausible because cAMP-increasing agents increase the phosphorylation state of both proteins in isolated hearts (11, 33) or in isolated cells (16). As delineated above, phosphorylation of both proteins is thought to mediate, in part, the positive inotropic and relaxant effects of cAMP-increasing agents. Hence, a decrease in their phosphorylation state should impair relaxation and inotropy. The reduced phosphorylation state of phospholamban will reduce the Ca$^{2+}$ sensitivity of SERCA (7). Therefore, less Ca$^{2+}$ is pumped into the SR. This might lead to the Ca$^{2+}$ overload in the cytosol of the stunned myocardium. However, data on Ca$^{2+}$ transients during regional stunning in conscious animals are not available. In isolated perfused hearts unchanged levels of Ca$^{2+}$ were reported (for review see Ref. 9). Whether the situation is different in conscious animals or whether decreased uptake of calcium into the SR is counterbalanced by a reduced release of Ca$^{2+}$ from the SR needs to be elucidated. In addition, reduced phosphorylation of TnI would lower the rate of cardiac relaxation (11, 47).

Several caveats are in order. We only measured biochemical parameters at the time when 50% of regional contractile function was restored. Moreover, we did not measure regional blood flow. To prove a cause-and-effect relationship it is necessary to establish a correlation between blood flow and phosphorylation. Likewise, the detailed time course of phosphorylation during reperfusion needs to be elucidated.

In summary, the present study shows that altered posttranslational modifications, i.e., diminished phosphorylation of cardiac regulatory proteins, occurred during stunning in conscious dogs.

The excellent technical assistance of Christina Burhoi is gratefully acknowledged. We thank Dr. G. S. Bodor (Denver) for the gift of TnI 1E11.3 and 2F6.6.51 antibodies and Dr. L. R. Jones (Indianapolis) for the gift of SERCA and calsequestrin antibodies.

This work was supported by the Innovative Medizinische Forschung, the Interdisziplinares Zentrum für Klinische Forschung Münster, and the Deutsche Forschungsgemeinschaft.

REFERENCES


The work was supported by the Innovative Medizinische Forschung, the Interdisziplinares Zentrum für Klinische Forschung Münster, and the Deutsche Forschungsgemeinschaft.