Reduced Reperfusion–Induced Ins(1,4,5)P₃ Generation and Arrhythmias in Hearts Expressing Constitutively Active α₁B-Adrenergic Receptors

Sharon N. Harrison, Dominic J. Autelitano, Bing Hui Wang, Carmelo Milano, Xiao-Jun Du, Elizabeth A. Woodcock

Abstract—Reperfusion of globally ischemic rat hearts causes the generation of inositol(1,4,5)trisphosphate [Ins(1,4,5)P₃] and the initiation of arrhythmias. These responses are mediated by α₁-adrrenergic receptors (ARs), but the subtype of receptor involved has not been identified. Under normoxic conditions, hearts from transgenic animals expressing constitutively active α₁B-ARs in heart (α₁B-constitutively active mutant [CAM]) showed higher [³H] inositol phosphate responses to norepinephrine (2.3-fold) than hearts from nontransgenic animals (α₁B-WT) (1.6-fold). α₁B-WT hearts responded to 2 minutes of reperfusion after 20 minutes of global ischemia by generation of Ins(1,4,5)P₃ (5301±1310 to 11 413±1597 CPM/g tissue; mean±SEM; n=6; P<0.01) labeling studies and 3.8±0.2 to 6.3±0.6 nmol/g by mass analysis, n=6; P<0.05). In contrast to findings in normoxia, hearts from α₁B-CAM animals showed no Ins(1,4,5)P₃ response in early reperfusion. In parallel studies, α₁B-WT hearts developed ventricular tachycardia and ventricular premature beats (VPB) during 5 minutes of reperfusion after 20 minutes of ischemia. The incidence of these arrhythmias was reduced in the α₁B-CAM hearts (95% to 62% for VPB and 47% to 12% for ventricular tachycardia; both P<0.05). The resistance of the α₁B-CAM hearts was due to α₁B-AR–mediated preconditioning, as the Ins(1,4,5)P₃ response to thrombin receptor activation during reperfusion was not different between the 2 groups. To investigate the possibility of reduced α₁B–receptor activity in the α₁B-CAM hearts, expression of the mRNA for α₁A* and α₁B-receptors was measured. α₁B-WT hearts contained mRNA for both receptor subtypes, but the levels of α₁B-receptor mRNA were 5-fold higher than α₁A-receptor mRNA. α₁B-CAM hearts contained very high levels of α₁A-receptor mRNA (26-fold increase), but the expression of mRNA for the α₁A-receptors (0.141±0.035 amol/µg RNA; mean±SEM; n=6) was reduced by 50% relative to α₁B-WT controls (0.276±0.046 amol/µg RNA; n=6; P<0.01). The reduction in arrhythmogenic and Ins(1,4,5)P₃ responses in α₁B-CAM hearts provides evidence that these response are not mediated by α₁B-receptors. (Circ Res. 1998;83:1232-1240.)

Key Words: α₁B-adrrenergic receptor ■ α₁A*-adrrenergic receptor ■ Ins(1,4,5)P₃ ■ reperfusion ■ arrhythmia

Sympathetic control of cardiac function involves most importantly β-ARs that mediate their effects primarily by activation of adenyl cyclase and consequent stimulation of the protein kinase A cascade. The heart also contains α-ARs that do not play a major functional role under normal physiological conditions but increase in relative importance under pathological conditions, including ischemia and post-ischemic reperfusion, as well as some forms of heart failure. α₁-Receptors have been implicated in the genesis of arrhythmias under these pathological conditions. α₁-Adrenergic receptors activate the phosphatidylinositol (PtdIns)-specific phospholipase C with the generation of inositol phosphates (InsPs) and sn-1,2-diacylglycerol. Unlike most other cell types, however, large increases in inositol(1,4,5)-trisphosphate [Ins(1,4,5)P₃] are not observed when intact heart tissue is stimulated with α₁-adrenergic agonists. The heart contains a relatively low concentration of Ins(1,4,5)P₃ receptors, and these do not appear to be localized primarily on Ca²⁺ stores. Responses to Ins(1,4,5)P₃ are slow and weak, and the Ca²⁺ so generated does not contribute to calcium-induced calcium release. Thus Ins(1,4,5)P₃ is unlikely to be important in regulating beat-to-beat changes in Ca²⁺ under physiological conditions.

In contrast to findings under physiological conditions, reperfusion after global ischemia causes a rapid, transient generation of Ins(1,4,5)P₃. This response is dependent on norepinephrine, either exogenously added or released from sympathetic nerves, and is mediated via α₁-ARs. More importantly, generation of Ins(1,4,5)P₃ was shown to be necessary for the initiation of reperfusion arrhythmias under...
these conditions.\textsuperscript{11,12} Inhibition of the \( \alpha_1 \)-receptor–mediated Ins(1,4,5)P\(_3\) response thus provides a suitable target for the development of antiarrhythmic agents.

Heart contains both \( \alpha_{1A} \) and \( \alpha_{1B} \)-ARs expressed at the protein level. In rat, \( \approx 80\% \) of the expressed receptor proteins are of the \( \alpha_{1A} \)-subtype, whereas in human tissue, the receptors are mostly \( \alpha_{1A} \)-subtype. \( \alpha_{1B} \)-Receptors do not appear to be expressed in heart at the protein level.\textsuperscript{13–16} Both the \( \alpha_{1A} \)- and \( \alpha_{1B} \)-receptor classes can generate InsPs in isolated cell systems.\textsuperscript{17} In intact rat and rabbit heart, under normoxic conditions, InsP\(_3\) generation appears to be mediated primarily by \( \alpha_{1B} \)-receptors,\textsuperscript{18} but the contribution from the \( \alpha_{1A} \)-subtype increases with cardiomyocyte isolation and culture.\textsuperscript{15,19} Furthermore, \( \alpha_{1A} \)-receptor activity has been associated with pathological changes leading to hypertrophy in isolated cells and in hearts in vivo.\textsuperscript{19,20} The current studies were undertaken to investigate the subtype of \( \alpha_{1} \)-receptor involved in mediating inositol phosphate responses in heart under conditions of postischemic reperfusion. Studies were performed using transgenic mice with cardiac targeted expression of constitutively active \( \alpha_{1B} \)-ARs. These mutant receptors have high activity in the absence of agonist and, in addition, show increased agonist affinity and increased maximal activation by added agonist.\textsuperscript{21} It was reasoned that if the reperfusion response is mediated via \( \alpha_{1B} \)-ARs, then a heightened activity would be expected in \( \alpha_{1A} \)-constitutively active mutant (CAM) hearts. Thus these experiments investigated the activity of the constitutively active \( \alpha_{1B} \)-ARs under normoxic conditions and under conditions of postischemic reperfusion.

Materials and Methods

Animals

Parent \( \alpha_{1B} \)-CAM mice were generated at the Howard Hughes Medical Institute, Duke University. The mutant receptor is expressed under an \( \alpha_1 \)-myosin heavy chain promoter, as described previously.\textsuperscript{22} Female mice derived from \( F_1 \) crosses made from SJL and C57 strains were used in equal numbers, ranging in age from 12 to 30 weeks. No differences in responses were observed between male and female animals. Where indicated, mice were treated with reserpine (3 mg/kg IP) 24 hours before experimentation to deplete endogenous catecholamines. The procedure resulted in a reduction of cardiac responses to exogenous catecholamines. The procedure resulted in a reduction of cardiac responses to exogenous catecholamines.

\[ ^{3}H \]InsPs in Isolated Perfused Mouse Hearts

Adult mice were injected with heparin (5 IU/g) for 30 minutes before killing by cervical dislocation. Hearts were removed and chilled immediately in ice-cold saline. Cancellation of hearts was via the aorta and carried out in ice-cold HEPES-buffered Krebs medium according to the Langendorff method. The medium contained the following (in mmol/L): HEPES buffer (pH 7.4) 20, glucose 11, Na\(^+\) 138, K\(^+\) 4.5, Mg\(^2+\) 1.2, HCO\(_3\)\(^--\) 25, PO\(_4\)^{3-} 1.2, and Ca\(^{2+}\) 2. Hearts were perfused with medium at 2 mL/min in 5 mL organ baths, kept at 37°C, and gassed constantly with 95% O\(_2\)/5% CO\(_2\). After 5 minutes of equilibration, inositol phospholipids were labeled with \( ^{3}H \)inositol (40 \( \mu \)Ci/mL) for 2 hours. Labeled medium was removed, and hearts were perfused with medium containing lithium chloride (LiCl), 10 mmol/L and propanolol (1 \( \mu \)mol/L) for 10 minutes.

For experiments under normoxic conditions, hearts were perfused with norepinephrine (100 \( \mu \)g/mL) for 20 minutes and then were frozen in liquid N\(_2\) and subsequently weighed, and InsPs were extracted.

For experiments investigating ischemia and reperfusion, hearts were subjected to global ischemia by turning off the perfusion pump and the oxygen flow. Reperfusion was achieved by restarting flow of medium and oxygen. After 2 minutes of reperfusion, hearts were frozen in liquid N\(_2\) and weighed subsequently and InsPs extracted.

Extraction and Quantitation of \([^{3}H]\)InsPs

InsPs were extracted from frozen ventricles in 2 mL of 5% trichloroacetic acid (TCA) containing 2.5 mmol/L EDTA and 5 mmol/L phytic acid using a "Polytron" homogenizer followed by sonication as described previously.\textsuperscript{20} After centrifugation at 5000g for 10 minutes (4°C) supernatants were removed, and TCA pellets were re-extracted with 1 mL TCA. The combined aqueous phases were pooled and extracted with a 1:1 mixture of freon and tri-N-octylamine (0.75 mL/mL of supernatant). The final aqueous phase was collected and treated with proteinase K (50 \( \mu \)g/mL; 2 hours; 50°C), and the samples then were passed through a 1 mL Dowex-50 column (4% cross-linked 4-400 mesh size) and eluted with 1 mL of water. Urea (0.05 mol/L final) was added and samples lyophilized before high-performance liquid chromatography (HPLC) analysis.\textsuperscript{24} \([^{3}H]\)-Labeled InsPs were separated using anion-exchange HPLC and quantitated using an on-line \( \beta \)-counter (Radiomatic Instruments Model CR) as described previously.\textsuperscript{24} Recoveries of \([^{3}H]\)Ins(4)P\(_3\), \([^{3}H]\)Ins(1,4,5)P\(_3\), and \([^{3}H]\)Ins(1,4,5,6)P\(_4\) were determined by adding authentic standards to unlabeled tissue and extracting as described. In all cases, recovery of \([^{3}H]\)label was >90%.

Extraction and Quantitation of \([^{3}H]\)Inositol Phospholipids

TCA pellets remaining after InsP extraction were extracted with 3 mL of chloroform:methanol:HCI (200:100:1) using sonication and vigorous vortexing. EDTA (1 mL of 1 mmol/L) was added and the phases separated by centrifugation. The interface was re-extracted, and the final organic phase evaporated under N\(_2\). The dried lipids were deacylated by treatment with methanolic:mebutanol (42:47:9) for 45 minutes at 50°C, followed by evaporation under vacuum. The residue was dissolved in water (1 mL) and extracted with butanol:pentane:ether:ethyl formate (20:40:1). Phases were separated and the organic phase re-extracted. The efficiency of the deacylation procedure was checked by counting the organic phase together with any remaining insoluble residue. On this basis, <5% of the \([^{3}H]\) lipids remained unhydrolyzed. The combined aqueous phases were pooled and applied to 1-mL columns of Dowex-1 (formate form). Columns were washed with 20 mL water. Glycerophosphoinositol (deacylated PtdIns) was eluted with 20 mL of 180 mmol/L ammonium formate and 5 mmol/L sodium tetraborate. After an additional 20 mL of this solution, glycerophosphoinositol(4)mono phosphate [deacylated PtdIns(4)P\(_3\)] was eluted with 20 mL of 400 mmol/L ammonium formate, 0.1 mol/L formic acid, and glycerophosphoinositol(4,5)bisphosphate [deacylated PtdIns(4,5)P\(_3\)] was eluted with 7 mL of 1 mol/L ammonium formate and 0.1 mol/L formic acid. Samples were counted using a \( \beta \)-counter. The \([^{3}H]\)labeled lipids were identified as PtdIns, PtdIns(4)P, and PtdIns(4,5)P\(_2\) by removing the mobile phase with repeated lyophilization and then performing anion-exchange HPLC as described above.

Measurement of Ins(1,4,5)P\(_3\) and PtdIns(4,5)P\(_2\) Mass

For measurement of Ins(1,4,5)P\(_3\) and PtdIns(4,5)P\(_2\), mass, unlabeled tissue was extracted in 2 mL of 5% TCA, 2.5 mmol/L EDTA, and 5 mol/L ATP as described above. The procedure was similar to that described for \([^{3}H]\)InsPs, except that the proteinase K step was omitted and urea was not added before lyophilization.\textsuperscript{24} Lyophilized samples were dissolved in water and neutralized with NaHCO\(_3\) plus NaOH, as required. Ins(1,4,5)P\(_3\) content of the neutralized samples was quantitated using an on-line \( \beta \)-counter (Radiomatic Instruments Model CR) as described previously.\textsuperscript{24} Recoveries of \([^{3}H]\)Ins(4)P\(_3\), \([^{3}H]\)Ins(1,4,5)P\(_3\), and \([^{3}H]\)Ins(1,4,5,6)P\(_4\) were determined by adding authentic standards to unlabeled tissue and extracting as described. In all cases, recovery of \([^{3}H]\)label was >90%.

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For experiments under normoxic conditions, hearts were perfused with norepinephrine (100 \( \mu \)g/mL) for 20 minutes and then were frozen in liquid N\(_2\) and subsequently weighed, and InsPs were extracted.
titiated by using a commercial competitive binding assay involving an
Ins(1,4,5)P₃ receptor preparation and [³H]Ins (1,4,5) P₃, according to
the manufacturer’s instructions (Amersham).

For measurement of PtdIns(4,5)P₂ mass, lipids were extracted
from the TCA pellets remaining after Ins(1,4,5)P₃ extraction from
unlabeled tissue and deacylated after the method described above.
The deacylated lipids were deglycerated by adding sodium periodate
(10 mmol/L) for 30 minutes, followed by 15 minutes of incubation
with ethylene glycol (10% vol/vol). The reaction was completed
using dimethylhydrazine (0.4%) treatment for 3 hours. Samples were
neutralized and lyophilized. Mass assay of the Ins(1,4,5)P₃, result-
ing from deacylation and deglyceration of PtdIns(4,5)P₂ was carried
out as above.

The identity of the compound measured in the assay as
Ins(1,4,5)P₃, [and thus the progenitor lipid as PtdIns(4,5)P₂] was
validated by treating aliquots of representative samples with pure
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out as above.

**Measurement of mRNA**

Total RNA was prepared using the acid guanidinium thiocyanate
procedure.²⁹ RNA concentration was determined by absorbance at
260 nm. Levels of α₁₃C- and α₁₆α-mRNA transcripts were measured by
RNase protection analysis. A 746-nucleotide fragment of the rat
α₁₃C-receptor cDNA corresponding to nucleotides 1 to 746 of the
published sequence,²⁷ and a 471-nucleotide fragment of the hamster
α₁₆α-C DNA corresponding to nucleotides 1 to 471²⁶ were subcloned
into PGEM-4Z vectors for generation of cRNA probes as previously
described.²⁹ Sense RNA was synthesized in vitro from each template
and served to generate a standard curve for each RNA species so that
the absolute level of these RNA transcripts could be determined in
heart RNA extracts. RNA standards and samples (5 µL), 20 µL of
hybridization buffer (80% formamide; 40 mmol/L PIPES, pH 6.7;
0.4 mol/L NaCl; 1 mmol/L EDTA) and 5 µL of antisense probe
(reconstituted in hybridization buffer) were hybridized overnight at
45°C. After hybridization, samples were digested at 37°C for 60
minutes by addition of 300 µL of RNase buffer (300 mmol/L NaCl;
10 mmol/L Tris, pH 7.5; 5 mmol/L EDTA) and 20 µL of antisense probe
(10 mg/mL) to 50% was observed. The perfusion flow rate was adjusted
reoccluded, and the hearts were perfused with Evans Blue (10% 0.05
mL). A nonstained area of left ventricle (LV), indicative of lack of
perfusion, was visualized. The incidence of reperfusion arrhythmias
was not altered by propranolol or LiCl alone, or in combination.
During the 20 minutes of ischemia and 5 minutes of reperfusion, the
epicardial ECG and the coronary perfusion pressure were monitored.
Unlike our previous findings in rat heart,¹¹ and in agreement with the
work of other laboratories,³² ventricular fibrillation was not observed in
the mouse hearts either during the ischemic or reperfusion periods.
Ventricular arrhythmias expressed as ventricular premature beats
(VPB) and ventricular tachycardia ([VT] defined as at least 5
consecutive ectopic beats) were quantified according to the Lambeth
Convention guidelines and as depicted in Figure 1.¹³

**Coronary Artery Occlusion and Reperfusion**

*Mice were anesthetized with a mixture of ketamine (100 mg/kg)
and xylazine (20 mg/kg IP). After tracheal intubation, artificial ventila-
tion with a Harvard ventilator was started (0.5 mL tide volume; 80
strokes/min). A left thoracotomy was performed to expose the heart,
and after 10 minutes of stabilization, the left coronary artery was
ligated using 7-0 silk suture enclosing 2 releasing rings. The chest
was then closed, leaving the suture and the releasing rings accessible
from outside the chest. After 40 minutes of occlusion, the ligature
was released from outside the chest to allow 60 minutes of
reperfusion.***

**Measurement of Area at Risk and Infarct Zone**

The zone at risk (RZ) and the infarcted zone (IZ) were determined as
described elsewhere.⁹ In brief, at the end of the reperfusion period,
the ascending aorta was cannulated and the heart isolated. After
washing the coronary vasculature with cold saline, the coronary
artery was reoccluded, followed by infusion of 0.1 mL 10% Evans
Blue to stain the nonischemic area. The excess dye was washed
away, and large vessels, atria, and the right ventricle were removed.
The LV was then frozen, cut transversely into ~1-mm thick slices (6
to 7 slices per heart), and incubated with 1.5% triphenyltetrazolium
in 100 mmol/L PBS (pH 7.4) for 15 minutes at 37°C. The viable
myocardium were stained brick red, and the infarcted myocardium
came pale white. The stained slices were mounted on a glass plate,
photographed, and enlarged. The 3 areas, nonischemic area, RZ, and
IZ, were outlined and quantified using a software program (BioScan
Optimas).

**Statistics**

Values presented are mean±SEM. Statistical analysis of inositol
phosphate data involved the use of 1-way ANOVA followed by
Student unpaired t test. Significance was determined at P<0.05.
Chi-square or Fisher exact test was used for percentages of VPB and
TABLE 1. Characteristics of Hearts from α1B-WT and α1B-CAM Mice

<table>
<thead>
<tr>
<th></th>
<th>BW, g</th>
<th>HW, mg</th>
<th>HW/BW, mg/g</th>
<th>ANP mRNA, Phosphorimager Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>α1B-WT</td>
<td>28±2</td>
<td>126±6</td>
<td>4.4±0.2</td>
<td>875±276</td>
</tr>
<tr>
<td>α1B-CAM</td>
<td>26±2</td>
<td>122±6</td>
<td>4.6±0.1</td>
<td>2909±688*</td>
</tr>
</tbody>
</table>

Shown are heart weights of transgenic mice and controls expressed as tissue weight and as heart weight (HW) relative to body weight (BW) together with the contents of mRNA for ANP. Values are mean±SEM, n=6 (3 male, 3 female).

*P<0.05 relative to α1B-WT. The animals were between 3 and 6 months old.

Materials

[2-3H]myo-Inositol (18 Ci/mmol), [3H]Ins(1,4,5)P3, [14C]Ins(1)P1, and mass assay kits for Ins(1,4,5)P3 were obtained from the Radiochemical Center (Amer sham). [3H]-labeled Ins(1,4)P2 and Ins(4)P1 were obtained from New England Nuclear and were supplied by Auspep. Norepinephrine, reserpine, phytic acid, and nucleotides were obtained from the Sigma Chemical Co. Thrombin receptor–activating peptide (TRAP) was supplied by Auspep. Triphenyltetrazoliun was obtained from New England Nuclear and were supplied byAuspep.

Results

Effects of the α1B-CAM Receptors on Heart Weight and ANP Gene Transcription

Hearts from the α1B-CAM animals bred in our animal facility did not show signs of hypertrophy in terms of tissue weight (Table 1), even though a mild 10% to 20% increase in heart weight has been reported previously in this strain.23 Increased ANP gene transcription was observed, as reported previously, suggesting a sensitivity to the development of hypertrophy. Differences in diet or housing conditions most likely explain these differences.

InsP Responses in α1B-WT and α1B-CAM Hearts Under Physiological Conditions

The effect of the CAM α1B receptors on the contents of InsPs and inositol phospholipids was investigated in perfused mouse hearts under normoxic conditions. Isolated mouse hearts were labeled with [3H]inositol, pretreated with propranolol and LiCl, and then subjected to 20 minutes of global, zero flow ischemia followed by 2 minutes of reperfusion. Ischemia (20 minutes) did not cause any alteration in the total [3H]InsP content (66 973±5965 CPM/g wet wt versus 60 332±9479 CPM/g; mean±SEM; P=NS) nor in the content of [3H]Ins(1,4,5)P3 (7407±1073 CPM/g wet wt versus 5376±1392 CPM/g; P=NS). There was a decrease in [3H]Ins(4)P1, as described previously in rat heart.10,30 In α1B-WT hearts, 2 minutes of reperfusion after 20 minutes of ischemia caused generation of [3H]InsPs (Figures 3 and 4). As described previously in rat heart, increases in norepinephrine as described previously for this mutant expressed in COS cells.23 Although both α1B-WT and α1B-CAM hearts responded to norepinephrine with an increase in [3H]InsPs, there was no detectable increase in [3H]Ins(1,4,5)P3 or in Ins(1,4,5)P3 mass in either preparation (Figure 2). Also, although increases in [3H]InsPs were observed with 20 minutes of stimulation with norepinephrine, no significant increase was observed in either α1B-WT or α1B-CAM hearts when norepinephrine (100 μmol/L) was perfused for 2 minutes under these normoxic conditions (data not shown).

InSp Responses During Early Reperfusion in α1B-WT and α1B-CAM

Isolated perfused mouse hearts were labeled with [3H]inositol, pretreated with propranolol and LiCl, and then subjected to 20 minutes of global, zero flow ischemia followed by 2 minutes of reperfusion. Ischemia (20 minutes) did not cause any alteration in the total [3H]InsP content (66 973±5965 CPM/g wet wt versus 60 332±9479 CPM/g; mean±SEM; P=NS) nor in the content of [3H]Ins(1,4,5)P3 (7407±1073 CPM/g wet wt versus 5376±1392 CPM/g; P=NS). There was a decrease in [3H]Ins(4)P1, as described previously in rat heart.10,30 In α1B-WT hearts, 2 minutes of reperfusion after 20 minutes of ischemia caused generation of [3H]InsPs (Figures 3 and 4). As described previously in rat heart, increases in norepinephrine as described previously for this mutant expressed in COS cells.23 Although both α1B-WT and α1B-CAM hearts responded to norepinephrine with an increase in [3H]InsPs, there was no detectable increase in [3H]Ins(1,4,5)P3 or in Ins(1,4,5)P3 mass in either preparation (Figure 2). Also, although increases in [3H]InsPs were observed with 20 minutes of stimulation with norepinephrine, no significant increase was observed in either α1B-WT or α1B-CAM hearts when norepinephrine (100 μmol/L) was perfused for 2 minutes under these normoxic conditions (data not shown).

InSp Responses During Early Reperfusion in α1B-WT and α1B-CAM

![Figure 2. Inositol phosphate responses in hearts from α1B-WT and α1B-CAM mice. Top. Hearts were labeled with [3H]inositol and subsequently incubated for 20 minutes with propranolol (1 μmol/L) and LiCl (10 mmol/L) in the presence or absence of norepinephrine (100 μmol/L). [3H]InsPs were extracted and quantitated. Bottom. Similar experiments were performed using unlabeled ventricles and measuring Ins(1,4,5)P3 mass. Animals were between 3 and 6 months old, and the groups contained equal numbers of male and female animals. Values shown are [3H]InsPs in CPM/g tissue wet wt, mean±SEM, n=4 (labeling studies) or Ins(1,4,5)P3 nmol/g tissue wet wt, n=6 (mass analysis). *P<0.01 relative to no norepinephrine; #P<0.01 relative to α1B-WT. nor indicates norepinephrine.](image-url)
Top, [3H]-labeled InsPs. Values shown are [3H]CPM/g tissue and 5.3±1.6×10^3 CPM/g tissue. Addition of norepinephrine (100 μmol/L) to the medium used for reperfusion caused a return of the reperfusion response (total [3H]InsPs 102±9.7×10^3 and [3H]Ins(1,4,5)P_3 12±2×10^3; n=6; P<0.01 in both cases). Similar findings have been reported previously in rat heart. This shows that the reperfusion-induced InsP response is dependent on release of norepinephrine from the sympathetic nerves under these conditions.

In contrast to findings in α_1B-WT hearts, reperfusion of ischemic hearts from α_1B-CAM mice did not cause an increase in any of the InsPs (total [3H]InsPs, 128±17×10^3 CPM/g; n=8; compared with 131±16×10^3 CPM/g). There was no increase in [3H]Ins(1,4,5)P_3 and no change in Ins(1,4,5)P_3 mass (Figure 4). Furthermore, no increase in total [3H]InsPs (128±17 to 119±4×10^3 CPM/g tissue) or in [3H]Ins(1,4,5)P_3 (10.9±1.6 to 9.1±2×10^3 CPM/g tissue; mean±SEM; n=4; P>0.05 in both cases) was detected when hearts were reperfused in the presence of added excess norepinephrine (100 μmol/L). This eliminates the possibility of differences related to norepinephrine availability.

**Figure 4.** Reperfusion-induced InsP responses in hearts from α_1B-WT and α_1B-CAM animals. Hearts were labeled with [3H]inositol and subjected subsequently to 20 minutes of global zero flow ischemia followed by 2 minutes of reperfusion. [3H]InsPs were extracted and quantitated as depicted in Figure 3. Top, [3H]-labeled InsPs. Values shown are [3H]CPM/g tissue wet wt, mean±SEM, n=8. Bottom, Ins(1,4,5)P_3 mass. Animals were between 3 and 6 months old, and the groups contained equal numbers of male and female animals. Values shown are Ins(1,4,5)P_3 mass, nmol/g tissue wet wt, mean±SEM, n=6. *P<0.01 relative to 20 minutes of ischemia. ##P<0.05 relative to preischemia.

**Effect of Thrombin Receptor Activation During Reperfusion**

Failure to detect a reperfusion response in α_1B-CAM hearts could mean either that α_1B-receptors are not involved in mediating the InsP response to norepinephrine under reperfusion conditions or that the presence of the active α_1B-receptors has had a preconditioning effect. In previous studies, we have shown that under reperfusion conditions, thrombin receptor activation causes similar Ins(1,4,5)P_3 and arrhythmogenic responses. Any preconditioning effect of α_1B-CAM receptors would be expected to affect responses to α_1-receptor or thrombin receptor activation. Thus responses...
to thrombin receptor stimulation were investigated in α_{IB}-WT and α_{IB}-CAM hearts. Catecholamine-depleted hearts from α_{IB}-WT or α_{IB}-CAM mice were subjected to 20 minutes of ischemia followed by reperfusion in the presence of thrombin receptor activating peptide (TRAP, SFLLRN; 50 μmol/L). Ins(1,4,5)P₃ content was measured after 2 minutes. As shown in Figure 5, addition of TRAP to catecholamine-depleted α_{IB}-WT hearts caused generation of Ins(1,4,5)P₃. The TRAP response was quantitatively similar to the response to norepinephrine. Similar experiments were performed using α_{IB}-CAM hearts. Addition of TRAP (50 μmol/L) to α_{IB}-CAM hearts for the 2-minute reperfusion period caused an increase in Ins(1,4,5)P₃ similar to that observed in α_{IB}-WT hearts. Thus the α_{IB}-CAM receptor reduces the reperfusion-induced Ins(1,4,5)P₃ response only when this is activated by α₁-adrenergic agonists. This argues against preconditioning as an explanation.

Table 3. Incidence of VPB and VT in Perfused Hearts From α_{IB}-WT and α_{IB}-CAM Mice Over a 5-Minute Reperfusion Period After 20 Minutes of Regional Ischemia

<table>
<thead>
<tr>
<th></th>
<th>α_{IB}-WT (n=19)</th>
<th>α_{IB}-CAM (n=18)</th>
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<tbody>
<tr>
<td>VPB, %</td>
<td>95</td>
<td>62*</td>
</tr>
<tr>
<td>VT, %</td>
<td>47</td>
<td>12†</td>
</tr>
<tr>
<td>VPB, No.</td>
<td>46±13</td>
<td>16±7*</td>
</tr>
<tr>
<td>VT, s</td>
<td>8±4</td>
<td>1±1</td>
</tr>
</tbody>
</table>

Values shown are the percentages of animals developing VPB and VT (as defined in Materials and Methods) as well as total number of VPB (mean±SEM) and total time in VT (mean±SEM) over the 5-minute reperfusion period.

*P<0.01 and †P<0.05 relative to α_{IB}-WT by χ² test or Fisher exact test for percentages and by a Mann-Whitney rank sum test for VPB number.

Infarct Size Measurements in α_{IB}-WT and α_{IB}-CAM Hearts

Preconditioning would be expected to reduce infarct size after coronary artery ligation as well as reducing arrhythmias. Experiments were performed to measure infarct size in hearts from α_{IB}-WT and α_{IB}-CAM animals after ischemia/reperfusion in vivo. There was no difference between control and α_{IB}-CAM groups (n=7 per group) in the RZ (45±4% versus 50±4% of LV; P=NS) or IZ (32±2% versus 31±3% of LV; P=NS). The infarct size, calculated from the ratio of IZ/RZ (%) was also similar between the 2 groups (67±4% versus 62±6%; P=NS).

Expression of α_{IA}-Receptors in α_{IB}-CAM Hearts

α_{IB}-CAM hearts did not generate Ins(1,4,5)P₃ in early reperfusion, providing evidence that the response is not α_{IB}-receptor-mediated. As hearts express only α_{IB}- and α_{IA}-receptors with minimal expression of α_{IA}-receptors at the protein level, this implicates α_{IA}-receptor involvement in mediating the reperfusion response and implies that α_{IA}-receptor activity is depressed in α_{IB}-CAM hearts. Thus the effect of the α_{IB}-CAM receptors on the expression of α_{IA}-receptors was investigated. RNA was extracted from α_{IB}-CAM and α_{IB}-WT hearts, and the content of mRNA for α_{IA}- and α_{IB}-receptors was quantitated by RNase protection. The content of α_{IA}-receptor mRNA in the α_{IB}-CAM hearts, expressed under the myosin heavy chain promoter, was markedly increased (26-fold) relative to α_{IB}-WT hearts. The level of expression of α_{IA}-receptor mRNA under the α_{IB}-CAM promoter in the α_{IB}-CAM hearts was very constant between animals, but there was greater variation between animals in expressions of α_{IA}- and α_{IB}-receptor mRNAs expressed under their own promoters. Hearts from α_{IB}-WT animals contained mRNA for both receptor classes, but the α_{IB}-receptors were expressed more strongly, accounting for 5 times more mRNA than the α_{IA}-receptors. More importantly, although there was considerable variation between animals, the expression of the mRNA for α_{IA}-receptors on average was depressed in the α_{IB}-CAM hearts (Figure 6), raising the possibility of lower levels of expression of α_{IA}-receptors in the α_{IB}-CAM hearts. The expression of mRNA for GAPDH was similar in α_{IB}-WT and α_{IB}-CAM hearts (Figure 6).

Discussion

Reperfusion of rat hearts after global ischemia causes release of norepinephrine from the sympathetic nerves, the transient generation of Ins(1,4,5)P₃ within the myocardium, and the initiation of arrhythmias. As increases in Ins(1,4,5)P₃ are not detectable in intact heart under physiological conditions in response to norepinephrine, this marks a major change in the operation of the inositol phosphate pathway. Delineating the relative roles of the α₁-receptor subtypes in mediating InsP responses under normoxic and reperfusion is thus of central importance.

Under normoxic conditions, the constitutively active α₁-receptors conferred on the expressing hearts a heightened InsP response in the presence or absence of norepinephrine (Figure 2). If α_{IB}-receptors mediated the norepinephrine response during reperfusion, then generation of very large amounts of Ins(1,4,5)P₃ would be expected in α_{IB}-CAM...
hearts. However, this was not found to be the case. Hearts from α₁B-WT animals responded to reperfusion by generation of Ins(1,4,5)P₃ as demonstrated previously in rat hearts but, in marked contrast to findings under normoxic conditions, the α₁B-CAM hearts showed no detectable Ins(1,4,5)P₃ response during early reperfusion. Together with the reduced Ins(1,4,5)P₃ response, the α₁B-CAM hearts had a reduced incidence of reperfusion arrhythmias. This agrees with previous findings from our laboratory of a relationship between Ins(1,4,5)P₃ generation and arrhythmias in rat heart. Rat hearts develop more substantial arrhythmias under these conditions of early reperfusion than mouse hearts, with ventricular fibrillation being observed in addition to VPB and VT. Ischemia has been shown to cause the translocation and activation of PKC, and this together with any sn-1,2-diacylglycerol generated along with Ins(1,4,5)P₃, might be important in arrhythmogenesis. However, our studies in rat hearts showed no antiarrhythmic action of inhibitors of protein kinase C during reperfusion. More importantly, recent studies from other laboratories have demonstrated a direct proarrhythmic action of Ins(1,4,5)P₃ when applied intracellularly. Thus it seems most likely that Ins(1,4,5)P₃ itself, presumably via perturbations in cytosolic Ca²⁺, initiates electrophysiological changes that culminate in the development of arrhythmias.

The reduced Ins(1,4,5)P₃ response during reperfusion of α₁B-CAM hearts suggests that the norepinephrine response is not mediated by α₁B-receptors under these conditions. However, it is also possible that the α₁B-CAM receptors have caused a preconditioning effect. We have shown previously that preconditioning can reduce the reperfusion Ins(1,4,5)P₃ response, and others have demonstrated a preconditioning effect of α₁B-receptor activation under some conditions. However, 2 different experiments

Figure 6. Expression of α₁A- and α₁B-AR mRNA in whole mouse heart. Left, Solution hybridization/RNase protection of (A) in vitro synthesized sense RNA standards, (B) α₁A-AR mRNA, (C) α₁B-AR mRNA, and (D) GAPDH mRNA after polyacrylamide gel electrophoresis and phosphorimaging analysis. Total RNA (20 μg) derived from either α₁B-WT or α₁B-CAM mouse heart was used for analysis of α₁A-AR mRNA, whereas 4 μg RNA was hybridized to the GAPDH probe. Exposure times were 4 h for GAPDH and α₁A-AR and 24 h for α₁B-AR mRNA. Right, Quantitation of α₁A- and α₁B-AR mRNA expressed as amol-specific mRNA transcript per microgram total RNA and GAPDH mRNA expressed as percentage of control. Data represent mean±SEM, n=6. *P<0.05 compared with respective control. Regression analyses of sense RNA standards showing linear standard curves used for the quantitation of α₁A-AR mRNA are shown in the top right panel.
argue against preconditioning as an explanation of the reduced reperfusion response in the α1B-CAM hearts. First, α1B-CAM hearts responded to thrombin receptor activation under reperfusion conditions in a similar manner to α1B-WT hearts. Preconditioning would be expected to reduce responses to all effectors similarly. Second, preconditioning would also be expected to reduce infarct size after coronary artery ligation,25 but infarct sizes were found to be similar in the 2 groups. Thus no evidence was found for a preconditioning effect of the α1B-CAM receptors under the conditions of our experiments.

The data presented indicate the involvement of α1-receptors other than α1A-subtype as mediators of the reperfusion-induced Ins(1,4,5)P3 response to norepinephrine. Hearts, at least human and rat, express only the α1A- and α1B-subtypes of α1-receptors at the protein level.26 If also true of mouse heart, this implies an involvement of α1A-receptors. Such an interpretation however, suggests a reduction in α1A-receptor activity in the α1B-CAM hearts. Some evidence for this was supplied by the experiments showing reduced α1A-receptor mRNA expression in the α1B-CAM hearts (Figure 1). However, it must be stressed that an involvement of α1B-receptors cannot be discounted at this stage. We have reported previously that stimulation of α1A-receptors causes a reduction in α1A-receptor mRNA.43 Together with data reported here, this points to a reciprocal relationship between these 2 receptor subclasses, at least in the myocardium.

An involvement of receptors with α1A-specificity in the generation of arrhythmias under ischemic/reperfusion conditions has been suggested previously on the basis of effects of α1B-receptor antagonists. In addition, increased activity of α1B-receptors cannot be discounted at this stage. We have reported previously that stimulation of α1A-receptors causes a reduction in α1A-receptor mRNA expression.20 Together with data reported here, this points to a reciprocal relationship between these 2 receptor subclasses, at least in the myocardium.

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