Inositol phospholipids localized to caveolae in rat heart are regulated by α₁-adrenergic receptors and by ischemia-reperfusion

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Inositol phospholipids localized to caveolae in rat heart are regulated by α₁-adrenergic receptors and by ischemia-reperfusion. Am J Physiol Heart Circ Physiol 290: H2059–H2065, 2006. First published December 22, 2005; doi:10.1152/ajpheart.01210.2005.—Postischemic reperfusion of rat or mouse hearts causes generation of inositol (1,4,5)trisphosphate [Ins(1,4,5)P₃] and the initiation of arrhythmias. In the current study we investigated the possibility that the enhanced Ins(1,4,5)P₃ generation in postischemic reperfusion was associated with an increased availability of the precursor lipid phosphatidylinositol(4,5)bisphosphate (PIP₂) for α₁-adrenergic receptor-activated phospholipase C (PLC). Isolated-perfused rat hearts were labeled with [³H]inositol and subjected to ischemia-reperfusion or stimulation with norepinephrine under normoxic conditions. Caveolar fractions were prepared by buoyant density sucrose gradient centrifugation. [³H]PIP₂ was concentrated in caveolae, along with Gq and PLCβ₁b. Caveolae contained only 27.3 ± 6.9% (means ± SE, n = 6) of the total α₁-adrenergic receptor complement of the heart. These did not migrate to PIP₂-containing caveolar fractions with norepinephrine stimulation under normoxic conditions, even though caveolar PIP₂ was depleted. In contrast, [³H]PIP₂ in caveolae increased during 2 min of reperfusion, independently of norepinephrine release and thus of α₁-adrenergic receptor activation. The increased PIP₂ in the caveolar fractions where signaling proteins are concentrated may be critical for the heightened generation of Ins(1,4,5)P₃ in early reperfusion.

BRIEF PERIODS OF ISCHEMIA-REPERFUSION in the rat and mouse heart cause norepinephrine release, activation of α₁-adrenergic receptors (α₁-AR), and substantial generation of inositol (1,4,5)trisphosphate [Ins(1,4,5)P₃] associated with the onset of arrhythmogenesis (1, 2, 13, 24, 41). Ins(1,4,5)P₃ is generated from sarcolemmal phosphatidylinositol(4,5)bisphosphate (PIP₂) following activation of phospholipase C (PLC). We addressed the possibility that increased availability of PIP₂ or factors required for PLC activation contributed to the heightened generation of Ins(1,4,5)P₃ during postischemic reperfusion.

PIP₂ is generated from phosphatidylinositol most commonly by phosphorylation to PIP(4)P, followed by further phosphorylation to PI(4,5)P₂ (9) and to a lesser extent by sequential 5’ and 4’ phosphorylation (23). PIP₂ is critically involved in myocardial responses. In addition to Ins(1,4,5)P₃, PLC cleavage of PIP₂ generates sn-1,2-diacylglycerol, an activator of PKC isoforms (31). PIP₂ is also the precursor of PIP₃, an important factor in cardiomyocyte development and cytoprotection (43). Further to these roles as precursor to other active molecules, PIP₂ itself acts as a second messenger by activating phospholipase D activity in the heart (25). PIP₂ is critically important in stabilizing various ion channels and transporters (22, 32) and also regulates the cytoskeleton by virtue of its ability to interact specifically with actin-binding proteins via their plextrin homology (PH) domains (32). With this complexity in mind, we sought to define the localization of PIP₂ in the heart in relation to signaling proteins involved in Ins(1,4,5)P₃ generation, specifically α₁-AR, Gq, and PLCβ, and to establish how the localization of these various factors was influenced by ischemia and reperfusion.

The sarcolemma is highly organized, and it is now recognized that many signaling responses are localized to cholesterol-rich, sphingolipid-rich regions called light lipid rafts to reflect their low buoyant densities (20, 37). Important among this heterogeneous fraction are the caveolae, which in the heart are characterized by the presence of caveolin-3 (47). The concentration of receptors and downstream factors within caveolae facilitates regulation and potentially enhances specificity by physically limiting available signaling partners (20, 28, 37). In the heart, the caveolar fraction has been reported to contain α₁-AR, Gq, and the subtypes of PLCβ (15), but the localization of PIP₂ and its precursors have not been defined.

In the current study, we show that PIP₂, as well as its precursors PIP and PI, is highly enriched in the caveolar fraction along with the lower molecular weight “b” splice variant of PLCβ1 (4). Other proteins potentially involved in α₁-AR signaling were not exclusively localized in caveolae, suggesting a previously unrecognized level of specificity for PLCβ1b. We show that caveolar-localized PIP₂ is rapidly depleted by α₁-AR activation, but surprisingly that brief ischemia followed by reperfusion increases the content of PIP₂ in caveolae independently of norepinephrine release and α₁-AR activation. This rise in PIP₂ in the vicinity of PLCβ1b may contribute to the enhanced Ins(1,4,5)P₃ generation observed under these conditions.

METHODS

Preparation of perfused rat hearts, ischemia-reperfusion, and norepinephrine depletion. All procedures were approved by the Alfred Medical and Education Precinct animal ethics committee and all followed the guidelines of the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Adult male Sprague-Dawley rats were killed by decapitation. Hearts were isolated and perfused by the Langendorf method with HEPES-buffered Krebs.
medium, pH 7.4, at 7 ml/min, as previously described in detail (1). The medium contained the following (in mM): 126 NaCl, 25 NaHCO3, 1.85 CaCl2, 1.05 MgCl2, 0.5 NaH2PO4, 4 KCl, 11 glucose, and 20 HEPES buffer, pH 7.4. Inositol phospholipids were labeled by perfusing the hearts with medium containing 2 μCi/ml [3H]inositol for 2 h, at 7 ml/min, followed by washing with nonradioactive medium. LiCl (10 mM) and propranolol (1 μM) were then added to the perfusate to inhibit inositol phosphate metabolism and β-adrenergic receptor activation, respectively (1).

For studies of ischemia and reperfusion, [3H]inositol-labeled hearts were subjected to 20 min global zero-flow ischemia by turning off the perfusion pump for 20 min. Reperfusion was initiated by restarting flow at 7 ml/min. For studies of norepinephrine, stimulation under normoxic conditions norepinephrine (100 μM) was added to [3H]inositol-labeled hearts for 2 min. After treatment, ventricles were excised at the atrioventricular junction and were rapidly frozen in liquid N2 and stored at −80°C. Protocols are outlined in Fig. 1.

Depletion of norepinephrine was achieved by treating the animals with reserpine (5 mg/kg body wt ip) for 18 h. This reduced norepinephrine levels in the hearts from 24.4 ± 10.4 pmol/mg protein (means ± SE, n = 4) to undetectable levels. Norepinephrine was measured as described previously (14).

Separation of different membrane fractions from isolated perfused rat hearts. All solutions for preparation of membrane fractions contained the following proteinase and phosphatase inhibitors: 10 μg/ml leupeptin, 5 μg/ml aprotinin, 1 μg/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 20 mM NaF, 1 mM Na3VO4, and 1 mM sodium pyrophosphate. Frozen hearts were homogenized in 2.5 ml of 0.5 M NaClO4, pH 11, by using a Polytron homogenizer at maximum speed. Homogenates were sonicated three times for 20 s each and then mixed with an equal volume of 80% sucrose in MES-buffered saline (25 mM MES, pH 6.5, 0.15 M NaCl), as described elsewhere (39). Tubes were overlaid with 4 ml of 35% sucrose and 3 ml of 5% sucrose and centrifuged at 38,000 rpm for 24 h at 4°C using a SW-41 rotor in a Beckman L-90K ultracentrifuge. Gradients were fractionated from the bottom using a peristaltic pump.

Fractions (1 or 2 ml) were collected. The same fractions were used for lipid analysis and for estimation of protein content. The fractions were divided into two equal aliquots, one being used for lipid extractions, as described above, were solubilized in SDS-PAGE sample buffer (8). Protein concentration was measured by a modified Lowry estimation (33). Proteins (100 μg) were separated on gradient SDS-PAGE gels.

Measurement of [3H]inositol phospholipids. Phospholipids were extracted from the fractions using an equal volume of CHCl3/CH3OH/H2O (200/100/1 vol/vol/vol). Phases were separated by adding 2 mM EDTA, and the lipid phase was evaporated under vacuum. Dried lipids were deacylated using methyamine/CH3OH/butanol (42/47/9 vol/vol/vol) for 45 min at 50°C, followed by evaporation. Dried deacylated lipids were extracted with petroleum spirit-butanol-ethyl formate, as described previously (49). The deacylated lipids were separated into PI, PIP, and PIP2 fractions using 1-ml columns of Dowex-1 (formate form), as described previously (49). Samples were counted in a beta counter.

Fig. 1. Protocols used for perfused rat heart experiments. [3H]Inositol-labeled hearts are treated with 10 mM LiCl and 1 μM propranolol (pro) at the times indicated by shaded arrows. Addition of 100 μM norepinephrine (Nor) is indicated by hatched arrows. Collection of the ventricles is indicated by open arrows. Isch, ischemia; reper, reperfusion.
RESULTS

Reperfusion of ischemic rat hearts increases PIP2 independently of norepinephrine release. Isolated perfused [3H]inositol-labeled rat hearts were subjected to 20 min global zero-flow ischemia followed by reperfusion for 0.5–3 min and then snap frozen (1). [3H]Ins(1,4,5)P3 and [3H]PIP2 were extracted and quantified. Reperfusion caused an increase in [3H]Ins(1,4,5)P3 that was maximal 1–2 min after reinitiation of flow (Fig. 2A). Despite the clear activation of PLC and Ins(1,4,5)P3 generation, there was no decrease in [3H]PIP2 over this time period. Loss of PIP2 associated with generation of Ins(1,4,5)P3 might have been masked by increased generation of PIP2 caused by the ischemia-reperfusion procedure itself. To investigate this possibility, hearts were depleted of norepinephrine to prevent α1-adrenergic receptor-mediated PLC activation. Depletion of norepinephrine prevented the [3H]Ins(1,4,5)P3 response over 3 min of postischemic reperfusion, as reported previously (1), and caused a marked increase in [3H]PIP2 after reinitiation of flow (Fig. 2B).

To ensure that the rise in PIP2 in norepinephrine-depleted hearts reflected a loss of PLC activity, a number of experiments were performed. First, 100 μM norepinephrine was added to the perfusate of the norepinephrine-depleted hearts during 2 min of reperfusion, resulting in the generation of Ins(1,4,5)P3 with no increase in PIP2 (Fig. 3A). Second, 1.5 mM gentamicin was added during reperfusion of intact hearts (not reserpine treated) to inhibit PLC (24). Treatment with gentamicin prevented Ins(1,4,5)P3 generation, as reported previously (13), and also resulted in increased PIP2 (Fig. 3B). Thus the ischemia-reperfusion procedure generates PIP2 independently of norepinephrine release. When PLC is activated by α1-adrenergic receptor activation, this PIP2 increase is not observed, implying that the increased PIP2 provides a substrate for the activated PLC.

Fig. 2. Postischemic reperfusion causes generation of inositol (1,4,5)trisphosphate [Ins(1,4,5)P3] (B) as well as increases in phosphatidylinositol(4,5)-bisphosphate (PIP2) (A). Isolated perfused rat hearts, both intact and norepinephrine depleted, were labeled with [3H]inositol and subsequently subjected to 20 min of zero-flow ischemia followed by reperfusion at 7 ml/min for the indicated time. [3H]Ins(1,4,5)P3 and [3H]PIP2 were extracted and quantified. Values shown are expressed as [3H]counts/minute (CPM)/g wet wt; means ± SE, n = 6. Open symbols, norepinephrine-depleted hearts; filled symbols, intact hearts. *P < 0.05 and **P < 0.01 relative to 20 min ischemia.

Fig. 3. A: PIP2 increases when phospholipase C (PLC) is inhibited during postischemic reperfusion. B: isolated perfused rat hearts, both intact and norepinephrine depleted, were labeled with [3H]inositol and subsequently subjected to 20 min of zero-flow ischemia followed by reperfusion at 7 ml/min for 2 min. [3H]Ins(1,4,5)P3 and [3H]PIP2 were extracted and quantified. Open bars, values after 20 min ischemia (isch); solid bars, catecholamine-depleted hearts (reserpine); gray bars, catecholamine-depleted hearts with added 100 μM norepinephrine (res + nor); hatched bars, intact hearts, no reserpine (intact); crosshatched bars, intact hearts treated with 1.5 mM gentamicin. Values are expressed as [3H]CPM/g wet wt; means ± SE, n = 6. *P < 0.05 and **P < 0.01 relative to 20 min ischemia.
PLCβ1b (150 and 140 kDa mol mass, respectively) described in other cells types (4) are detectable in neonatal rat cardiomyocytes but not in caveolae from adult rat heart (Fig. 4). Gaq was found in the caveolar fraction as well as in higher density membranes, although relative to protein content Gaq was highly enriched in the caveolar fraction (Fig. 4, Table 1). In marked contrast to PLCβ1b, membrane fractions contained relatively little PLCβ3 (only 1–5% of the total cellular content of PLCβ3), and this was not highly enriched in caveolae (Fig. 4). Other proteins found in the caveolar fractions included PLCβ1, epidermal growth factor receptors, and PI(4)P 5-kinase1α. PI(4)P 5-kinase1β was not detected in rat hearts (data not shown).

α1-AR are not concentrated in caveolae in rat hearts. The caveolar localization of α1A- and α1B-AR was then assessed. Overall rat heart membranes contained 745.1 ± 161.8 fmol/heart of α1-AR, and of these, 79.3 ± 7.3% were the α1B-subtype (means ± SE; n = 6). However, only 27.3 ± 6.9% of the total α1-AR complement was found in the caveolar fractions (Fig. 5, Table 1) where [3H]PIP, [3H]PIP2, and PLCβ1b are concentrated (Fig. 4, Table 1). There was no difference between the α1A- and α1B-subtypes in terms of localization (Fig. 5). The small percentage of α1-AR located in the caveolar

Table 1. Percentage of Gaq, PLCβ1, and α1-AR in caveolae relative to total membrane content

<table>
<thead>
<tr>
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<th>Normoxia</th>
<th>Normoxia/Nor</th>
<th>Ischemia</th>
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<tr>
<td>Gaq</td>
<td>56.0 ± 8.1 (3)</td>
<td>61.1 ± 6.4 (3)</td>
<td>46.6 ± 11.7 (3)</td>
<td>40.0 ± 7.7 (3)</td>
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<td>PLCβ1</td>
<td>91.0 ± 8.6 (4)</td>
<td>84.1 ± 6.8 (3)</td>
<td>89.8 ± 2.1 (3)</td>
<td>85.2 ± 4.6 (4)</td>
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<tr>
<td>α-ARs</td>
<td>27.3 ± 6.9 (6)</td>
<td>39.3 ± 12.9 (4)</td>
<td>35.5 ± 7.4 (5)</td>
<td>16.6 ± 5.8* (6)</td>
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[3H]Inositol-labeled rat hearts were subjected to 20 min ischemia followed by 2 min ischemia-reperfusion (I/R) or to 2 min stimulation with 100 μM norepinephrine (Nor) in normoxia. Membrane fractions of different buoyant densities were separated. Content of the α1-adrenergic receptor (AR), Gaq, and PLCβ1 were measured in the caveolar fractions (9–12 ml from the bottom of gradient) and in noncaveolar fractions (1–8 ml). Values shown are the total content of the protein in caveolae expressed as a percentage of total membrane content (not the total cellular content). Values shown are % in caveolae, means ± SE; n values are indicated in parenthesis. *P < 0.05 relative to 20 min ischemia.
fraction along with its substrate PIP2 and its immediate signaling proteins Goq and PLCβ1 suggested that localization may limit the activity of α1-AR signaling.

We next examined whether norepinephrine stimulation caused movement of α1-AR into caveolae or alternatively moved Goq or PLCβ1 to membranes of higher density where most of the α1-AR are located. [3H]inositol-labeled rat hearts were subjected to ischemia-reperfusion, and membrane fractions were separated on the basis of their buoyant densities. α1-AR (ligand binding), PLCβ1, and Goq (Western blots) were measured in the same membrane fractions as used for inositol phospholipid quantifications (shown below). Western blots were quantified, and the percentage of the particular protein in the caveolar fractions was estimated relative to the content of all membrane fractions added together. As shown in Table 1, stimulation with 100 μM norepinephrine for 2 min in normoxia did not cause any detectable change in the distribution of any of these proteins. Reperfusion for 2 min after 20 min ischemia, however, caused a selective loss of α1-AR from the caveolar fraction. Loss of α1-AR from caveolae, rather than providing an explanation for the heightened response in reperfusion, most likely reflects receptor desensitization (45).

Ischemia-reperfusion increases PIP2 in caveolar fractions. The localization of the [3H]PIP2 that increases during postischemic reperfusion was then assessed. [3H]inositol-labeled hearts were subjected to 20 min ischemia, followed by 2 min reperfusion, and membrane fractions were prepared as described above. Neither 20 min ischemia nor 2 min postischemic reperfusion caused any change in the caveolar content of PIP2 in norepinephrine replete hearts (Fig. 6B). However, when catecholamine-depleted hearts were used, [3H]PIP2 increased in the caveolar fractions over the 2-min period of reperfusion (Fig. 6A), but there was no increase in [3H]PIP2 in noncaveolar fractions. This implies that activation of PLC under reperfusion conditions reduces the [3H]PIP2 in the caveolar fractions by hydrolyzing it to [3H]Ins(1,4,5)P3. To confirm that norepinephrine stimulation for 2 min reduces [3H]PIP2 in caveolae, similar experiments were performed using [3H]inositol-labeled hearts under normoxic conditions. Stimulation with 100 μM norepinephrine for 2 min caused a reduction in [3H]PIP2 in the caveolar fractions (Fig. 6C). Thus our data suggest that postischemic reperfusion increases the generation of PIP2 in caveolae independently of norepinephrine release and α1-AR activation. This increase in PIP2 in caveolae, where Goq and PLCβ1 are concentrated, potentially provides substrate for activated PLC, thereby reducing the caveolar PIP2 content by generating Ins(1,4,5)P3.

DISCUSSION

Reperfusion of ischemic rat or mouse hearts causes norepinephrine release from the cardiac sympathetic nerves (40), activation of α1-AR (42), and consequent generation of Ins(1,4,5)P3 by PLC hydrolysis of PIP2 (1). The rise in Ins(1,4,5)P3 is associated with the onset of arrhythmias that can be prevented by inhibiting PLC activity (13, 24) or by depleting the hearts of norepinephrine. α1-AR-mediated Ins(1,4,5)P3 generation is markedly enhanced over the first 3 min after initiation of reperfusion compared with responses in normoxic myocardium, and we sought to define the mechanisms responsible. Ins(1,4,5)P3 is generated from the sarcemembral phospholipid PIP2 following activation of α1-AR, Goq, and PLCβ. In the current study we addressed the possibility that ischemia and reperfusion alter the availability of PIP2 for PLCβ-mediated hydrolysis to Ins(1,4,5)P3.

In heart membranes, we showed that PIP2 and its precursors PIP and PI are localized in the caveolar fractions along with caveolin-3 (Fig. 4). Membrane-bound PLCβ1b was also localized exclusively in these fractions (Fig. 4, Table 1) where it is well placed to hydrolyze PIP2 to Ins(1,4,5)P3. Rat heart caveolae exhibited only the lower molecular weight “b” splice variant of PLCβ1 (4), in contrast to neonatal rat cardiomyocytes that express both PLCβ1a and PLCβ1b (3). In the current study we addressed the possibility that ischemia and reperfusion alter the availability of PIP2 for PLCβ-mediated hydrolysis to Ins(1,4,5)P3.
brane fractions, although relative to protein content, it was highly concentrated in the caveolar fractions (Fig. 4, Table 1). Thus in the rat heart, most of the factors required for generation of Ins(1,4,5)P$_3$ are concentrated in caveolae. For this reason it was surprising that $\alpha_1$-AR were largely located outside of these fractions (Fig. 5, Table 1). This is especially puzzling in light of a recent study showing close association between PIP$_2$ and fractions (Fig. 5, Table 1). This is especially puzzling in light of a recent study showing close association between PIP$_2$ and PLC-coupled receptors in atrial myocytes (10). Whereas heavier membrane fractions containing $\alpha_1$-AR also contained Goq, they were essentially devoid of PLC$_{B1}$ as well as the lipid substrates PIP and PIP$_2$. One possible consequence of this is that the majority of the $\alpha_1$-AR population in the heart is unable to activate PLC. If this is so, it may explain the comparatively weak PLC response to $\alpha_1$-AR activation in heart preparations compared with other cell types (6, 34, 49). Another possibility is that either the $\alpha_1$-AR or the lipids translocate with stimulation. Our studies, however, provided no evidence to suggest movement of $\alpha_1$-AR into caveolae or movement of PLC$_{B1}$ or PIP$_2$ to membranes of higher density when $\alpha_1$-AR were stimulated.

The current studies involve the use of a perfused rat ventricle that contains a number of other cell types in addition to cardiomyocytes. We have previously shown that PLC responses in this model reflect primarily responses in cardiomyocytes (52). Specifically, we detected no measurable response to angiotensin II or compound 48/80, strong stimuli of vascular smooth muscle and mast cell, respectively. $\alpha_1$-AR are not detected on mast cells or cardiac fibroblasts and thus contribution to norepinephrine-dependent responses from these cell types can be discounted. Furthermore, PLC responses in cardiomyocytes differ in terms of rate and in inositol phosphate isomers generated from responses in other cell types (51).

Having identified caveolae as the site where PIP$_2$ is hydrolyzed by PLC$_{B1}$ to generate inositol phosphates, we next examined how signaling factors in caveolae were influenced by ischemia and reperfusion. Importantly, we found that postschismic reperfusion, but not ischemia itself, caused a selective loss of both $\alpha_1$-AR from caveolae (Table 1), even though Ins(1,4,5)P$_3$ generation is maximal under these conditions (Fig. 2B). Rather than contributing to the heightened activity, this loss of $\alpha_1$-AR most likely reflects desensitization, although treatment with maximal concentrations of norepinephrine did not cause such changes during normoxia (Table 1). Such a conclusion is consistent with reports showing movement of $\beta_2$-AR out of caveolae following agonist stimulation of isolated cardiomyocytes (38).

The current studies show that postschismic reperfusion causes heightened generation of PIP$_2$ in caveolae independently of norepinephrine release and $\alpha_1$-AR activation (Fig. 6). This PIP$_2$ is available for $\alpha_1$-AR initiated hydrolysis when stimulated by norepinephrine released under these pathological conditions. Thus the heightened availability of caveolar PIP$_2$ may contribute to the enhanced generation of Ins(1,4,5)P$_3$ and thereby to the initiation of arrhythmias under conditions of ischemia and reperfusion. Ins(1,4,5)P$_3$ causes Ca$^{2+}$ release from receptors localized on the sarcoplasmic reticulum and the perinuclear membrane (5, 29). Subsarcolemmal IP$_3$ receptors on the sarcoplasmic reticulum are clearly present in atrial myocytes (29), but their expression is undetectably low in ventricle. The possibility remains that ischemia causes an increase in subsarcolemmal IP$_3$ receptor. The conducting myocytes express a higher concentration of IP$_3$ receptor (type 1) than working myocytes (46, 19), and some of these are subsarcolemmal and thus may contribute to arrhythmogenesis. Even though the expression of IP$_3$ receptor is low in heart, there is evidence that IP$_3$ receptor activation can perturb the Ca$^{2+}$-induced Ca$^{2+}$ release program orchestrated by the more prevalent ryanodine receptors and lead to increased Na$^+$/Ca$^{2+}$ exchange (30), a possible mediator of the observed arrhythmias. Furthermore, removal of the type 2 IP$_3$ receptor, the subtype expressed in working cardiomyocytes (17), prevented arrhythmogenic responses to PLC activation in mouse atrial myocytes (27). Importantly, increased IP$_3$ receptor expression is seen in failed human ventricular tissue (18) and in atrial samples from patients predisposed to atrial fibrillation (54), suggesting a contribution of Ins(1,4,5)P$_3$ to human pathology. Whereas our studies and those of others have pointed to Ins(1,4,5)P$_3$ as an initiator of arrhythmias, some recent studies have suggested the possibility of a protective role against postschismic infarction (35, 36, 48). This may explain our previous finding that the absence of Ins(1,4,5)P$_3$ generation in mouse hearts expressing constitutively active $\alpha_1$B-AR reduced arrhythmias but did not limit infarction (16, 21).

In conclusion, the current studies show that brief periods of ischemia and reperfusion in rat hearts cause a number of changes in the caveolar fraction of rat heart sarcolemma that may be related to enhanced Ins(1,4,5)P$_3$ generation. Of these, the loss of caveolar $\alpha_1$-AR likely reflects heightened receptor activation under these conditions. However, the increased synthesis of PIP$_2$ in caveolae in early reperfusion may be important as a source of substrate for the generation of the Ins(1,4,5)P$_3$ when $\alpha_1$-AR are activated following release of norepinephrine from the sympathetic nerves.

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