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Phosphatidylinositol-bisphosphate regulates intercellular coupling in cardiac myocytes

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Abstract

Changes in the lipid composition of cardiac myocytes have been reported during cardiac hypertrophy, cardiomyopathy, and infarction. Because a recent study indicates a relation between low phosphatidylinositol-bisphosphate (PIP_2) levels and reduced intercellular coupling, we tested the hypothesis that agonist-induced changes in PIP_2 can result in a reduction of the functional coupling of cardiomyocytes and, consequently, in changes in conduction velocity. Intercellular

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coupling was measured by Lucifer Yellow dye transfer in cultured neonatal rat cardiomyocytes. Conduction velocity was measured in cardiomyocytes grown on microelectrode arrays. Intercellular coupling was reduced by angiotensin II (43.7±9.3%, *N*=11) and noradrenaline (58.0±10.7%, *N*=11). To test if reduced intercellular coupling after agonist stimulation was caused by PIP_2-depletion, myocytes were stimulated by angiotensin II (57.3±5.7%, *N*=14) and then allowed to recover in medium with or without wortmannin (an inhibitor of PIP_2 synthesis). Intercellular coupling fully recovered in control medium (102.1±8.9%, *N*=10), whereas no recovery occurred in the presence of wortmannin (69.3±7.8%, *N*=12). Inhibition of PKC, calmodulin, or arachidonic acid production did not affect the response to either angiotensin II or noradrenaline. Furthermore, decreasing or increasing PIP_2 also decreased and increased intercellular coupling, respectively. This supports the role of PIP_2 in the regulation of intercellular coupling. In beating myocytes, conduction velocity was reduced by angiotensin II stimulation, and recovery after wash out was prevented by inhibition of PIP_2 production. Reductions in PIP_2 inhibit intercellular coupling in cardiomyocytes, and stimulation by physiologically relevant agonists reduces intercellular coupling by this mechanism. The reduction in intercellular coupling lowered conduction velocity.

Keywords

Connexin; Gap junctions; PIP2; Cardiomyocytes

Introduction

Arrhythmias after acute myocardial infarction can be divided into distinct phases based on the time of their occurrence. The acute arrhythmias are divided into phase 1a (early) and phase 1b (late) arrhythmias. Phase 1b arrhythmias occur after approximately 12–30 min of ischemia [23] and correlate with compromised intercellular coupling [25, 46]. It has been suggested that changes in the intracellular milieu (for example calcium and pH [6, 34, 39]), lipid metabolites [19, 33, 55], and in phosphorylation [1, 4] status cause this reduction in coupling.

Another important event during phase 1b is the release of catecholamines by reversal of the reuptake mechanism. The role of this release is unclear, but its prevention or the inhibition of α-adrenergic receptors reduces the occurrence of arrhythmias [40, 44]. The proarrhythmic effect of α-adrenergic stimulation has been suggested to partly rely on inhibition of intercellular coupling [8]. The mechanisms by which Gαq-coupled receptors, such as α-adrenergic receptors, reduce coupling are unclear. These receptors are expressed in the heart; their agonists include noradrenaline, endothelin, thrombin, and angiotensin II. The receptors activate phospholipase C (PLC), which breaks down PIP_2, resulting in generation of diacylglycerol (DAG) and inositol trisphosphate (IP_3). These messengers activate protein kinase C (PKC) and releases calcium from intracellular stores, respectively.

Besides acting as precursor for the production of IP_3 and DAG, PIP_2 also acts as a signaling molecule itself. Hilgeman and coworkers showed that PIP_2 directly regulates the activity of cardiac Na/Ca exchangers [21]. Since then, several ion channels and transporters have been found to be regulated by PIP_2 (for review see [47]). PIP_2 is reported to regulate a number of channels that are expressed in cardiac tissues. These include inward rectifier potassium channels [22], hERG [5], and KCNQ channels [30].

Several studies indicate that PIP_2 levels are significantly reduced in the heart during stimulation by agonists such as adenosine [38], endothelin, bradykinin, and phenylephrine [10]. Furthermore, pathophysiological conditions such as ischemia are associated with

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reductions in PIP_2 [36], and similar decreases are observed during cardiac hypertrophy [13], cardiomyopathy [58], and heart failure subsequent to chronic infarction [49]. The pathophysiological role and significance of these findings for the electrophysiological properties of the heart has not yet been determined.

Moolenaar and coworkers [51] recently showed that connexin 43 (Cx43), the main protein subunit of cardiac gap junctions, is regulated by PIP_2, and reductions in the PIP_2 level resulted in reduced coupling in Rat-1 fibroblasts. Whether PIP_2 regulates intercellular coupling in cardiac myocytes is unknown, but if it does, it would contribute to the increased risk of arrhythmia in states of reduced PIP_2.

Therefore, the aim of this study was to investigate whether PIP_2 levels affect intercellular coupling in cardiac tissue. Furthermore, we tested whether stimulation of Gαq-coupled receptors relevant to cardiac tissue affect intercellular coupling and thereby conduction velocity via this mechanism.

Methods and materials

Isolation and culture of neonatal rat cardiomyocytes

Ventricular myocytes were isolated by a method modified from Simpson and Savion [45] by multiple rounds of trypsin digestions of ventricles from neonatal Wistar rats. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Detailed description of isolation procedure and culture conditions can be found in [7]. Experiments were performed on myocytes between days 4 and 8 in culture.

Cardiomyocytes were seeded onto collagen-coated slides (140,000 cells/cm^2). For collagen coating, collagen type VI (0.142 mg/mL in 0.1% acetic acid and 30% ethanol) was added to coverslips and incubated for 3 h at 37°C. Excess solution was removed and coverslips left to dry for 2–3 h.

Measurements of intercellular coupling by localized electroporation

Principle of EpiZap dye diffusion

Intercellular coupling in neonatal rat cardiomyocytes was measured using the EpiZap system (ASK Science, Kingston, Ontario, Canada). Coupling is measured as spread of the fluorescent dye Lucifer Yellow (LY) after localized electroporation of a subset of cells [43]. The principle is similar to that used in dye injections, but electroporation ensures delivery of dye to many cells. Therefore, the measurements reflect the coupling of a large population of cells instead of just one cell. The myocytes were beating spontaneously after electroporation, indicating that the treatment was well tolerated.

In the example shown in Fig. 1, the slide surface left of the white line is conductive, and cells growing on this surface are electroporated. The bright fluorescence of the cells in this area results from uptake of LY during electroporation. Dye was allowed to spread into neighboring myocytes for 2 min before images were captured at four different locations along the border line. The position of the border line was set to zero in each horizontal line of the image. Fluorescence was summed for each position and plotted. The resulting profile (Fig. 1, middle panel) shows high intensity in the electroporated cells (negative position values) which decays with increasing distance from the border. Intercellular coupling was quantified as mean intensity in region A divided by mean intensity in area B for comparison between experiments with different levels of electroporation. In 20 determinations, the ratio

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was 0.163±0.012 in control cells and 0.050±0.006 in cells treated with the gap junction uncoupler carbenoxolone (100 μmol/L, 30 min, $P<0.0001$ versus control in paired t test).

On each experimental day, double determination was performed in untreated cells and in carbenoxolone-treated (100 μmol/L, 30 min) cells. Relative coupling was determined as:

$$\text{Relative coupling} = 100\% \cdot \frac{\text{Ratio}_x - \text{Ratio}_{\text{carb}}}{\text{Ratio}_{\text{control}} - \text{Ratio}_{\text{carb}}},$$

where Ratio_X is the ratio of interest, $\text{Ratio}_{\text{carb}}$ is the ratio in carbenoxolone-treated cells, and $\text{Ratio}_{\text{control}}$ is the ratio in untreated cells. Thereby, 100% corresponds to the level of untreated myocyte cultures, and 0% corresponds to complete uncoupling.

Using this method, we determined the dose-inhibition curve to carbenoxolone (Fig. 1, bottom panel). This yielded an IC_{50} of 26 μmol/L which is in the expected range [11].

Experimental procedure

For each experimental condition, cardiomyocytes were incubated (37°C, 5% CO_2) in serum-free medium with test reagents. A 23-μl drop of LY solution (12 mg/mL) was applied on the cells and the electrode placed on top of the slide. Cells were electroporated three times by discharging a capacitor (1 μF, 15 V). After electroporation, slides were rinsed and images acquired 2 min later, using a Micromax camera (Princeton Instruments, Trenton, NJ, USA) mounted on a DMRE microscope with an I3 filter cube (Leica Microsystems, Heidelberg, Germany). Electroporation was performed in calcium-free buffer to avoid calcium overload. Buffer contained (in mmol/L): NaCl (135), KCl (4), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (10), $MgCl_2$ (0.8), NaH_2PO_4 (0.5), Glucose (5), ethylene glycol tetraacetic acid (1) and 10% fetal calf serum, pH adjusted to 7.4.

Measurements of conduction velocity

Multielectrode arrays (MEAs; Multi Channel Systems, Reutlingen, Germany) were used for field potential recordings from spontaneously active monolayers of neonatal rat cardiomyocytes [3, 20]. The MEAs used consisted of 60 electrodes (diameter 30 μm) which had an inter-electrode distance of 200 μm. MEAs were fibronectin coated (2.5 μg/ml in H_2O). Experiments were conducted at 37°C and data stored online and analyzed offline with a customized toolbox programmed for MATLAB (The Mathworks, Natick, MA, USA) [16, 20]. Activation time contour plots revealed direction of excitation spread in cultures, and conduction velocity was obtained perpendicularly to the excitation wave front.

Chemicals

All chemicals were from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. Candesartan was a gift from AstraZeneca (Gothenburg, Sweden).

Statistical analysis

Data are displayed as mean±SEM, and N denotes number of experiments on separately grown cell cultures. Statistical significance was tested by one-way analysis of variance (ANOVA) followed by Tukey's or Dunnet's Multiple Comparison Test (using GraphPad Prism 4.00) as indicated in figure legends.

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Results

Stimulation of $G_{\alpha q}$-coupled receptors reduces intercellular coupling

To stimulate Gαq-coupled receptors, cardiomyocytes were exposed to either angiotensin II (1 μmol/L) or noradrenaline (0.5 μmol/L) for periods ranging from 5 to 60 min. In all experiments with noradrenaline, propranolol (0.5 μmol/L) was added to prevent activation of β-adrenergic receptors. As shown in Fig. 2, both angiotensin II (N=10–11) and noradrenaline (N=9–11) decreased intercellular coupling in a time-dependent manner with maximal uncoupling after 10 min. Maximal uncoupling was 43.7±9.3% of control for angiotensin II and 58.0±10.7% for noradrenaline.

To test the specificity of the response, cardiomyocytes were pre-incubated with receptor antagonists, and the response to 10 min of agonist stimulation was evaluated. Pre-incubation with the AT1-receptor antagonist candesartan (20 nmol/L) abolished the response to angiotensin II (91.2±13.9%, N=9), showing that Gαq-coupled AT1-receptors mediate the observed response.

To evaluate the noradrenaline-induced signaling pathway, cells were pre-incubated with the α_1-adrenergic receptor antagonist prazosin (0.5 μmol/L). This abolished the effect of noradrenaline stimulation (116.3±15%, N=17 with prazosin) supporting a Gαq-coupled signaling pathway. Neither candesartan (106.1±6.5%, N=8) nor prazosin (104.8±14.0%, N=7) alone affected intercellular coupling.

Intercellular coupling is regulated by PIP_2

To test whether changes in PIP_2 regulate intercellular coupling per se, cardiomyocytes were exposed to a high concentration of wortmannin (20 μmol/L) for 1 h before electroporation. At this concentration, wortmannin inhibits the PI4 kinase [37] and thereby synthesis of PIP_2, which will result in a reduction of the plasma membrane pool of PIP_2 over time. To accelerate this process, serum (5%) was added to the medium. After wortmannin treatment, intercellular coupling was reduced to 52.4±8% (N=9, P<0.01, see Fig. 3), indicating that a reduction in PIP_2 inhibits intercellular coupling. This result shows that depletion of PIP_2 by wortmannin resulted in an inhibition comparable to that induced by agonist stimulation and could therefore, in principle, underlie the agonist responses.

In contrast, exposure of cardiomyocytes to a low concentration of wortmannin (0.5 μmol/L), which completely inhibits the PI3 kinase but not the PI4 kinase [37], did not affect intercellular coupling (94.0±9.92%, N=8, Fig. 3). This result indicates that inhibition was likely caused by a reduction in PIP_2 synthesis and not by PI3 kinase and its downstream signaling.

Hypertonic shock increases PIP_2 by activation of PI kinase in cell lines as well as in cardiac cells [38, 56]. To test whether an increase in PIP_2 increases intercellular coupling, cardiomyocytes were exposed to mannitol (250 mmol/L; 10 min), which increased intercellular coupling (145.0±14.3%, N=13, P<0.01, Fig. 3). Pre-incubation with wortmannin (20 μmol/L; 5 min) before the hypertonic shock, prevented the increase in intercellular coupling (98.3±17.4%, N=7). This result indicates that stimulation of PIP_2 synthesis was involved in the upregulation of intercellular coupling. Wortmannin treatment alone (15 min) did not reduce intercellular coupling. These experiments indicate that intercellular coupling is sensitive to both increasing and decreasing levels of PIP_2.

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The uncoupling by $G_{\alpha q}$-coupled receptor stimulation is mediated by PIP_2

To evaluate whether PIP_2 was involved in the uncoupling after receptor stimulation, experiments were conducted where cardiomyocytes recovered for 10 min after angiotensin II stimulation (1 μmol/L; 10 min) in angiotensin II-free medium. As shown in Fig. 4, stimulation by angiotensin II resulted in a reduction of intercellular coupling to 57.3±5.7% (*N*=14). This effect was reversible and intercellular coupling recovered completely within 10 min after removal of angiotensin II (102.1±8.9%, *N*= 10). However, when wortmannin (20 μmol/L) was added to the recovery medium, intercellular coupling remained reduced (69.3±7.8%, *N*=12). In contrast to prolonged exposures to wortmannin and serum (Fig. 2), wortmannin treatment for 10 min did not significantly affect intercellular coupling (94.7±11.4%, *N*=14). This result indicates that the recovery from inhibition by angiotensin II is dependent on the resynthesis of PIP_2, underlining that the response to this agonist is at least partly mediated by a reduction of PIP_2.

The role of PKC in uncoupling by Gαq-coupled agonists

Stimulation with angiotensin II and noradrenaline also activates PKC. Specific inhibition of PKC can be obtained by bisindolylmaleimide (BIM). To evaluate the role of PKC, cardiomyocytes were pretreated with BIM (100 nmol/L) for 5 min before stimulation with angiotensin II (1 μmol/L; 10 min) in the continued presence of BIM (Fig. 5). No significant change in the angiotensin II response was detected, with a coupling of 47.8±7.2% in the presence of angiotensin II + BIM (*N*=10) versus 42.7±5.6% with angiotensin II alone (*N*=10). Similar experiments showed that the reduction in intercellular coupling induced by noradrenaline was also unaffected by inhibition of PKC (51.9±14.5%, *N*=8 with BIM, versus 49.8±14.5% with noradrenaline alone, *N*=8, Fig. 5). Figure 5 also shows that propranolol alone does not affect intercellular coupling (102.5±16.6%, *N*=8). BIM itself did not affect intercellular coupling (96.7±11.8%, *N*=9 in medium and 86.0±24.5%, *N*=7 in medium with propranolol). These data indicate that the decrease in intercellular coupling after Gαq-coupled receptor stimulation is not mediated by PKC.

The role of calcium in uncoupling by Gαq-coupled agonists

Activation of phosholipase C also leads to the generation of IP_3, which induces the release of calcium from intracellular stores. Because gap junctions are sensitive to calcium, this could inhibit coupling. Lurtz and Louis [31] showed that inhibition by calcium is mediated by calmodulin, and to evaluate the role of this pathway, we used the calmodulin-inhibitor calmidazolium (CDZ, 5 μmol/L). Pretreatment with CDZ had no effect on the response to angiotensin II (1 μmol/L, 10 min; see Fig. 6). Angiotensin II reduced coupling to 45.9±19.0% (*N*=5), and the response in CDZ-treated cultures was not significantly different (59.9±8.5%, *N*=6). CDZ alone did not affect coupling (93.0±10.5%, *N*=6). Thus, we conclude that the decrease in intercellular coupling after agonist stimulation is not due to increased intracellular calcium.

Role of arachidonic acid in the response to Gαq-coupled agonists

An alternative explanation to the observed responses could be a build up of arachidonic acid (AA), which is known to directly inhibit intercellular coupling [19, 33]. AA could increase after stimulation of PLA_2, which releases AA directly from phospholipids, or by release from DAG by the action of DAG lipase and monoacylglycerol lipase [9].

To test the involvement of PLA_2 and DAG lipase, we employed the respective inhibitors arachidonyl trifluoromethyl ketone ($AACOCF_3$) and RHC80267. As shown in Fig. 7, 5 min pre-incubation with $AACOCF_3$ (50 μmol/L) did not affect the response to angiotensin II which was 47.9± 8.1% for angiotensin II alone (*N*=8) and 42.3±10.1% in the presence of

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$AACOCF_3$ (*N*=8). Similar results were observed with RHC80267 (25 μmol/L), where the response to angiotensin II was 53.7±14.9% in the presence of RHC80267 (*N*=8).

Also, the effect of noradrenaline was insensitive to inhibition of PLA_2 and DAG lipase. Noradrenaline reduced intercellular coupling to 61.7±3.9% alone (*N*=9), 50.9± 12.2% with $AACOCF_3$ (*N*=9), and 44.9±4.8% in the presence of RHC80267 (*N*=8).

Neither $AACOCF_3$ nor RHC80267 affected coupling when applied alone (data not shown). Data are summarized in Fig. 7, showing that AA is unlikely to mediate the observed reduction in coupling after stimulation of Gαq-coupled receptors.

Effect of Gαq-coupled agonists on conduction velocity

Neonatal cardiomyocytes grown on MEAs form spontaneously active monolayers. We determined the excitation spread and conduction velocity under control conditions and after 10 min incubation with angiotensin II (1 μmol/L, Fig. 8). Under control conditions, conduction velocity was 16.1±2.6 cm/s (*N*=8), but stimulation with angiotensin II reduced conduction velocity to 13.0±2.9 cm/s (*N*=8). This decrease was reversible after wash out of angiotensin II with a recovery of conduction velocity to 17.8±3.2 cm/s (*N*=8). Because changes in conduction velocity can also result from changes in the excitability of the cells, we further analyzed the effect of angiotensin II on the spontaneous activity, but we observed no significant change in the beating frequency during the application of angiotensin II (control=2.25±0.29 Hz; angiotensin II=2.16±0.30 Hz). These data indicate that the reduction in intercellular coupling after agonist stimulation had functional consequences by reducing action potential propagation. To test if, also in this case, the effect was mediated by metabolism of PIP_2, experiments were conducted in which wortmannin was included in the wash out medium. In these experiments, conduction velocity was 22.8±3.2 cm/s (*N*=5) in untreated cells, and stimulation with angiotensin II reduced conduction velocity to 17.4±2.2 cm/s (*N*=5). Wash out of angiotensin II in the presence of wortmannin only resulted in a partial recovery to 19.0±3.0 cm/s (*N*=5), indicating that resynthesis of PIP_2 was needed to reestablish normal conduction velocity. Thus, the decrease in intercellular coupling after reducing PIP_2 was sufficiently robust to affect the propagation of the impulse wave front.

Discussion

The aim of this study was to investigate whether PIP_2 levels affect intercellular coupling in cardiac tissue. We provide evidence that agonists acting on Gαq-coupled receptors reduce intercellular communication by lowering the levels of PIP_2 in the cell membrane. This is a novel mechanism that may partly explain the uncoupling observed during acute ischemia and other conditions where PIP_2-levels are reduced. The resulting reduction in conduction velocity could increase the susceptibility to cardiac arrhythmia.

All tested conditions expected to change the level of PIP_2 were associated with corresponding changes in intercellular coupling. This was the case not only for interventions expected to reduce PIP_2 such as prolonged wortmannin treatment or stimulation of Gαq-coupled receptors, but also for hypertonic shock which has been shown to increase PIP_2 [38] via stimulation of PIP5 kinase [56]. These independent approaches indicate that PIP_2 regulates intercellular coupling.

Stimulation of Gαq-coupled AT1 and α_1-adrenergic receptors reduced intercellular coupling in a time-dependent manner. Maximal reduction was seen after 10 min of incubation and was of the same magnitude as observed when PIP_2 was reduced by wortmannin. The partial recovery beyond the 10-min time point likely reflects internalization of receptors or

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activation of compensatory resynthesis of PIP_2. In the case of angiotensin II, its low stability may also have reduced its effective concentration over time.

The inhibition of intercellular coupling after stimulation of Gαq-coupled receptors was likely caused by reduced PIP_2 levels in the plasma membrane because recovery from inhibition of intercellular coupling and conduction velocity was sensitive to the PI4-kinase inhibitor wortmannin. Wortmannin was originally found to be an inhibitor of the PI3-kinase when used in concentrations in the nanomolar range [42]. However, it was later discovered that higher concentrations led to inhibition of the PI4-kinase [37]. This kinase is essential in the synthesis of PIP_2 and mediates phosphorylation of PI in the 4′ position, generating PIP, which is further phosphorylated to PIP_2. At lower concentrations, wortmannin inhibits only the PI3-kinase, an essential component in the activation of the akt/PKB signaling cascade. However, the inhibitory effect of wortmannin was not mediated by PI3-kinase, as low concentrations of the compound did not affect intercellular coupling. Wortmannin inhibits other proteins besides the PI3 and -4 kinases. Myosin light chain kinase and Polo-like kinase can be inhibited by wortmannin; but as for PI3 kinase, they are inhibited by low concentrations [2] and therefore unlikely to be involved.

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Wortmannin has been used extensively to document the involvement of PIP_2 in the regulation of many membrane proteins. In some of these studies, other approaches, such as application of PIP_2 to the inside of macropatches [21, 57] or expression of components of the PIP_2 metabolic pathways [48, 54], confirmed the involvement of PIP_2. Moolenaar and coworkers recently showed that activation of Gαq-coupled receptors in Rat-1 fibroblasts reduced Cx43-mediated intercellular coupling [51]. This reduction was dependent on a ZO-1 mediated interaction between Cx43 and PLC-β3. Furthermore, they showed that specific activation of PIP5-phosphatase reduced coupling and that overexpression of PIP5-kinase reduced the ability of Gαq-coupled receptors to inhibit intercellular coupling. The study thus supports the concept that PIP_2 regulates intercellular coupling, which is further substantiated by the present study on intercellular coupling in cardiac myocytes.

The suggestion that the sole effect of wortmannin is prevention of the resynthesis of PIP_2 has important implications. The lack of recovery after angiotensin II stimulation in the presence of wortmannin indicates that the inhibition of intercellular coupling is mainly due to a reduction of PIP_2. This is a surprising conclusion because of the large volume of evidence that activation of PKC leads to hyperphosphorylation of Cx43 and inhibition of coupling measured by dye diffusion (for review see [27]).

Originally, uncoupling due to PKC activation was shown using phorbol esters as activators [18], and many subsequent studies have used this approach. Phorbol esters such as TPA activate a number of PKC subtypes [32] by mimicking DAG binding. The reduction in dye transfer after stimulation with TPA correlates with phosphorylation of serine 368 in the Cx43 C-terminus, and mutation of this serine to alanine abolishes the effect of TPA [28].

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We found that stimulation via specific Gαq-coupled receptors reduces intercellular coupling independently of PKC, as the response to angiotensin II and noradrenaline was unaffected by PKC inhibition. The reason for this lack of PKC involvement could be that stimulation via specific receptors leads to a subthreshold level of activation of PKC or that these receptors activate specific subtypes of PKC that either do not affect intercellular coupling or have opposing effects. Studies have shown that different PKC subtypes may exert different effects on intercellular coupling. Along these lines, PKC-α reduces intercellular coupling in cardiac cells after stimulation by growth factors [15], whereas PKC-α increases intercellular coupling after stimulation with anti-arrhythmic peptide analogs [14, 53]. Lack of PKC

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involvement after stimulation of Gαq-coupled receptors was also reported by Postma et al. [41].

Electroporation was performed in calcium-free solution to avoid calcium overload. This means that calcium was likely to be low during the actual measurement of coupling. This does, however, not exclude that IP_3 could increase calcium by activation of IP_3 receptors in the sarcoplasmic reticulum. Other studies show that calcium increases in cultured neonatal rat cardiac myocytes after stimulation with, for example, angiotensin II [24, 50]. Lurtz and Louis [31] showed that the effect of calcium is mediated by calmodulin. Therefore, we tested the possible involvement of calcium by inhibiting calmodulin with CDZ. This did not affect the response to angiotensin II, and therefore, we conclude that calcium is not involved in the observed inhibition of intercellular coupling.

Activation of Gαq-coupled receptors can activate release of AA either directly by activating PLA_2 or indirectly by producing DAG which can be further metabolized to AA in a two-step reaction by diacylglycerol lipase and mono-acylglycerol lipase [9]. AA directly inhibits intercellular coupling [19, 33] and could represent an alternative to PIP_2 as a mediator of the response to Gαq-coupled receptors. However, inhibition of PLA_2 and DAG lipase did not affect the reductions in intercellular coupling seen after stimulation with angiotensin II or noradrenaline, making it unlikely that AA plays a role in the response.

Previous studies have shown that the ability to pass dyes and current do not necessarily correlate [17, 26]. In the present study, however, changes in intercellular coupling after angiotensin II stimulation, measured as dye diffusion, were accompanied by a conduction velocity reduction, indicating that electrical coupling was also reduced. This is supported by a study by De Mello showing that acute angiotensin II stimulation reduces electrical coupling in freshly isolated hamster cardiomyocytes [12].

Decreased conduction velocity can also be induced by inhibition of voltage-dependent sodium or calcium channels. The constant beating frequency of the monolayers even in the presence of angiotensin II implies that voltage-dependent ion channels are not affected by agonist stimulation. Although we suspect that noradrenaline would decrease conduction velocity, as angiotensin II does, this was not tested in the current study.

An acute and massive stimulation of Gαq receptors occurs during ischemia, where reversal of uptake mechanism for noradrenaline leads to liberation of large amounts of this agonist. This condition could be worsened by release of other substances such as endothelin, ATP, and thrombin. On top of these agonists that can potentially reduce PIP_2, other factors such as lysophosphatidylcholine [29] and oxidative stress [35] reduces PIP_2 levels. The risk of reentry arrhythmia is increased whenever the wavelength (conduction velocity×refractory period) of the excitation is reduced. We did not investigate the effect of agonist stimulation on refractory period, but the slowing of conduction velocity would decrease the wavelength. If this occurs, it could increase the risk that the heart can harbor a reentrant circuit. Also, in the case of adrenergic stimulation during phase 1b, Verkerk and coworkers suggested that the reduced coupling enhances development of afterdepolarizations [52]. This way, we speculate that changes in intercellular coupling downstream of PIP_2 changes may also contribute to occurrence of phase 1b arrhythmias by ectopic mechanisms.

We conclude that stimulation of Gαq-coupled receptors reduces intercellular coupling in cultured neonatal rat cardiac myocytes by reducing levels of PIP_2 in the plasma membrane. Whether this mechanism plays a physiological role in cardiac cells in situ remains to be investigated. But if so, it could play an important role in several pathological conditions where the risk of arrhythmias is increased by impaired coupling.

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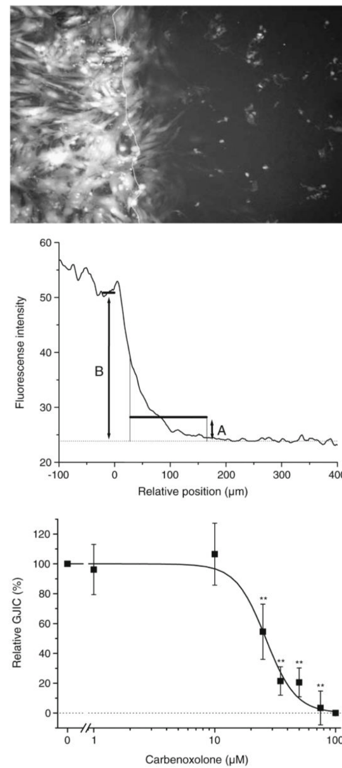

Fig. 1.

Measurements of intercellular coupling by electroporation of Lucifer Yellow. *Top panel*: Fluorescence image showing cardiomyocytes after electroporation. The *white line* indicates the border between the conductive (*left*) and *nonconductive* surface of the slide. Cells growing on the conductive layer are electroporated and show bright fluorescence. The *fluorescence right of the line* is caused by diffusion of dye through gap junctions. *Middle panel*: Quantification of images. The border position was set to zero in each *horizontal line* in the image. Fluorescence was summed for each position and plotted in the graph. The ratio between average intensity in region A and average intensity in region B was used to calculate relative coupling. *Bottom panel*: Carbenoxolone dose-inhibition curve. Cardiomyocytes were exposed to varying carbenoxolone concentrations and coupling determined (N=4–9). IC_{50} was 26 μmol/L (Hill coefficient 3.5). Statistical analysis was performed by one-way ANOVA with Dunnet's test post hoc (versus control). ** signifies P<0.01

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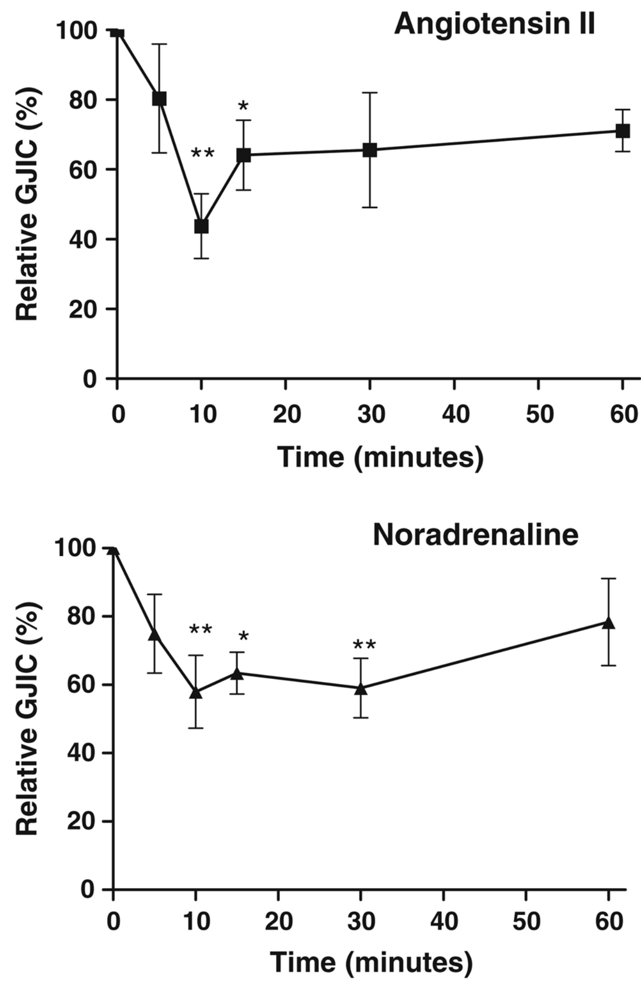

Fig. 2.

Angiotensin II and noradrenaline time-dependently reduce intercellular coupling. *Top panel*: Intercellular coupling as function of time during angiotensin II stimulation (1 μmol/L, *N*=10–11). *Bottom panel*: Intercellular coupling as function of time during noradrenaline stimulation (0.5 μmol/L, *N*=9–11). Propranolol (0.5 μmol/L) was included in noradrenaline experiments to prevent activation of β-adrenergic receptors. Statistical analysis was performed by one-way ANOVA with Dunnet's test post hoc (versus control). * signifies $P<0.05$ and ** $P<0.01$

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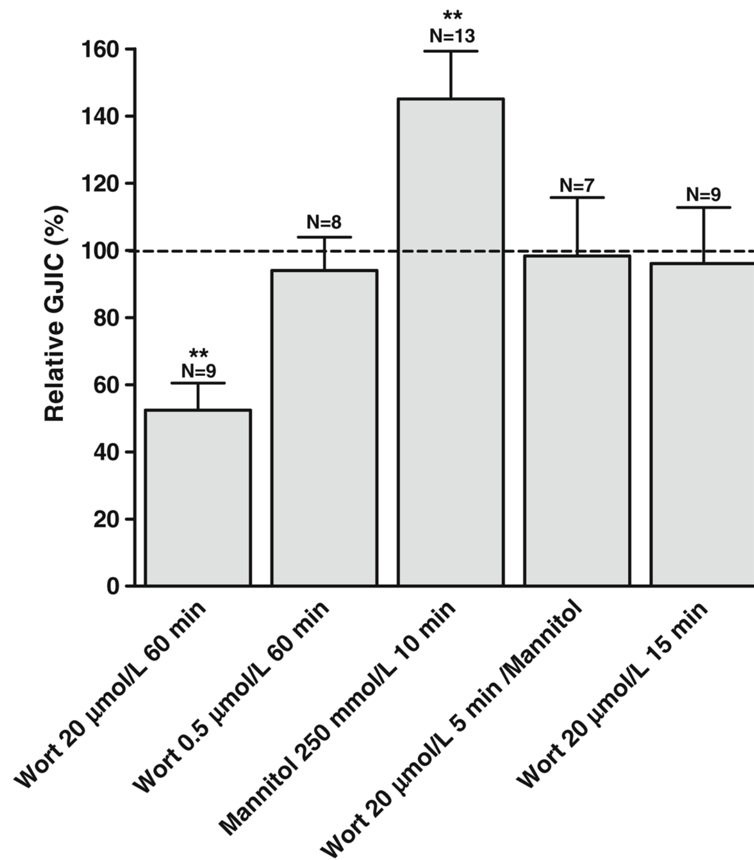

Fig. 3.

Effect of wortmannin and hypertonic shock on intercellular coupling. Wortmannin treatment for 60 min significantly reduced intercellular coupling at 20 μmol/L, but had no effect at 0.5 μmol/L. Hypertonic shock for 10 min increased intercellular coupling, and this effect was prevented by wortmannin (20 μmol/L, 5 min pre-incubation and during hypertonic shock). Incubation with wortmannin (20 μmol/L) alone for 15 min had no effect on intercellular coupling. *N* indicates number of experiments in each group. Statistical analysis was performed by ANOVA with Dunnet's test post hoc (versus control). ** signifies $P<0.01$

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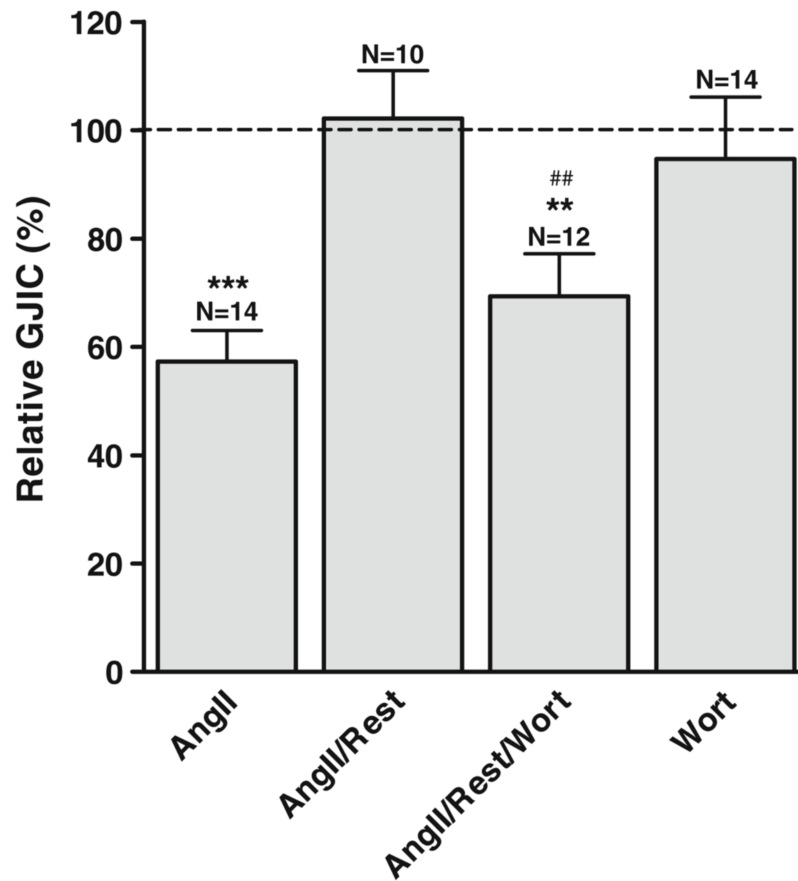

Fig. 4.

Recovery from angiotensin II stimulation is dependent on resynthesis of PIP_2. Stimulation by angiotensin II (1 μmol/L) reduced intercellular coupling, but 10 min of rest in angiotensin II-free medium resulted in full recovery. When wortmannin (20 μmol/L) was added to the medium during rest, recovery was prevented. Wortmannin alone for 10 min did not affect intercellular coupling. *N* indicates number of experiments in each group. Statistical analysis was performed by ANOVA with Tukey's multiple comparison test post hoc. ** signifies $P<0.01$ and *** $P<0.001$ versus control and ## $P<0.01$ versus angiotensin II/rest

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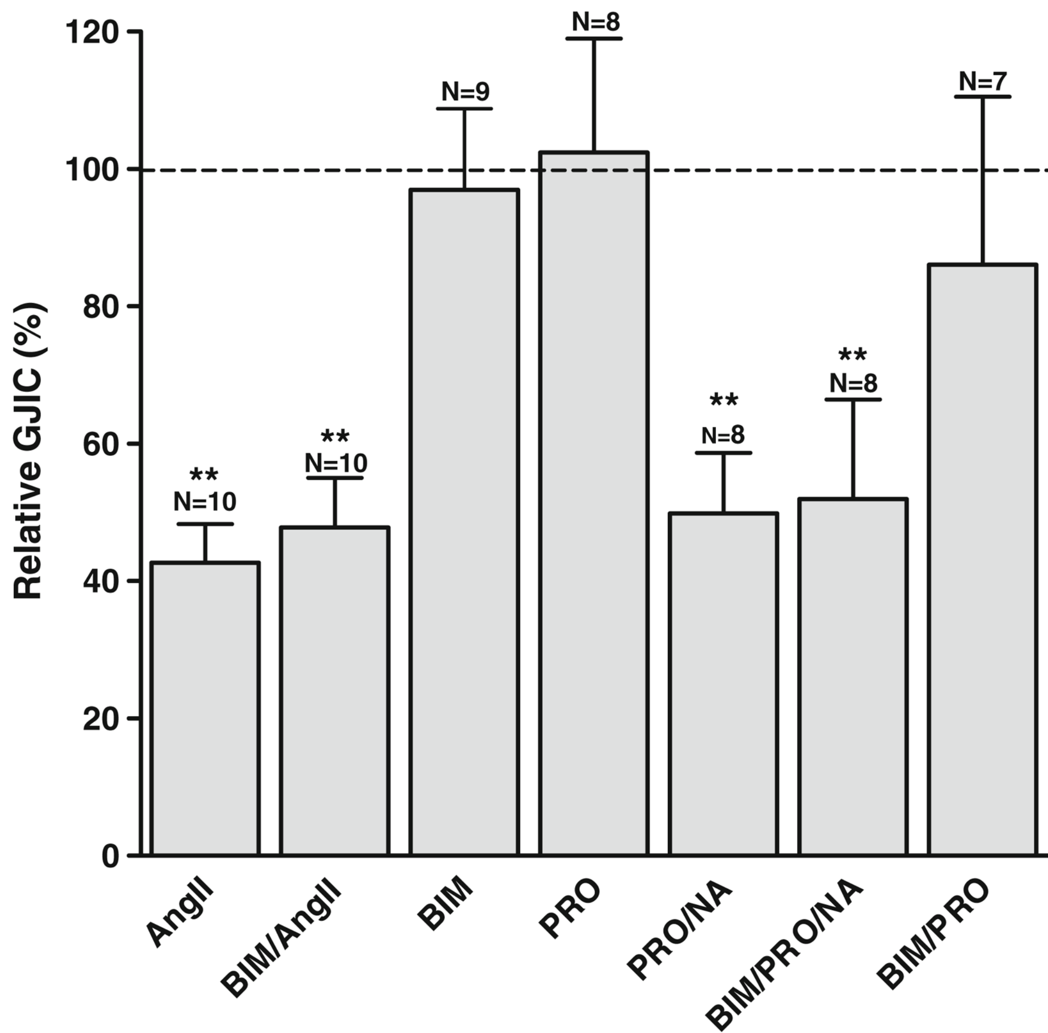

Fig. 5.

Inhibition of PKC does not affect the response to angiotensin II and noradrenaline. Angiotensin II (1 μmol/L) and noradrenaline [0.5 μmol/L, with propranolol (0.5 μmol/L)] reduced intercellular coupling, and this reduction was unaffected by inhibition of PKC by BIM (100 nmol/L). BIM alone did not affect intercellular coupling with or without propranolol (0.5 μmol/L). *N* indicates number of experiments in each group. Statistical analysis was performed by ANOVA with Dunnet's test post hoc (versus control). ** signifies $P<0.01$

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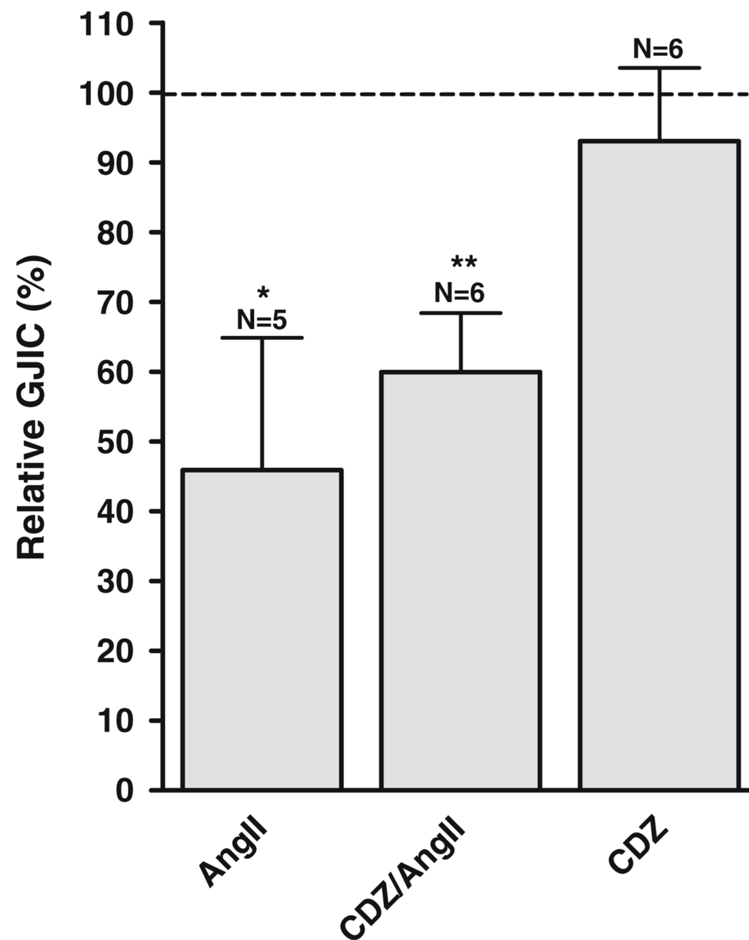

Fig. 6.
The decreased coupling after angiotensin II stimulation is not mediated by calmodulin. Angiotensin II (1 μmol/L) reduced intercellular coupling, and this reduction was unaffected by inhibition of calmodulin by calmidazolium (CDZ, 5 μmol/L). *N* indicates number of experiments in each group. Statistical analysis was performed by ANOVA with Dunnet's test post hoc (versus control). * signifies $P < 0.05$ and ** $P < 0.01$

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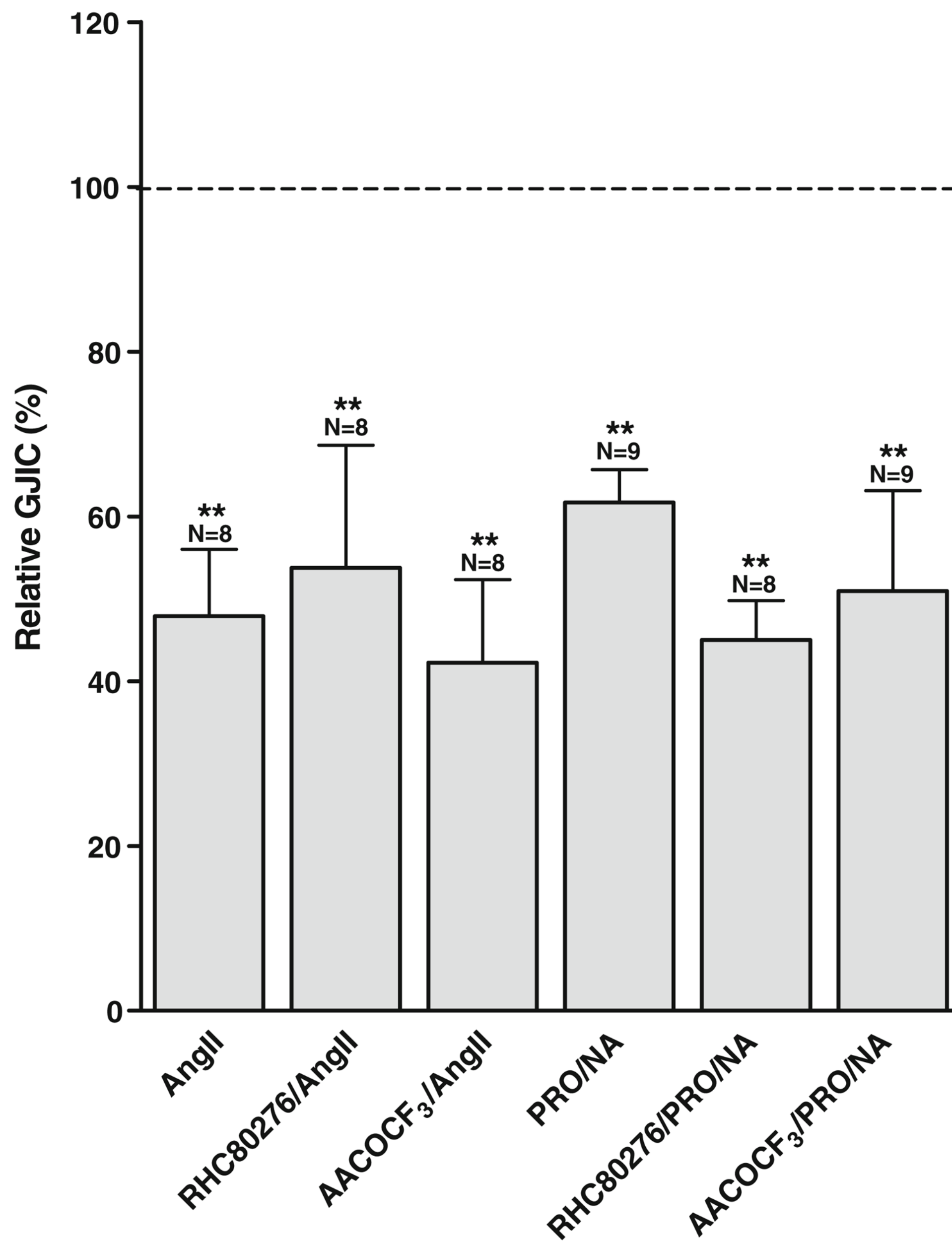

Fig. 7.

Arachidonic acid is not involved in the response to angiotensin II and noradrenaline. Angiotensin II (1 μmol/L) and noradrenaline (0.5 μmol/L, with propranolol (0.5 μmol/L)) reduced intercellular coupling, and this reduction was unaffected by inhibition of PLA_2 by $AACOCF_3$ (50 μmol/L) or DAG lipase by RHC80267 (25 μmol/L). *N* indicates number of experiments in each group. Statistical analysis was performed by ANOVA with Dunnet's test post hoc (versus control). ** signifies $P<0.01$

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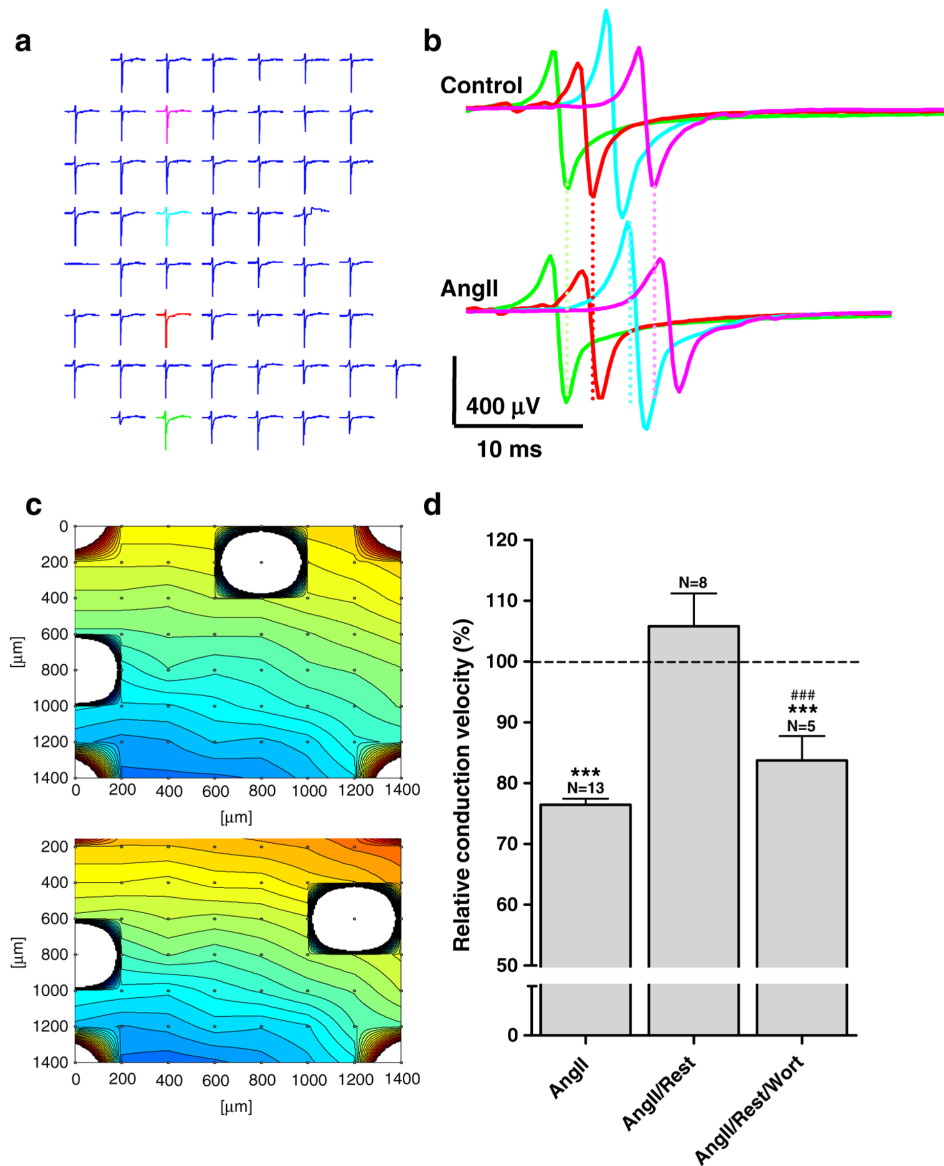

Fig. 8.

Angiotensin II reduces conduction velocity, and recovery is dependent on resynthesis of PIP_2. **a** 8×8 plot showing original voltage recordings from all 60 MEA electrodes. **b** The *electrodes marked in color* are superimposed for control (*top*) and angiotensin II conditions (*bottom*); *lines* illustrate the increasing delay of excitation spread between the two conditions. **c** Contour plots for the two conditions also show the decreased conduction velocity (0.18 m/s vs. 0.13 m/s) in the presence of angiotensin II. **d** Stimulation by angiotensin II (1 μmol/L) reduced conduction velocity, but 10 min of rest in angiotensin II-free medium resulted in full recovery. When wortmannin (20 μmol/L) was added to the medium during rest, recovery was prevented. Statistical analysis was performed by ANOVA with Tukey's multiple comparison test post hoc. *** signifies $P<0.001$ versus control and ### $P<0.001$ versus angiotensin II/rest