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Immobilization stress elevates IP_3 receptor mRNA in adult rat hearts in a glucocorticoid-dependent manner

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Abstract Gene expression of the type 1 and 2 inositol 1,4,5-trisphosphate (IP_3) receptors in the rat cardiac atria and ventricles and their possible modulation by single immobilization stress was studied. Single immobilization stress significantly elevated mRNA levels for both types of these receptors. To evaluate the involvement of glucocorticoids in the modulation of the gene expression of IP_3 receptors by immobilization stress, we used adrenalectomized and/or hypophysectomized rats. Since adrenalectomy and/or hypophysectomy completely abolished increase in IP_3 receptor's mRNA levels after the immobilization, we conclude that immobilization stress elevates mRNA of type 1 and 2 IP_3 receptors, mainly through the glucocorticoid responsive element.

Key words: Inositol 1,4,5-trisphosphate receptors; Rat heart; Atrial function; Ca-channels; Glucocorticoid modulation

1. Introduction

Variety of G-protein coupled receptors activates phospholipase C, thereby causing the intracellular production of the second messengers 1,2-diacylglycerol and inositol 1,4,5-trisphosphate (IP_3). IP_3 triggers the release of calcium from intracellular stores through IP_3 receptors (IP_3Rs). The heart contains a relatively low concentration of IP_3Rs [1]. By RNase protection assay it was shown that individual types of these receptors are not distributed uniformly. In the whole heart, type 1 IP_3R was much more abundant than type 2, while in purified ventricular myocytes type 2 of IP_3R predominates [2]. There is significant evidence that the type 1 receptor is expressed at high levels in the conduction system in the whole heart [3]. It has been suggested that different IP_3R subtypes may have distinct biological roles within a cell [4].

Physiological function of IP_3Rs in the heart is not clear. Responses to IP_3 are slow and weak, and the calcium so generated does not contribute to calcium-induced calcium release. Thus IP_3 is less likely to be important in regulating beat-to-beat changes in intracellular calcium under physiological conditions. Recent results suggested that calcium released from IP_3Rs activates ryanodine receptors [5]. However, activation of these receptors during some cardiac diseases was proposed, e.g. relationship between the generation of IP_3, and development of reperfusion arrhythmias was suggested [6]. Also, chronic mechanical overload of the atrial myocardium increased IP_3R expression especially in patients with chronic atrial fibrillation [7].

Stress is one of major contributors to the development of cardiovascular disorders and psychiatric illnesses. The physiological response to stress varies among individuals, because many factors influence the threshold at which stress becomes excessive, including genetics and prior experience of stress. Therefore, it is crucial to clarify the mechanisms involved in the conversion of brief beneficial responses to stressors into prolonged detrimental consequences. Immobilization stress belongs to severe stressors, since it activates both pathways of the sympathoadrenal system. We have already shown that in cardiac atria, single immobilization for 2 h increases significantly the gene expression of phenylethanolamine-*N*-methyltransferase, the enzyme converting norepinephrine to epinephrine [8]. This increase was glucocorticoid-dependent [8].

In this study, we have examined the expression of IP_3Rs of type 1 and 2 in the rat heart and correlated it with the IP_3R immunoprotein and IP_3-induced calcium release. We also studied, how immobilization stress affects the gene expression of these receptors, and whether glucocorticoids might be involved in relative changes in IP_3R gene expression.

2. Material and methods

2.1. Animals and immobilization

Male Sprague–Dawley rats (280–320 g, Suzfeld, Germany) 3 months old were used. Prior to experiments, animals were for 1 week housed four per cage in a controlled environment (22 ± 2°C, 12 h light/dark cycle, light on at 6:00 a.m.). Food and water were available ad libitum. The Ethic Committee of the Institute of Experimental Endocrinology approved all presented experiments.

Immobilization stress was performed as described previously [9]. Briefly, animals were immobilized tightly to a board for 2 h, afterwards transferred to their home cages and decapitated 3 h after the end of immobilization.

In specified experiments, bilaterally adrenalectomized (adrex) or hypophysectomized (hypox) Sprague–Dawley male rats were used. Adrenalectomized rats weighting about 252.0 ± 3.3 g and sham-operated rats (304.0 ± 3.4 g) were obtained from IFFA Credo Laboratories, France and were used for the experiment 10 days after the surgery. Hypophysectomy (body weight 237.0 ± 5.0 g) and sham-operation (body weight 362.0 ± 4.0 g) were performed by IFFA Credo Laboratories, France and animals were used for the experiment 20 days after surgery. All adrenalectomized and hypophysectomized rats received isotonic saline instead of drinking water. Appropriate sham-

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operated rats were used as a control group. Success of both ectomies was checked by visual control of adrenal or pituitary removal and by levels of plasma corticosterone. Both procedures clearly documented the complete extirpation of adrenals or pituitaries.

2.2. RNA isolation and relative quantification of mRNA levels by RT-PCR

RNA was isolated by the method of Chomczynski and Sacchi [10]. Reverse transcription was performed using Ready-To-Go You-Prime First-Strand Beads (AP Biotech) and pd(N)$_6$ primer. PCR specific for IP$_3$R1 was carried out afterwards using primers IP3R1A: 5′-GTG GAG GTT TCA TCT GCA AGC-3′ (position 1171–1190 in exon 1) and IP3R1B: 5′-GCT TTC GTG GAA TAC TCG GTC-3′ (position 1904–1923 in exon 2) yielding a 410 bp fragment, corresponding to the non-neuronal form of the IP$_3$ receptor [11]. To avoid amplification of the genomic DNA, these primers were spanning intron (in position 1271–1763, sequence according to Genazzani et al.[12]). For IP$_3$R2, the following primers were used: IP3R2A: 5′-GCT CTT GTC CCT GAC ATT G-3′ and IP3R2B: 5′-CCC ATG TCT CCA TTC TCA TAG C-3′, giving a 361 bp fragment [12]. After initial denaturation for 5 min, the PCR program included 35 cycles of the denaturation at 94°C for 1 min, annealing at 60°C for 1 min and polymerization at 72°C for 1 min. The number of cycles was determined by testing 15, 20, 25, 30, 35, 37 and 40 cycles (not shown), in order to be within linear range of amplification. Gene expression of IP$_3$ receptors was evaluated relatively to the housekeeper glyceraldehyde 3-phosphate dehydrogenase. Primers GPH1 and GPH2 (GPH1: 5′-AGA TCC ACA ACG GAT ACA TT-3′; GPH2: 5′-TCC CTC AAG ATT GTC AGC AGC AA-3′) were used to amplify a 309 bp fragment from each first strand sample. After denaturation at 94°C for 5 min, 30 cycles of PCR at 94°C, 60°C and 72°C for 1 min each were performed [13].

For the semi-nested PCR, additional primers for IP$_3$R1 and IP$_3$R2 were used: IP3R1AA: 5′-AGA GGA GCT TGA ACC AAG TC-3′ and IP3R2AA: 5′-CAT GCA TGA CTA TGC TCC AC-3′. As a negative control, amplification was performed on mRNA omitting the reverse transcription. PCR products were analyzed on 2% agarose gels and visualized by ethidium bromide. Intensity of the individual bands was evaluated by Image software. PCR products were purified using a Qiagen purification kit (Qiagen) and sequenced as described in Wang et al. [14] using the ^{32}P end-labeled primers.

2.3. Western blot analysis

Protein concentration was determined according to Lowry [15]. 50, 100 and 150 μg of protein extract from cardiac atria and ventricles were used for Western blot analysis, as described in [16]. Specific anti-IP$_3$-receptor antibody (rabbit, specific for the C-terminal domain of the IP$_3$ receptor; Calbiochem) was used for IP$_3$R determination. This antiserum shows cross reactivity with IP$_3$Rs isolated from a variety of cell types and tissues in human, mouse, rat, dog, pig and cow. After the incubation with the secondary anti-rabbit antibody conjugated to horseradish peroxidase (dilution 1:5000), bands were visualized by ECL (enhanced chemiluminescence, AP Biotech).

2.4. Preparation of microsomes and calcium release

Microsomes were prepared as described by Krizanova et al. [17]. Prior to calcium release studies, microsomes were sonicated 2×30 s on ice to obtain unilamellar vesicles. Calcium release was measured as described in Kocan et al. [11] using $^{45}Ca^{2+}$. Radioactivity was measured on Rackbeta counter.

2.5. Statistical analysis

Each value represents an average of at least five, mostly 8–15 animals. Results are presented as mean ± S.E.M. Statistical differences among groups were determined by one-way analysis of variance (ANOVA). Values of $P < 0.05$ were considered to be significant. For multiple comparisons, an adjusted t-test with P-values corrected by the Bonferroni method was used (Instat, GraphPad Software, USA).

3. Results

We detected the signal of type 1 and 2 IP$_3$Rs mRNA in both, cardiac atria and ventricles (Fig. 1, upper part). In the left (LA) and right (RA) atrium, a clear 410 bp band for IP$_3$R1 mRNA and 361 bp band for IP$_3$R2 mRNA appeared after the simple RT-PCR (Fig. 1, lanes N). However, in the left (LV) and right (RV) ventricles, bands obtained by simple RT-PCR were very faint. Thus, we performed additional semi-nested PCR (Fig. 1, lanes S) and observed a 339 bp band for IP$_3$R1 mRNA and 231 bp band for IP$_3$R2 mRNA in both, cardiac atria and ventricles. Quantification of the IP$_3$R1 and IP$_3$R2 mRNA relatively to the housekeeper GAPDH revealed the highest amount in the LA (Fig. 1, lower part, $n = 15$). In the RA, the amount of IP$_3$R1 mRNA was 8–10-times lower compared to the LA, while the amount of IP$_3$R2 in LA was only two-times higher compared to the RA. Very low amounts of IP$_3$R1 mRNA were observed in ventricles. To ensure that amplified fragments are of IP$_3$R

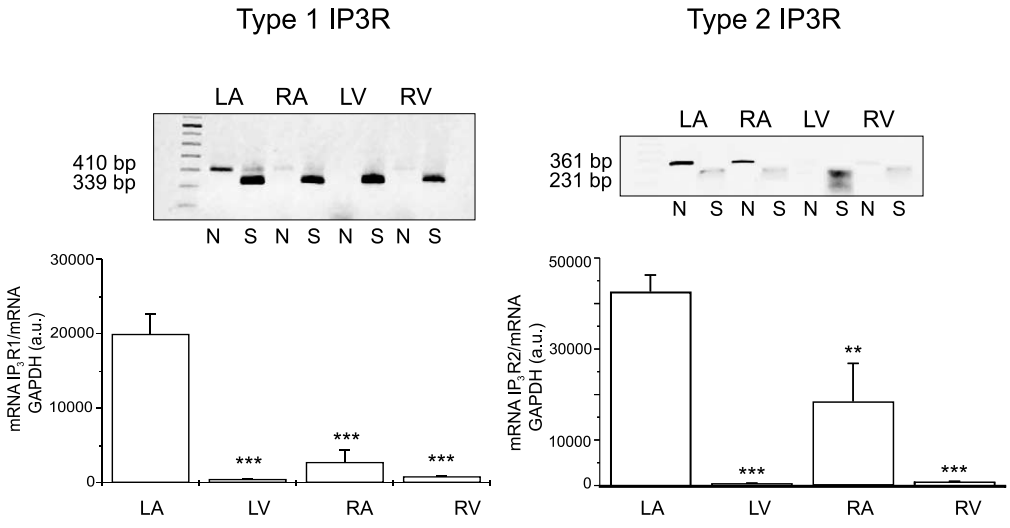

Fig. 1. Identification of type 1 (left) and type 2 (right) IP$_3$R mRNA in the LA, RA, LV and RV of the adult rat heart. In the upper part typical gels representing normal (N; IP$_3$R1 – 410 bp, IP$_3$R2 – 361 bp) and semi-nested (S; IP$_3$R1 – 339 bp, IP$_3$R2 – 231 bp) RT-PCR are shown. While in the atria (LA, RA) a clear visible signal was observed after simple RT-PCR, in ventricles the semi-nested PCR was required to prove the presence of both types of IP$_3$R mRNA. Quantification of the mRNA signal relatively to the housekeeper GAPDH revealed the highest amount of both types IP$_3$R mRNA in the LA. Results are presented as mean ± S.E.M. and each value is an average of 15 animals. Statistical significance represents $^{**}P < 0.01$ and $^{***}P < 0.001$. Significances were calculated relatively to the mRNA levels in the LA.

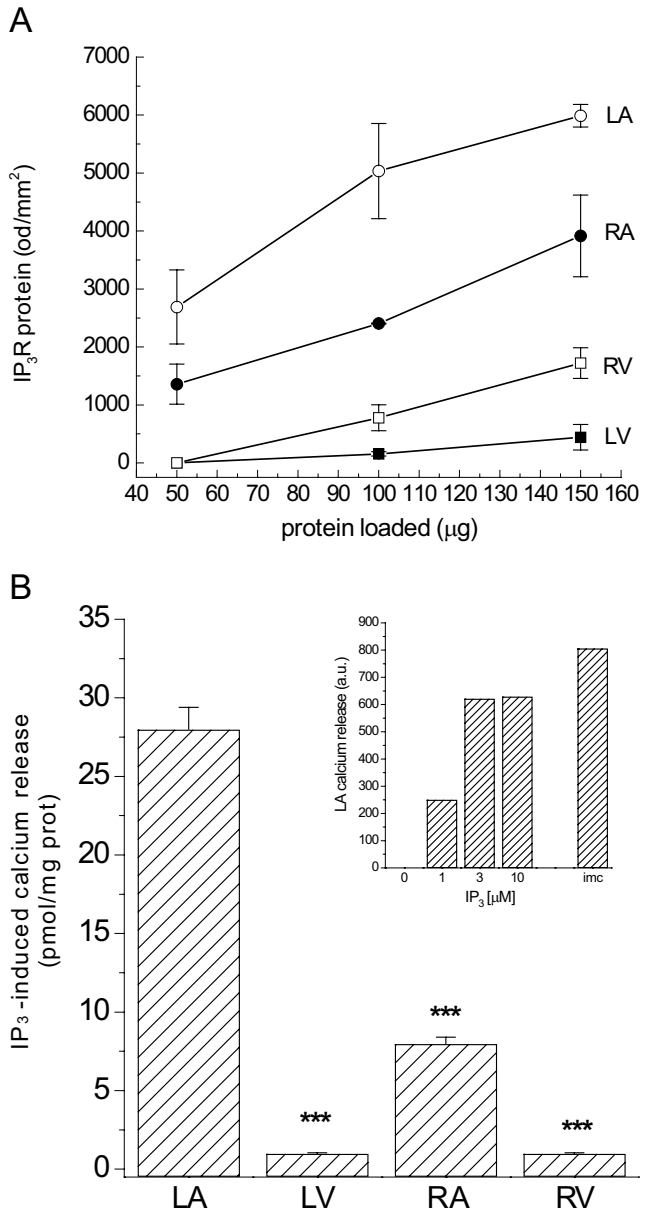

Fig. 2. Identification of IP_3R protein by Western blot analysis (A) and IP_3-induced calcium release (B) from microsomes of the LA, RA, LV and RV. For the Western blot analysis (A) we loaded three different concentrations of total protein (50, 100, 150 μg) on the gel. The highest amount of IP_3R we observed in the LA (empty circles), followed by the RA (solid circles). In the LV (solid square) and RV (empty square), significantly lower amounts of IP_3R were observed compared to atria. Inset in (B) shows the concentration-dependent IP_3-induced calcium release from the LA. In order to determine an optimal concentration of IP_3, we used 1, 3 and 10 μM IP_3. As a control of calcium release, 1 μM ionomycin (imc) was used. Calcium release was induced by 3 μM IP_3. Results are displayed as mean ± S.E.M. and each value represents an average of at least six animals. Statistical significance represents $***P < 0.001$. Significances were calculated relative to the values in the LA.

origin, we sequenced both PCR products and compared the obtained sequence with the sequences of type 1 and 2 IP_3R. We have found that PCR fragments were identical with these sequences (not shown).

Protein of IP_3R detected by Western blot analysis corresponds to the mRNA distribution (Fig. 2A). Since the antibody we used was not type-specific, the amount of IP_3R immunoprotein reflects the sum of all types of IP_3R, type 1, 2 and 3. In order to calculate the ratio of IP_3R protein in atria and ventricles, we loaded 50, 100 and 150 μg of total protein from each cardiac part (Fig. 2A). In the LA, we found approximately two-times more IP_3R protein compared to the RA. The significantly higher amounts of IP_3R were found in the LA and RA compared to the amount of these receptors in the LV and RV (Fig. 2A).

Finally, we measured the IP_3-induced calcium release from the cardiac microsomes isolated from each, LA, RA, LV and RV. To ensure that we used the proper concentration of IP_3, we tested different concentrations (1, 3 and 10 μM) of IP_3 on IP_3-induced calcium release (Fig. 2B, inset) from microsomes prepared from the LA. As a positive control, 1 μM concentration of ionophor ionomycin was used. Since 3 μM IP_3R caused the same release as 10 μM IP_3, for further experiments we used 3 μM IP3 to induce calcium release from cardiac vesicles. Similarly to protein levels, IP_3-induced calcium release was highest in the LA, followed with the RA. Very weak calcium release was observed from vesicles from the LV and RV (Fig. 2B).

We examined the effect of single immobilization stress for 2 h on mRNA levels of type 1 and 2 IP_3Rs in cardiac atria. Single immobilization stress for 2 h significantly increased levels of type 1 (Fig. 3, left) and type 2 (Fig. 3, right) IP_3R mRNA, both in the LA (empty columns) and RA (striped columns), compared to sham-operated animals (control). In cardiac ventricles, we were not able to measure the effect of immobilization on IP_3Rs, because of a low abundance of type 1 and 2 IP_3R mRNA in this tissue. Since the mechanism in the immobilization-induced increase of both types of receptors is not known yet, we examined the possible involvement of glucocorticoids in this process. Adrenal glucocorticoid production is regulated by the complex integration of stimulatory and inhibitory signals that maintain homeostasis during stress. The primary stimulus for glucocorticoid release is the secretion of adrenocorticotropic hormone (ACTH) by the pituitary. We used either adrenalectomized (adrex; removal of adrenals) or hypophysectomized (hypox; removal of pituitaries) rats, since they do not produce the adrenal glucocorticoids and compared them with the sham-operated control group of rats. Adrenalectomy eliminates all components produced by adrenals (e.g. glucocorticoids, mineralocorticoids, catecholamines, etc.), while hypophysectomy eliminates ACTH, which is a major regulator of glucocorticoid synthesis, as well as its release. Hypophysectomy practically eliminated the glucocorticoid levels in both, control and immobilized animals.

Adrenalectomy and hypophysectomy did not affect mRNA levels of type 1 and 2 (Fig. 4, control) IP_3R in control, unstressed rats, but they completely prevented immobilization-induced increase of mRNA in both LA and RA (Fig. 4, imo).

4. Discussion

We identified mRNA of both, type 1 and type 2 IP_3 receptors in the rat cardiac atria and ventricles. Both types of IP_3 receptors occur predominantly in cardiac atria, with the higher prevalence in the LA. These results concur with the levels of protein of IP_3 receptor, which is also much more abundant in atria compared to ventricles. Our results are in a good

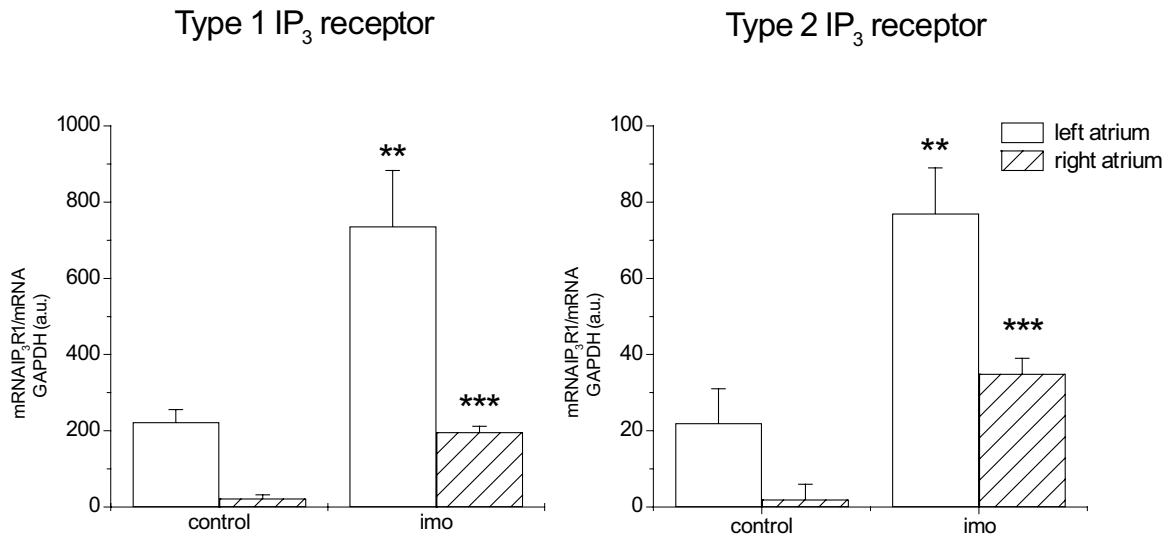

Fig. 3. Immobilization-induced increase in mRNA levels of type 1 and 2 IP_3 receptors. Immobilization for 2 h increased significantly mRNA levels of type 1 IP_3R in the LA (left graph, empty columns) and RA (left graph, striped columns). The mRNA levels of type 2 receptors (right graph) were also elevated. Results are presented as mean ± S.E.M. and each value is an average of at least 10 animals. Statistical significance represents $^{**}P < 0.01$ and $^{***}P < 0.001$. Significances were calculated relative to the mRNA levels in controls.

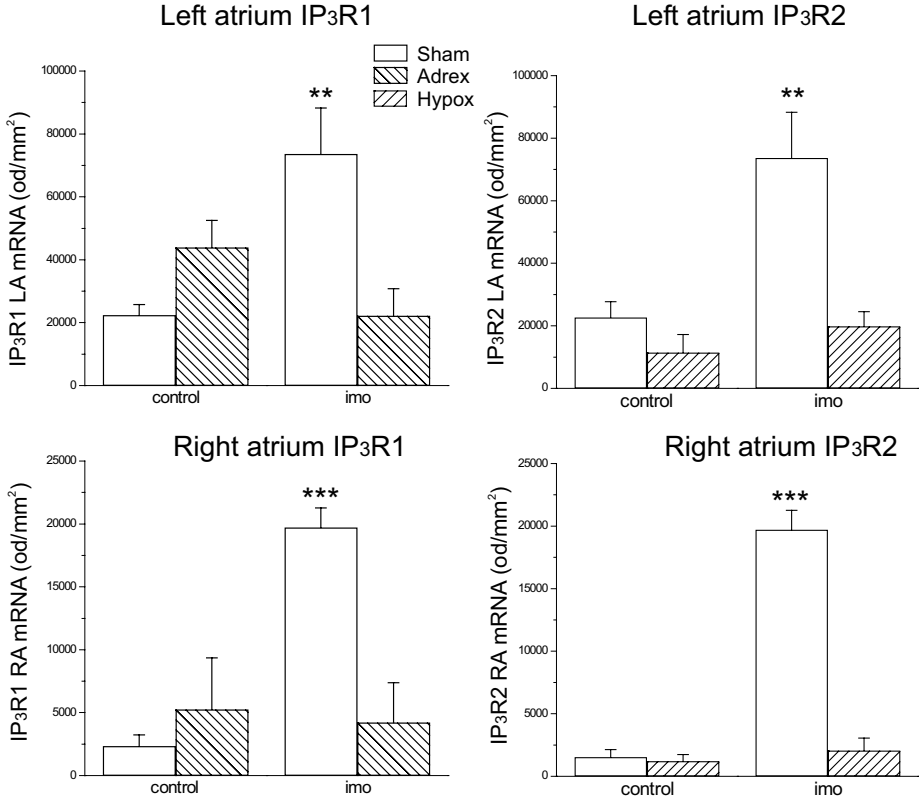

Fig. 4. Effect of the adrenalectomy (adrex; left striped) and hypophysectomy (hypox; right striped) on the immobilization-induced mRNA levels of type 1 and 2 IP_3Rs. Adrenalectomy and/or hypophysectomy did not change significantly the mRNA levels of IP_3R1 and IP_3R2 under control conditions in comparison to sham-operated rats (control, empty columns). Immobilization (imo, empty columns) for 2 h significantly increases levels of the IP_3R1 and IP_3R2 mRNA in both atria of sham-operated rats. Nevertheless, both ectomies prevented the immobilization-induced increase in both atria (imo, striped columns), which suggests that increase in the IP_3R1 and IP_3R2 mRNA was glucocorticoid-dependent. Results are displayed as mean ± S.E.M. and each column represents an average of at least five animals. Statistical significance represents $^{**}P < 0.01$.

agreement with those observed recently by Lipp et al. [5], who also found the higher amount of IP_3R type 2 protein in cardiac atria compared to ventricles. In addition, these authors also showed that in cardiomyocytes, type 2 IP_3Rs are predominant species from isolated atrial and ventricular myocytes.

In the heart, IP_3Rs of type 1 and 2 were detected also by other authors on the level of mRNA and protein [1]. IP_3R1 is also present at higher levels in Purkinje fibers [3] that conduct electrical signals from the atria to ventricles. This finding could explain higher levels of IP_3R in cardiac atria, although still remains to be elucidated, why the LA possess the highest level of IP_3R mRNA and protein.

In atria, gene expression of type 1 and 2 was upregulated by single immobilization stress for 2 h. Immobilization stress is known to elevate rapidly plasma glucocorticoids during stress [18]. Thus, we tested the hypothesis that glucocorticoids might affect the gene expression of IP_3Rs, especially during immobilization. We have observed that while under control conditions the mRNA levels of both types IP_3 receptors are not significantly changed in adrenalectomized and/or hypophysectomized rats compared to sham-operated controls; removal of the glucocorticoid source and/or synthesis prevented the increase in mRNA levels of both types IP_3 receptors. From these results it is clear that increase in IP_3R mRNA due to immobilization is regulated by glucocorticoids, possibly through the glucocorticoid responsive element. Since basal levels of the gene expression of both IP_3R1 and IP_3R2 were the same in sham-operated and adrenalectomized and/or hypophysectomized rats, it is likely that the gene expression of these receptors under normal, control conditions is not regulated by glucocorticoids.

Physiological function of IP_3Rs in heart remains to be elucidated. It is generally accepted that it does not play a major role in cardiac EC coupling, although it could be involved in physiological modulation of cardiac contractility in response to pharmacological agents and hormones [19]. IP_3 has been reported to be required for signaling pathways leading to apoptotic cell death in lymphoid cells, when apoptosis was induced by a number of different agents, including glucocorticoids [20]. Thus, it is tempting to speculate that increase in the gene expression of IP_3 receptors after immobilization stress has beneficial effects on the cardiac function during stress.

In conclusion, this is the first evidence that IP_3R gene expression is regulated by immobilization stress in cardiac atria and that glucocorticoids are involved in this process.

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