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Agonistic antibody to the α_1-adrenergic receptor mobilizes intracellular calcium and induces phosphorylation of a cardiac 15-kDa protein

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Abstract Hypertension is a major cause for hypertrophic remodelling of the myocardium. Agonistic autoantibodies to extracellular loops of the α_1-adrenergic receptor (α_1-AR) have been identified in patients with arterial hypertension. However, intracellular reactions elicited by these agonistic antibodies remain elusive. An anti-peptide antibody (anti-α_1) was generated against the second extracellular loop of the α_1-AR that bound to its peptide epitope with high affinity (K_D ~50 nM). We studied anti-α_1 effects on intracellular calcium (Ca_i), a key factor in cellular remodelling, and receptor-mediated cardiac protein phosphorylation. Anti-α_1 induced pronounced but transient increases in Ca_i in CHO cells expressing the human α_1-AR (CHO-α_1) and in neonatal cardiomyocytes. Preincubation experiments failed to demonstrate a tonic effect of anti-α_1 on Ca_i. However, preincubation with the antibody attenuated the effect of the α_1-AR antagonist prazosin. In neonatal cardiomyocytes anti-α_1 induced a robust phosphorylation of a 15-kDa protein that is involved in α_1-AR signalling. Our data support the notion that elevation of Ca_i is a general feature of agonistic antibodies' action and constitute an important pathogenic component of hypertension-associated autoantibodies. Furthermore, we suggest that agonistic antibodies to the α_1-AR contribute to hypertrophic remodelling of cardiac myocytes, and that the cardiac 15-kDa protein is a relevant downstream target of their action.

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Introduction

G-protein-coupled receptors (GPCR) represent the largest and the most important family of cell surface receptors. They mediate a diversity of physiological functions and consequently are the predominant targets of drugs. In the cardiovascular system, adrenergic receptors mediate catecholamine actions. Whereas β_1-adrenergic receptors (β_1-AR) dominate in regulating myocardial functions, α_1-adrenergic receptors (α_1-AR) mediate inotropic cardiac responses and vascular smooth muscle contraction. In the myocardium, signalling of α_1-AR leads to cellular remodelling, and hypertrophy which occurs independently of its inotropic effects [1]. The existence of three α_1-AR isoforms complicates the situation, and there are still some inconsistencies regarding their role in α_1-AR-mediated cardiovascular functions in health and disease [2].

Functional, agonistic autoantibodies to GPCR have been identified to be associated with serious cardiovascular diseases. For instance, a number of patients with idiopathic dilated cardiomyopathy harbour agonistic autoantibodies against the β_1-AR [3, 4]. The functional autoimmune epitopes were localized to the first and second extracellular loops of the receptor [5, 6]. These agonistic autoantibodies elicit functional alterations in intracellular calcium (Ca_i) handling and contractile function [7, 8]. In patients with hypertension, agonistic autoantibodies to the α_1-AR were found, and the functional autoimmune epitope was mapped to the second extracellular loop of the receptor [9–11]. Hypertension is a major cause of cardiac hypertrophy

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which in progression leads to heart failure. Hypertension-associated agAAB, in addition to their vascular action, also recognize myocardial α_1-AR and therefore may contribute to hypertrophic remodelling in cardiomyocytes.

It is suggested that binding of agonistic autoantibodies to the cognate receptor mimics agonist action leading to activation of down-stream signalling cascades. Thway et al. [12] demonstrated in Chinese hamster ovary (CHO) cells transfected with the human angiotensin II type 1 receptor (AT_1-R) that agonistic autoantibodies isolated from sera of preeclamptic patients led to Ca_i mobilization resulting in activation of the transcription factor NFAT. In vascular smooth muscle cells, these autoantibodies mobilize Ca_i, induce ERK1/2 kinase phosphorylation, increase the DNA binding activity of the transcription factors activator protein 1 and nuclear factor-κB and stimulate cell proliferation [13, 14]. Agonistic AT_1-R antibodies isolated from preeclamptic women produced the clinical signs of preeclampsia when injected in pregnant mice [15].

However, compared to the AT_1-R system, few data on intracellular reactions elicited by agonistic antibodies to the α_1-AR were available. Rats immunized with a peptide corresponding to the second extracellular loop of the α_1-AR produced antibodies and developed cardiac hypertrophy and signs of cardiomyocyte remodelling, but no increase in blood pressure [16]. These antibodies acutely increased Ca_i in isolated adult rat cardiomyocytes. Antibodies produced in animals against extracellular epitopes of the α_1-AR stimulated the L-type Ca^{2+} current and exerted a positive chronotropic action in spontaneously contracting neonatal cardiomyocytes [17].

This study aimed at elucidating intracellular reactions evoked by the interaction of antibodies with the α_1-AR. Using an anti-peptide antibody generated to the second extracellular loop of the α_1-AR (anti-α_1), we studied its potency to affect Ca_i in CHO cells transgenic for the human α_1-AR and in neonatal cardiomyocytes [9, 10, 17]. We found that this antibody to the α_1-AR was agonistic and induced a fast and transient rise in Ca_i. Furthermore, investigation of receptor-mediated anti-α_1 effects on downstream signalling in cardiomyocyte revealed the phosphorylation of a cardiac 15-kDa protein.

Materials and methods

Materials

Synthetic peptides were purchased from Biosyntan (Berlin, Germany). Fura 2-AM was obtained from Merck Biosciences (Bad Soden, Germany) and ε-aminocapryl agarose from Sigma–Aldrich (Deisenhofen, Germany). Phosphorylation motif-specific antibody (phospho-Akt substrate RXRXXS*/T* rabbit mAb) was purchased from New England Biolabs (Frankfurt, Germany). The 1-step Ultra TMB ELISA was from Perbio Science (Bonn, Germany). The CHO cell line transgenic for the human α_1-AR (CHO-α_1) was kindly provided by Dr. Gerd Wallukat, Max Delbrück Center for Molecular Medicine, Berlin-Buch.

Antibody production

Antisera against the α_1-AR were produced in rabbits using a peptide corresponding to the second extracellular receptor loop (PAPEDETICQINEE) by BioGenes GmbH (Berlin, Germany). The obtained antisera were affinity purified onto peptide antigen containing matrices by column chromatography. The specificity of the antibody fractions was verified by ELISA.

Affinity matrices and affinity purification of antibodies

The peptide corresponding to the second extracellular loop of the isoform A of the α_1-AR was covalently bound to ε-aminocapryl agarose using the glutaraldehyde method. The affinity beads were poured into a Bio-Rad column and stored at 4°C with 0.02% Na-azide in buffer A consisting of (mM): Tris/HCl 50; pH 7.4, and NaCl 500. The affinity beads were washed with buffer A. Serum samples from immunized rabbits (10–20 ml) were incubated with the affinity beads overnight at 4°C on a rotating wheel. The beads were washed with buffer A until the protein concentration in the effluent measured at 280 nm reached baseline levels. The antibodies were eluted at room temperature in 1-ml fractions with (mM): Tris/glycine 50; pH 2.5 and NaCl 500. Antibody fractions were immediately neutralized with 0.5 ml of (mM): Tris/HCl 500; pH 7.4, NaCl 500. The immunoglobulin (IgG) concentration was calculated from the absorbance at 280 nM. Affinity-purification yielded about 1–2 mg monospecific IgG from a given rabbit blood sample.

Cell cultures

Neonatal cardiomyocytes were prepared by tryptic digestion of the minced ventricles of 1–3-day-old rats and cultivated in Halle SM20.1 medium in gas-tight flasks essentially as described in [18]. For calcium measurements and protein phosphorylation experiments, neonatal cardiomyocytes were cultivated in Halle SM20-1 medium buffered with 10 mM HEPES at 37°C and 5% CO_2. CHO cells stably expressing the human α_{1A}-AR (CHO-α_1) were cultivated in DMEM/Ham's F-12 containing 10% FBS at 37°C and 5% CO_2.

Surface plasmon resonance measurements

Binding experiments were performed in a BIAcore 2000 Instrument (Uppsala Sweden) at 25°C. N-terminally biotinylated peptides corresponding to the first and second extracellular loops of the α_1-AR isoform A were immobilized at binding levels of 100 relative units (RUs) on parallel lanes of a SA-biosensor chip. Affinity-purified antibody fractions were injected into the flow cells at a rate of 20 µl/min in HBSE running buffer consisting of (mM): HEPES 10; pH 7.4, NaCl 150, EDTA 3. The binding surface was regenerated using (mM): Tris/glycine 5, NaCl 50; pH 2.5 with no decrease in signal intensities over the duration of an experiment. Data were analysed using the BIA evaluation 3.2 RC 1 program. The analysis software corrects for baseline drift during measurements. The curves were fitted to a single-site interaction model. K_D values were calculated by using the formula $K_D = k_{off}/k_{on}$ in which k_{off} and k_{on} are the rate constants of dissociation and association kinetics, respectively.

Calcium measurements

Cells were plated onto Labtek four chamber slides (Nunc, Wiesbaden, Germany) suited for fluorescence measurements at a density (cells per chamber) of 200,000 and 50,000 for neonatal cardiomyocytes and the CHO-α_1 cell line, respectively. After cultivation for 4 days, the medium was removed and cells were washed twice with prewarmed HBSS consisting of Hank's salt solution buffered with Hepes of (mM): 10, pH 7.4. Cells were incubated on HBSS for 60 min at 37°C. The solution was replaced by 0.5 ml HBSS containing Fura 2-AM at 2.5 µM final concentration for neonatal cardiomyocytes and 5 µM for CHO-α_1 cells. Loading was performed at room temperature in the dark for 30–45 min. Then, the loading solution was aspirated, the cells were washed and kept on 0.5 ml HBSS in the dark at room temperature for another 30 min before use. Cytosolic Ca^{2+} transients were measured on an IonOptix Fluorescence and Contractility System (Milton, USA) equipped with a Leica microscope by monitoring the fluorescence signal obtained by alternative excitation at 340 and 380 nm. All the measurements were carried out at 37°C. Neonatal cardiomyocytes were electrically stimulated at 1 Hz to ensure stable contractions because loading the cells with the fluorescence indicator Fura 2 affected the frequency and stability of spontaneous beating.

Protein phosphorylation

Neonatal cardiomyocytes were plated in six-well plates at a density of 900,000 cells per well and cultivated. After 4 days, the medium was removed, and the cells were rinsed two times with HBSS, pH 7.4. Then, cells were incubated with 1 ml HBSS; pH 7.4 for 1 h at 37°C before starting manipulations as indicated. At the end of the experiment, cells were fixed by 5% final concentration of ice-cold trichloro acetic acid (TCA). After twice carefully rinsing with 2.5%, TCA cells were transferred into Eppendorf caps, solubilized in sample buffer pH 6.8, neutralized by the addition of 1 M Tris-base and immediately heated at 95°C for 5 min. Aliquots were taken from clear solubilisates for protein measurements. Typically, 20 µg of cell protein were applied per lane of a 15% SDS- or a 7.5% urea-SDS-polyacrylamide gel. After electrophoretic separation, proteins were electro-transferred to polyvinylidenfluoride (PVDF) membranes by tank blotting. Membranes were processed for immunoblotting according to standard protocols. The primary phosphorylation motif-specific antibody as specified was used at a dilution of 1:1000. The secondary antibody was a horseradish peroxidase-conjugated anti-rabbit IgG. Immunoreactive proteins were visualized by enhanced chemoluminescence and autoradiography on X-ray film. Data for changes in phosphorylation of interesting proteins were obtained by densitometrically scanning the autoradiograms.

Data analysis

Statistical significance was determined by the unpaired *t*-test after data had been checked for normal distribution or by the non-parametric Mann–Whitney test where appropriate, using GraphPad Prism software (GraphPad Software, San Diego, CA). A P value < 0.05 was considered statistically significant.

Results

Anti-peptide antibody against the α_1 adrenergic receptor is selective and acts agonistic

The anti-α_1 antibody was produced against the second extracellular loop of isoform A of the α_1-AR, identified to be the target region of agonistic autoantibodies in hypertension [9, 10, 17, 19]. We investigated its specificity towards the extracellular first (L1) and second loop (L2) peptides of the α1-AR. Real time binding experiments by surface plasmon resonance (SPR) revealed a fast efficient binding to the L2 peptide (Fig. 1). In contrast, the interaction with the L1 peptide was negligible. Assuming a molecular mass of 150,000 for IgGs, the K_D value for anti-α_1 was calculated from SPR data to be 50 nM. The antibody anti-α_1 evoked a positive chronotropic reaction in the cardiomyocyte contraction assay. It increased the number

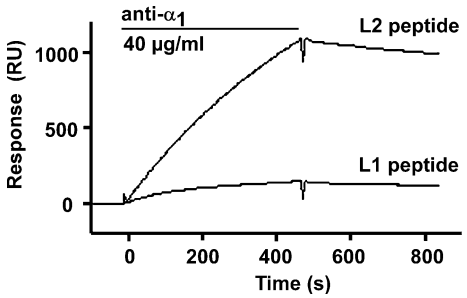

Fig. 1 Surface plasmon resonance measurements of α_1-adrenergic receptor antibody (anti-α_1) binding to peptides corresponding to extracellular loop 1 (L1) and extracellular loop 2 (L2) of the of α_1-adrenergic receptor. Biotinylated peptides, L1 and L2, were immobilized at a BIAcore SA sensor chip at 1,000 resonance units (RU). The anti-peptide antibody (90 µl; 40 µg IgG/ml of anti-α_1) flowed over the surface at 20 µl/min (the time of association is marked by the *straight line*) followed by buffer alone (dissociation). Anti-α_1 bound efficiently to its corresponding L2 peptide ($\sim$1,100 RU) with little cross-reactivity to the L1 peptide. Binding affinity for anti-α_1 is reflected by the K_D value amounting to 50 nM

of beats per minute by 13 ± 1 (mean ± S.E.M., basal contraction rate 167 ± 5, $n = 17$).

Anti-peptide antibody against the α_1-adrenergic receptor transiently elevates intracellular calcium

Stimulation of GPCR activates distinct signalling pathways which have in common the elevation of Ca_i. Furthermore, disturbed handling of Ca_i is a main mechanism underlying pathological changes at the cellular level. We therefore studied the effect of anti-α_1 on Ca_i transients in comparison to the GPCR agonist. We used CHO-α_1 cells stably expressing the isoform A of the human α_1-AR. Representative traces of Ca_i are shown in Fig. 2A; the summarized data are demonstrated in Fig. 2B. The α_1-AR agonist phenylephrine (PE) induced a fast and statistically significant increase in Ca_i, which was abolished when cells were pretreated with the receptor antagonist prazosin (PRAZ).

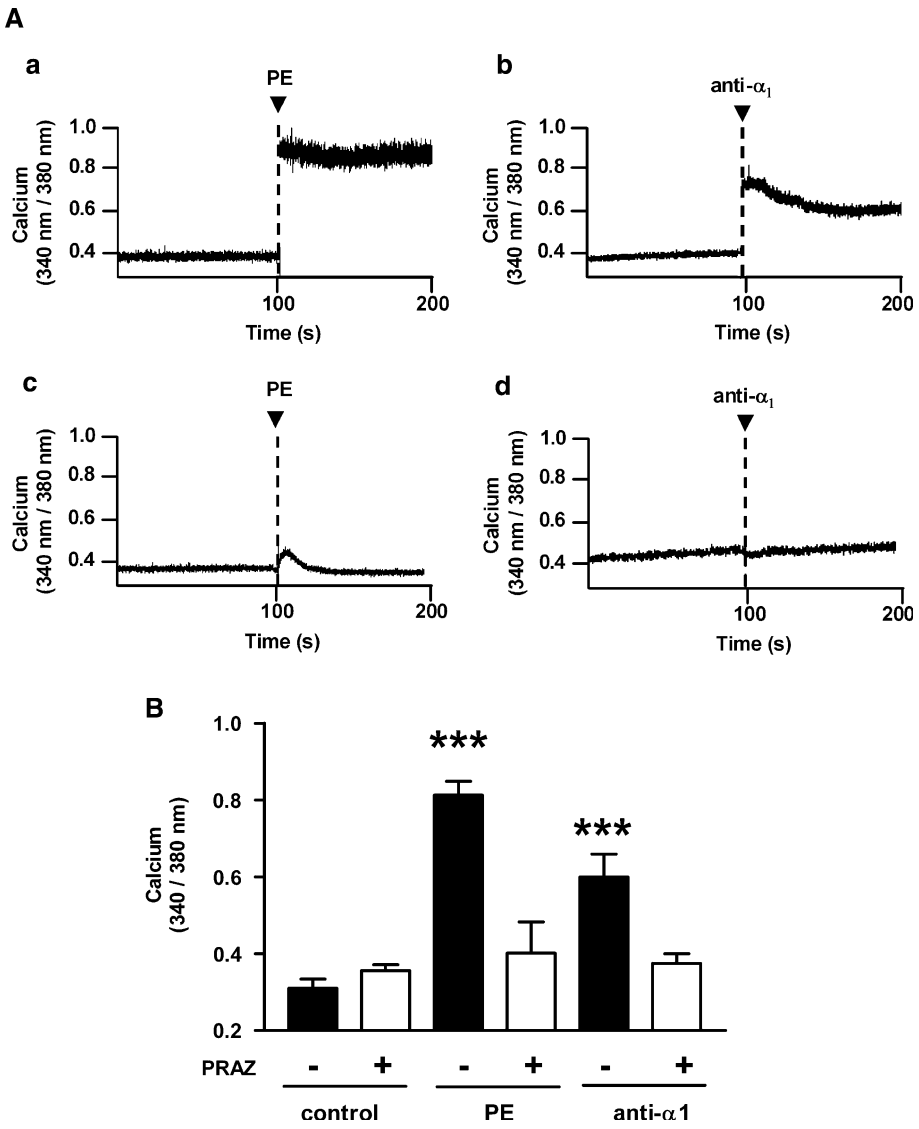

Fig. 2 Intracellular calcium transients in transgenic CHO-α_1 cells stably expressing the human isoform A of the α_1-adrenergic receptor. **A** Representative traces of comparing the action of phenylephrine (PE) and the α_1-adrenergic receptor antibody anti-α_1. Effect of PE (1 µM) without (**a**) and in the presence of prazosin (PRAZ, 1 µM) added 5 min prior to the administration of PE (**c**). Action of 2 µg/ml antibody anti-α_1 alone (**b**) and in the presence of PRAZ (1 µM) applied 5 min before the antibody was added (**d**). **B** Summarized data of intracellular calcium transients given as mean values plus SEM, *asterisks* denote statistical significance (*** $P < 0.001$; $n = 3$–8)

Anti-α_1 evoked a notably fast and also statistically significant rise in Ca_i. PRAZ prevented the calcium response elicited by anti-α_1 indicating a mode of action depending on α_1-AR activation. Next, we investigated consequences of a longer-lasting exposure of cells to the antibody (Fig. 3). After 1 h of preincubation with anti-α_1, there was no increase in Ca_i detectable (Fig. 3a). PE produced the same statistically significant rise in Ca_i in antibody-exposed and in untreated cells, indicating that there was no down-regulation of α_1-AR due to anti-α_1. However, in anti-α_1-pretreated cells the α_1-AR antagonist PRAZ was significantly less efficient in counteracting the PE-induced rise in Ca_i (Fig. 3b). This indicates a competition between the agonistic antibody and the receptor antagonist with respect to receptor function.

In order to address anti-α_1 action on Ca_i in a cardiac cell model, we used cultured neonatal cardiomyocytes from rat which express the isoform A of the α_1-AR constitutively [20]. Importantly, the first and second extracellular loops of rat α_1-AR share 100% sequence homology with those of the human receptor. The cardiomyocytes were electrically stimulated to contract stably with a constant frequency. Figure 4 compares peak Ca_i traces of representative recordings of cells exposed either to PE or to anti-α_1. PE elicited a fast statistically significant and notably transient elevation of Ca_i. Application of anti-α_1 resulted in a fast rise of Ca_i as well that was statistically significant. The anti-α_1 effect was pronounced transient and Ca_i returned to near baseline values within less than 1 min. The increase of Ca_i to both PE and anti-α_1 was attenuated in the presence of PRAZ. In order to study tonic effects of anti-α_1, cardiomyocytes were preincubated with the antibody as described above for CHO-α_1 cells. After 1-h preincubation. there was no effect of anti-α_1 on Ca_i detectable (data not shown). Basal levels of Ca_i and peak values in PE stimulated cells remained unaffected by the antibody. PE produced a significant increase in Ca_i in both untreated controls and antibody preincubated cardiomyocytes.

Signalling of anti-α_1 involves the phosphorylation of a cardiomyocyte 15-kDa protein

Next, we were interested in the downstream action of the agonistic antibody anti-α_1 in cardiomyocytes. GPCR couple to intracellular signalling cascades, in which protein phosphorylation plays a key role. We therefore used cultured neonatal cardiomyocytes and sought for proteins which underwent prominent phosphorylation in response to α_1-AR activation and thus were also candidates of anti-α_1 intracellular signalling. We employed a commercially available phosphorylation motif-specific antibody. This antibody recognized numerous proteins in rat neonatal cardiomyocytes and detected the phosphorylation of distinct proteins when cells were challenged with GPCR agonists (Fig. 5). Stimulation of neonatal cardiomyocytes with the β_1-adrenergic agonist isoprenaline increased the phosphorylation of proteins with the apparent molecular masses of 6, 15, 29 and 150 kDa. In contrast, when stimulating the cells with the α_1-AR agonist PE, a considerable phosphorylation only of the 15-kDa protein was detectable. Next, we studied the action of anti-α_1 on phosphorylation of those proteins. Anti-α_1 induced a significant phosphorylation of the 15-kDa protein (Fig. 6). The reaction was specifically mediated by the activation of the α_1-AR as demonstrated by its inhibition with the receptor antagonist PRAZ (Fig. 6b). The time course of the robust 15-kDa

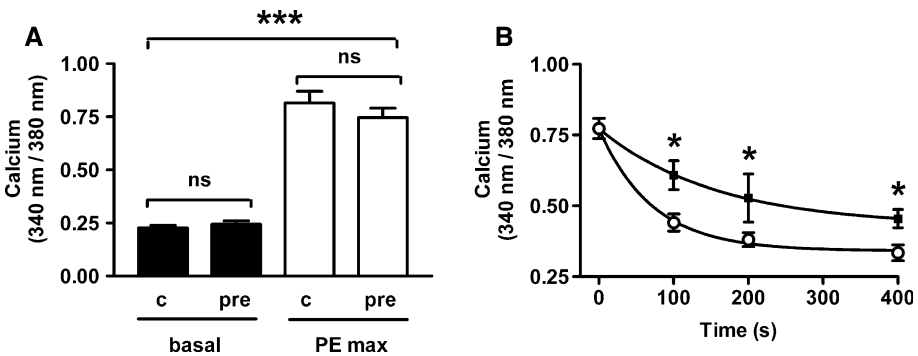

Fig. 3 Effect of preincubation of transgenic CHO-α_1 cells stably expressing the human isoform A of the α_1-adrenergic receptor with the α_1-adrenergic receptor antibody anti-α_1 on intracellular calcium (Ca_i). **a** Action of preincubation (pre) with anti-α_1 (2 μg/ml) for 60 min and control incubations (c) on basal Ca_i (*black bars*) and on the maximal response of Ca_i to phenylephrine (1 μM PE, *open bars*). **b** Effect of anti-α_1 on the efficacy of prazosin (1 μM) to lower Ca_i raised by 1 μM PE. Time zero represents the plateau of PE action on Ca_i (see also Fig. 2a) when prazosin was applied. The time course of Ca_i changes in cells preincubated with anti-α_1 (*closed squares*) and incubated in the absence of anti-α_1 (*open circles*) was monitored. Data are given as mean values plus SEM, *ns* not significantly different to controls, *asterisks* denote statistical significance (* $P < 0.05$; *** $P < 0.001$; $n = 5$–7)

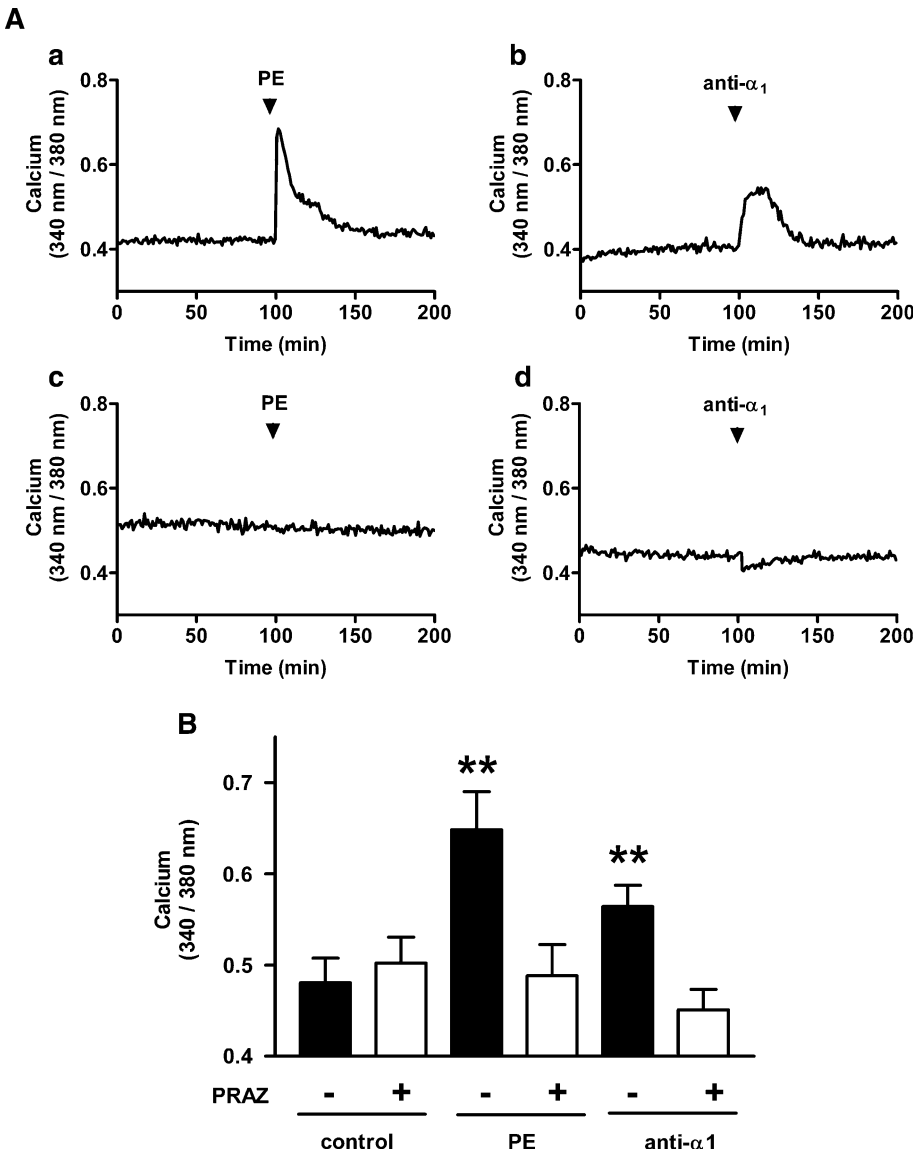

Fig. 4 Intracellular calcium transients in neonatal cardiomyocytes. **A** Representative recordings comparing the action of phenylephrine (PE) and the α_1-adrenergic receptor antibody (anti-α_1) on intracellular calcium (Ca_i). The cardiomyocytes were electrically stimulated and contracted with a frequency of 1 Hz. Traces of peak Ca_i achieved at maximum cell contraction were monitored. Effect of PE (1 μM) without (**a**) and in the presence (**c**) of prazosin (PRAZ, 1 μM) added 5 min prior to the administration of PE. Action of 2 μg/ml antibody anti-α_1 alone (**b**) and in the presence of PRAZ (1 μM) applied 5 min before the antibody was added (**d**). **B** Data of Ca_i measurements given as means plus SEM, *asterisks* denote statistical significance (** $P < 0.005$; $n = 3$–8)

protein phosphorylation elicited by the exposure to anti-α_1 was comparable to that evoked by the receptor agonist PE (Fig. 7).

Discussion

Anti-peptide antibodies to extracellular loops of GPCR are valuable model systems to study aspects of disease-associated agonistic antibodies' interactions with GPCR [17, 21–23]. This study focuses on isoform A of the α_1-AR because it is dominant in the human heart and plays a major role in cardiac physiology [24]. The anti-peptide antibody anti-α_1 was recently shown to induce hypertrophic signalling as agonistic antibodies isolated from sera of patients with refractory hypertension [19]. Here, we show that anti-α_1 bound with a K_D value in the nanomolar range that is comparable to the autoantibody isolated from hypertensive patients' sera [19]. Thus, anti-α_1 proved to be an appropriate tool to unravel cellular mechanisms of hypertension-associated agonistic autoantibodies to the α_1-AR.

Agonistic autoantibodies mimic agonists' action on GPCR and therefore are expected to raise Ca_i. Data on Ca_i mobilization in response to agonistic antibodies to the α_1-AR are limited [16, 17, 19]. We used cultures of neonatal cardiomyocytes and CHO cells stably expressing the human α_1-AR to study the ability of the anti-peptide antibody anti-α_1 to mobilize Ca_i. The anti-α_1 raised Ca_i with a time course close to that of the receptor agonist and comparable to that of agonistic antibodies from hypertensive patients [19]. This fast response suggests a high activation potency of the agonistic antibody. A comparable time course for Ca_i mobilization was reported when applying

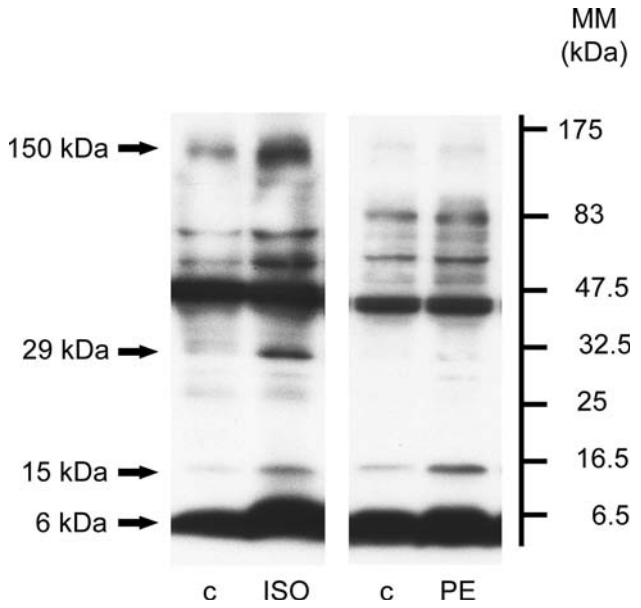

Fig. 5 Pattern of protein phosphorylation of neonatal cardiomyocytes challenged with adrenergic agonists. Cultures of neonatal cardiomyocytes were stimulated either with the β-adrenergic agonist isoprenaline (ISO) or the α_1-adrenergic agonist phenylephrine (PE). Final concentration was 1 μM for both agonists. Unstimulated cell cultures were used as controls (c). Phosphoproteins were detected using a phosphorylation-sensitive antibody specific for a serine or threonine residue containing motif. Proteins which underwent phosphorylation in response to the agonist treatment are indicated with their apparent molecular masses

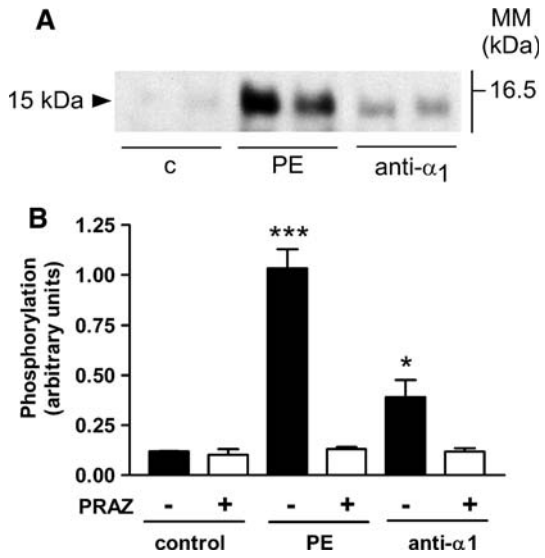

Fig. 6 Phosphorylation of the 15-kDa protein in neonatal cardiomyocytes exposed to the α_1-adrenergic receptor antibody anti-α_1. Phosphorylation was analysed by semiquantitative immunoblotting using a phosphorylation motif-specific antibody. **a** Autoradiograph of the phosphorylation of the 15-kDa protein. Cultures of neonatal cardiomyocytes were left untreated for controls (c), exposed to the α_1-adrenergic receptor agonist phenylephrine (PE, 1 μM) or to the α_1-adrenergic receptor antibody anti-α_1 (2.5 μg/ml) for 5 min. **b** Data of the 15-kDa protein phosphorylation derived from densitometrical scans of autoradiographs. Cultures of neonatal cardiomyocytes were left untreated (control), challenged with 1 μM phenylephrine (PE) or 2.5 μg/ml of the α_1-adrenergic receptor antibody (anti-α_1) without (−) or after preincubation (+) with the α_1-adrenergic receptor antagonist prazosin (PRAZ, 1 μM). Data are given as mean values plus SEM. *Asterisks* indicate statistical significance (* $P < 0.05$; *** $P < 0.001$; $n = 3$)

IgG-fractions from pre-eclamptic women containing agonistic autoantibodies to the AT_1-R [12]. The data presented here confirm that agonistic antibodies acutely elevate Ca_i and support the notion that this is a common property of their action [17].

The pathological potential of disease-associated agonistic autoantibodies comprises the induction of remodelling processes in the target cells [16]. Ca_i is critically involved in cellular remodelling processes such as hypertrophic growth of the myocardium [25]. Therefore, the question was for tonic effects of the agonistic antibody on Ca_i. However, after preincubating CHO-α_1 cells and cardiomyocytes with anti-α_1, we were not able to detect alterations in Ca_i, although the agonistic antibody was permanently present. This was not due to a down-regulation of the α_1-AR as indicated by the unchanged response to PE compared to untreated controls. In concurrence with our observation, Green et al. [26] reported no significantly altered basal Ca_i after 4-h incubation of vascular smooth muscle cells with serum from hypertensive preeclamptic women containing autoantibodies to the AT_1-R. The lack of a detectable long-lasting effect on Ca_i does not necessarily exclude the efficacy of the antibodies to affect Ca^{2+}-linked processes involved in cellular remodelling. The Ca^{2+}-dependence may be restricted to one or few upstream elements of the signalling cascade, which become temporarily activated. Antibody action may also modify Ca^{2+}-dependent systems by Ca_i independent mechanisms [27]. It was recently shown that the antibody anti-α_1 upregulated the level of cardiac L-type Ca^{2+} channel pore subunit mRNA [18]. Future study will be required to further unravel the role of Ca_i in the mechanism of agonistic antibody action linked to cellular remodelling.

Although in CHO-α_1 cells, after 1 h of incubation, anti-α_1 failed to keep the Ca_i level elevated, it significantly diminished the efficacy of the α_1-AR antagonist prazosin. There is obviously a competition of the agonistic antibody and the GPCR-antagonist with respect to the receptor activation-dependent cellular Ca_i response. The molecular mechanism of this functional antagonism of the GPCR antagonist and the agonistic antibody is unknown. Experimental approaches for its elucidation will be complicated by the existence of multiple conformations of the α_1-AR with multiple antagonist-binding sites [28]. We also showed that preincubation of cells with the α_1-AR antagonists was sufficient to prevent anti-α_1 from mobilizing

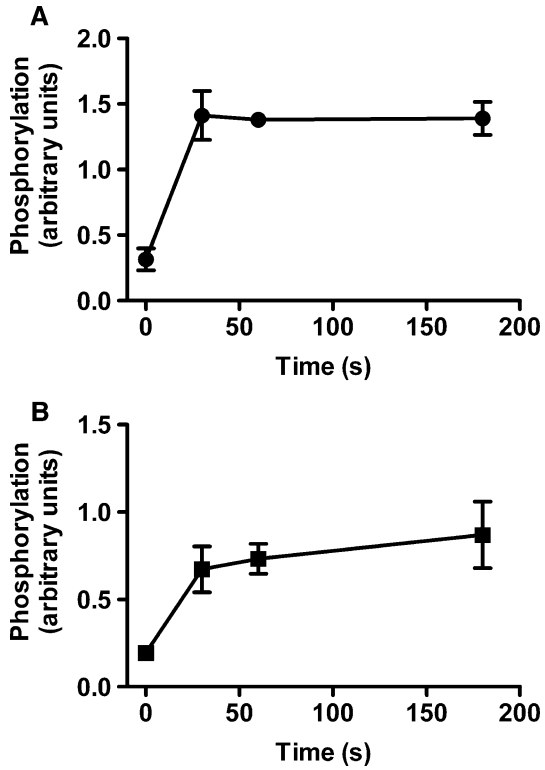

Fig. 7 Time course of the phosphorylation of the 15-kDa protein in neonatal cardiomyocytes. Phosphorylation was analysed by semi-quantitative immunoblotting using a phosphorylation motif-specific antibody. Data were obtained by densitometry of autoradiographs. **a** Phosphorylation in response to the α_1-adrenergic receptor agonist phenylephrine (PE, 1 μM). **b** Phosphorylation elicited by exposing the cells to the α_1-adrenergic receptor antibody anti-α_1 (2.5 μg/ml) Data are mean values plus SEM ($n = 3$)

Ca_i. Obviously the agonistic antibody is not able to dominate the blocking effect of the receptor antagonist. Thus, antagonists of GPCR have the potency to inhibit at least partially the cellular action of agonistic antibodies [15]. This sheds new light onto the therapeutic potential of GPCR antagonists in the treatment of agonistic autoantibody-associated complications in diverse diseases.

Protein kinase C and ERK1/2 kinases are established mediators of α_1-AR signalling in numerous cell types [29]. They recently have been identified by Wenzel et al. [19] to be also targets of agonistic autoantibodies to the α_1-AR isolated from patients with refractory hypertension and by the anti-α_1 used in this study. Here, we sought to examine typical cardiomyocyte phosphorylation substrates of the α_1-AR pathway. To our knowledge, we show for the first time that the anti-α_1 evoked phosphorylation of a cardiomyocyte 15-kDa protein. This phosphoprotein was absent in CHO-α_1 cells and the cardiac myoblast cell line H9c2 (Karczewski unpublished). The phosphorylation of a cardiac 15-kDa protein in response to β- and α-adrenergic receptor agonists was described earlier by various authors [30–33]. It was named phospholemman, and later recognized as a regulator of the Na^+/K^+ pump and the Na^+/Ca^{2+} exchanger, two important systems to regulate the cardiac Ca^{2+} homeostasis and critical in heart pathology [34–37]. The 15-kDa phosphoprotein described here shares similarities with phospholemman: (i) abundant small phosphoprotein in cardiomyocytes, (ii) migration at 15-kDa in SDS polyacrylamide gels, (iii) phosphorylation by β-adrenergic stimulation [31] and (iv) phosphorylation in response to α_1-AR activation [32]. However, its identification is presently hampered by the lack of specific antibodies to phospholemman.

The observed phosphorylation of the 15-kDa protein induced by the antibody anti-α_1 is relatively low when compared to that evoked by phenylephrine; however, it is statistically significant. Detailed dose–response studies will be required to assess the stoichiometry of its phosphorylation induced by anti-α_1. Neonatal cardiomyocytes express all three isoforms A, B and D of the α_1-AR [20]. When comparing the action of phenylephrine and anti-α_1, it has to be considered that this agonist acts through all isoforms of the α_1-AR, whereas anti-α_1 specifically targets isoform A. The α_1-AR signalling is cell type-dependent isoform-specific [29]. Although data for the link of α_1-AR isoforms to signalling molecules are controversial, isoform A seems to mediate the rise in intracellular calcium in cardiomyocytes [38]. Isoform A may be less efficiently coupled to the 15-kDa phosphorylation than the other isoforms of the α_1-AR. Furthermore, the 15-kDa protein may be phosphorylated at multiple sites in response to the receptor agonist, but at one site or fewer sites by the agonistic antibody.

In summary, anti-α_1 developed against the second extracellular loop of the α_1-AR elicited an agonistic mode of action with respect to its cognate GPCR. It induced intracellular signalling comprising acute but transient elevation of Ca_i and the phosphorylation of a 15-kDa protein in cardiomyocytes. We suggest that in addition to established α_1-AR downstream targets, the 15-kDa protein is part of the signalling pathway of agonistic α_1-AR antibodies linked to cardiomyocyte remodelling [16].

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