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Inhibition of PI3K improves contractility in alpha$_1$-adrenergically stimulated myocardium

Claudius Jacobshagen[1], Swantje Kortlepel[1], Bernhard W. Unsoeld[1], Thomas Sowa[1], Harald Koegler[1], Gerd Hasenfuss[1], Lars S. Maier[1]

[1]Department of Cardiology and Pneumology, Heart Center, Georg-August-University Goettingen, Robert-Koch-Strasse 40, 37075 Goettingen, Germany

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1. ABSTRACT

Recent studies have demonstrated that phosphoinositide 3-kinases (PI3Ks) play a fundamental role in regulating myocardial contractility. However, even though α_1-adrenergic receptor stimulation is known to activate PI3Ks, the impact of this pathway on the inotropic effects of alpha$_1$-stimulation is unclear. Isolated rabbit ventricular myocytes were preincubated with the PI3K inhibitor wortmannin (WM, 0.1µmol/L)). The alpha$_1$ agonist phenylephrine (PE, 10 µmol/L) induced a significantly stronger increase in contractility in WM-treated versus control myocytes (Fractional shortening in % of resting cell length: 6.14+/-0.33%; n=26 versus 4.85+/-0.33%; n=26, $P<0.05$). Furthermore, pretreatment with WM significantly increased the positive inotropic effect of PE in intact muscle strips from rabbit hearts. Mechanistically, we demonstrate that in WM-treated myocytes PE increased phospholamban (PLN) phosphorylation and intracellular Ca^{2+} transients to a significantly greater extent than in control myocytes. In summary, this is the first study to demonstrate that inhibition of PI3K by increasing PLN phosphorylation and Ca^{2+} transients significantly improves contractility in alpha$_1$-adrenergically stimulated myocardium. This may have clinical implications for the treatment of decreased cardiac function in acute heart failure.

2. INTRODUCTION

Phosphoinositide 3-kinases (PI3Ks) are a family of evolutionarily conserved lipid kinases that play a central role in the control of cell growth, proliferation, metabolism, apoptosis, membrane transport, and cell motility. Thus, PI3Ks regulate several physiological processes and are involved in the pathology of cancer, metabolic, inflammatory, and cardiovascular diseases (1-3).
In myocardium, PI3K activity is essential for both basal cell growth as well as adaptive (physiologic) and maladaptive (pathologic) hypertrophy (4-9). In this regard, the intensity and duration of Akt activation, a well characterized downstream target of PI3K, appear to determine the character of hypertrophy (8-10).

Furthermore, PI3Ks control glucose and amino acid metabolism in the heart (11,12). By inhibiting apoptosis, activation of PI3Ks plays a crucial role in cardiac myocyte survival. PI3K activation decreases infarct size after transient ischemia and prevents cardiac myocytes from oxidative stress-induced apoptosis (13-15).

Moreover, recent studies have demonstrated that PI3Ks play a fundamental role in regulating mechanotransduction and myocardial contractility (5,11,16,17). Cardiac-specific overexpression of IGF-1

receptor or IGF-1 infusion improve contractile function in mouse hearts by increasing PI3K activity (5,16). Consistently, knockout of insulin receptor results in reduced contractility (11). On the other hand, PI3Ks reduce beta-adrenergically mediated elevation in cyclic adenosine monophosphate (cAMP) levels and protein kinase A (PKA) activity, thereby counteracting the positive inotropic effects of beta-adrenergic signaling (17). Thus, modulating cardiac contractility by PI3Ks following insulin/IGF-1 and beta-adrenergic receptor stimulation has been described. In this regard, there is now growing evidence that the effect of PI3K on contractility depends on the specific PI3K isoform. PI3Kalpha and PI3Kgamma are the two main isoforms expressed in cardiac myocytes. PI3Kalpha predominantly regulates heart growth, but also exerts positive inotropic effects, whereas PI3Kgamma negatively influences cardiac contractility (2,17-20).

However, although alpha$_1$-adrenergic agonists are also known to increase PI3K activity the impact of PI3Ks on the inotropic effects of alpha$_1$-adrenergic stimulation is unclear (21). In the present study we investigate the effects of PI3Ks on contractile function and intracellular Ca^{2+} handling in alpha$_1$-adrenergically stimulated myocardium. Since growing evidence indicates that PI3Ks might qualify as drug targets for the treatment of cardiovascular diseases, cancer, and inflammation, exact knowledge of the coupling between the different cell-surface receptors, PI3Ks, and effectors is essential to predict how PI3K inhibitors could ameliorate disease and whether they might interfere with vital processes.

3. MATERIALS AND METHODS

3.1. Isolation and primary culture of rabbit ventricular myocytes

This study was designed and carried out in accordance with the *Guide for the Care and Use of Laboratory Animals* (NIH Publication No. 85-23, revised 1996). Female chinchilla bastard rabbits (1.5–2 kg, Charles River Laboratories, Kisslegg, Germany) were heparinized and anaesthetized with thiopental sodium (50 mg·kg^{-1} i.v.). Hearts were rapidly removed, mounted in a Langendorff perfusion setup, and perfused with Tyrode solution containing (in mmol/L): NaCl 137, KCl 5.4, Na_2HPO_4 1.2, HEPES 20, glucose 15, $CaCl_2$ 1, oxygenated with 100% O_2 for 5-8 min. Perfusion was then switched to nominally Ca^{2+}-free Tyrode solution for 12-15 min (30 mL/min) and digestion was performed by perfusion for 12-15 min (10 ml/min) with Tyrode-enzyme solution containing 250 U/mL collagenase type II (Biochrom), 0.04 mg/mL protease type XIV (Sigma-Aldrich) and (in mmol/L) Ca^{2+} 0.025, taurine 60, *D, L*-glutamic acid 8, and *D, L*-carnitine 2. Digestion was stopped by perfusion with 100 mL Tyrode solution containing 50 µmol/L Ca^{2+}, 2% fatty acid-free type V albumin, and 20 mmol/L 2,3-butanedione monoxime (BDM). Atria were cut off and the ventricles were immersed in Tyrode solution containing 20 mmol/L BDM, and 50 µmol/L Ca^{2+}. The heart was cut into chunks and myocytes were released by four rounds of mincing and gentle manual agitation. The myocytes were filtered through sterile nylon gauze (200 µm meshes) and progressively exposed to increasing Ca^{2+} concentrations in Tyrode solution. The final suspension was laid on top of a 6% albumin/M199 medium (1.75 mmol/L Ca^{2+}) supplemented with 5 mmol/L *D, L*-carnitine, 5 mmol/L taurine, 5 mmol/L creatine, 100 IU/mL penicillin and 70 µmol/L streptomycin. Myocytes viability was assessed using the Trypan-blue exclusion method. Cells were counted and plated at a density ~$4.2x10^3$ rod-shaped cells/cm^2 on culture dishes (55 mm) and incubated in supplemented M199 tissue culture medium (Sigma-Aldrich).

3.2. Single myocyte shortening and intracellular Ca^{2+} measurements

To inhibit PI3Ks, myocytes were preincubated with wortmannin (WM, Sigma-Aldrich, 0.1 µmol/L), a well established and widely used PI3K inhibitor, for 20 min. Since WM was dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich), control myocytes were incubated with DMSO (highest final concentration of DMSO in all experiments: 0.1 mL/L). Both groups of myocytes (WM and control) were stimulated with the alpha$_1$ agonist phenylephrine (PE, 10 µmol/L). Shortening and $[Ca^{2+}]_i$ measurements were performed as reported previously (22,23). Briefly, myocytes were loaded with the Ca^{2+}-sensitive dye indo-1/AM (10 µmol/L, Molecular Probes) to measure $[Ca^{2+}]_i$. Fluorescence was excited at 360+/-5 nm using a 75 W xenon arc lamp (Ushio, Japan) on the stage of a Nikon Eclipse TE200-U inverted microscope. Emitted fluorescence was measured using photomultipliers (at 405+/-15 nm and 485+/-12.5 nm; IonOptix Corp., Milton, MA). From the raw fluorescence, the indo-1 ratio was calculated (405 nm/485 nm) after subtraction of autofluorescence. Myocytes were electrically field-stimulated (voltage 25% above threshold) at 1 Hz and 37°C until myocyte shortening reached a steady state.

3.3 Preparation and contractile testing of intact muscle strips

Rabbits were heparinized and anesthetized as described above. Hearts were rapidly excised and retrogradely perfused with a modified Krebs-Henseleit buffer solution containing (in mmol/L): Na^+ 140.5, K^+ 5.1, $Mg2^+$ 1.2, Ca^{2+} 0.25, Cl^- 124.9, $SO4^{2-}$ 1.2, $PO4^{3-}$ 2.0, HCO_3^- 20, glucose 10, and BDM 20, equilibrated with carbogen (95% O_2 / 5% CO_2), pH 7.4. Right ventricular trabeculae and intact papillary muscles were isolated from the free wall and mounted in a superfusion bath (37°C) between a force transducer (Scientific Instruments, Heidelberg, Germany) and a hook connected to a micromanipulator for length adjustment. Preparations were superfused with BDM-free Krebs-Henseleit solution, starting with 0.25 mmol/L Ca^{2+}. The Ca^{2+}-concentration was raised stepwise (0.25 mmol/L every 2 min) up to 1.25 mmol/L. Isometric contractions were elicited using electrical field stimulation (1 Hz, amplitude 3 to 5 V; stimulator Stim1, Scientific Instruments). The force transducer signals were amplified and analyzed using software custom-made on the LabVIEW® platform (National Instruments, U.S.A.). After a stabilization phase (30 min), muscle strips were carefully stretched until diastolic resting tension reached a level of 2 mN/mm^2. This

reflects a physiological sarcomere length below L_{max} (sarcomere length at which developed force is maximum) and produces the most stable long-time performance of a preparation (24).

To inhibit PI3Ks, the bath solution was supplemented with WM (0.1 µmol/L) or DMSO solution without WM. After 40 min of preincubation with WM, both groups of muscle strips (WM and control) were alpha$_1$-adrenergically stimulated with PE (10 µmol/L). Reaching a new steady state, developed forces were measured and normalized to the baseline forces before PE-stimulation (relative developed tension, F_{dev}/F_0).

3.4. Myofilament Ca^{2+} responsiveness in semi-skinned muscle fibres

Right ventricular papillary muscles and trabeculae were excised as described above. The muscle strips were split longitudinally in sections measuring about 200-300 µm in width and thickness and 2-4 mm in length.The muscle strips were semi-permeabilized by incubation with 35 µmol/L beta-escin (Sigma-Aldrich) in relaxation solution for 30 min. This technique allows the control of intracellular ion concentrations but maintains sarcolemmal receptors and leaves subcellular signaling mechanisms intact. The muscle strips were mounted for isometric force measurement using aluminum T-clips and stretched to the length at which passive tension just began to increase. Measurements were carried out at room temperature. Relaxation solution contained (in mmol/L): imidazole 20, adenosine triphosphate 10, phosphocreatine 10, NaN_3 5, ethylene glycol-bis-(beta-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) 5, $MgCl_2$ 12.5. Activation solution additionally contained $CaCl_2$ at a nominal concentration of 5 mmol/L. The pH of both solutions was adjusted to 6.7 by addition of KOH. Differences in ionic strength between activation and relaxation solutions resulting from different amounts of KOH needed to adjust pH to 6.7 were corrected by adding KCl. Intermediate levels of $[Ca^{2+}]$ were obtained by mixing appropriate amounts of relaxation and activation solutions. The free $[Ca^{2+}]$ was calculated using the computer program WinMAXC (25) (http://www.stanford.edu/~cpatton/maxc.html). The study protocol was adapted to the experiments performed with single myocytes and intact muscle strips. Semi-skinned fibers were preincubated with WM (0.1 µmol/L) or DMSO for 30 min, respectively. Concentration-response curves for Ca^{2+} (tension-Ca^{2+} relationship) were obtained in PE-stimulated semi-skinned muscle strips, either with or without WM pretreatment.

3.5. Protein expression and phosphorylation level

Western blot analysis were performed as described previously (22,23). Briefly, cardiac myocytes were homogenized mechanically in 200 µL ice cold lysis buffer (containing 1% nonidet P 40, 10% glycerol, 137 mmol/L NaCl, 20 mmol/L Tris, pH 7.4, 20 mmol/L NaF, 1 mmol/L Na_3VO_4, 1 mmol/L $Na_4P_2O_7$, 50 mmol/L β-glycerophosphate, 10 mmol/L EDTA, pH 8.0, 1 mmol/L EGTA, pH 7.0, 1 mmol/L phenylmethyl-sulfonyl-fluoride (PMSF), 4 µg/mL aprotinin, 4 µg/mL leupeptin, 4 µg/mL pepstatin). After elimination of the cell debris by centrifugation, protein concentrations in the supernatant were determined by the bicinchoninic acid (BCA) method (Pierce, Bonn, Germany). Proteins were subjected to SDS-PAGE and electroblotted to nitrocellulose membranes. Blots were probed with antibodies against SERCA 2a (Affinity BioReagents, Golden, USA) or phospholamban (PLN, Upstate, Lake Placid, USA) and normalized to glycerol aldehyde phosphate dehydrogenase (GAPDH, Biotrend Chemikalien, Cologne, Germany). Phosphorylation levels were detected with phosphospecific antibodies against PLN-Ser16 and PLN-Thr17 (Badrilla, Leeds, UK). Immunoreactive bands were visualized using enhanced chemiluminescence (Pierce) and quantified by two-dimensional scans using a CCD camera system (Multi-Imager, AlphaInnotech Inc., San Leandro, USA).

3.6. Statistical Analysis

Data are presented as mean+/-SEM. Differences between experimental groups were evaluated for statistical significance by use of paired or unpaired Student's *t*-test where appropriate. Every muscle strip experiment followed a paired design with two preparations first dissected from immediately adjacent areas of the same rabbit heart and then examined in parallel in a dual-chamber superfusion system. Therefore, a paired t-test was considered appropriate to assess the level of statistical significance in this data set. EC_{50} values of concentration-response curves for Ca^{2+} in semi-skinned fibers were compared with extra sum-of-squares F test. A value of $P<0.05$ was considered significant.

4. RESULTS

4.1. Effect of PI3K inhibition on alpha$_1$-adrenergically stimulated single myocytes

In order to sufficiently inhibit PI3K activity, isolated cardiac myocytes were preincubated with WM (0.1 µmol/L) or solvent (DMSO), respectively, for 20 min. Subsequently, electrical field stimulation (1 Hz, 37°C) was started and contractility parameters were analyzed. Basal twitch contraction amplitude (in % of resting cell length) was not significantly altered in WM-treated versus control myocytes (1.91+/-0.19%; n=26 versus 2.01+/-0.21%; n=29; Figure 1). The mean resting cell length were 1.87+/-0.01 µm in WM-treated versus 1.85+/-0.01 µm in control myocytes (P=0.33).

After 20 min of incubation with WM, cardiac myocytes were alpha$_1$-adrenergically stimulated with PE (10 µmol/L). As expected, both WM-treated and control myocytes exhibited a significant increase in contractility (Figure 1A). However, in WM-treated myocytes, PE induced a significantly stronger increase in contractility compared to untreated myocytes (6.14+/-0.33%; n=26 versus 4.85+/-0.33%; n=29, $P<0.05$; Figure 1A-B). The mean resting cell length of PE-stimulated myocytes were 1.81+/-0.01 µm in WM-treated versus 1.81+/-0.01 in untreated myocytes (P=0.84).

4.2. Effect of PI3K inhibition on alpha$_1$-adrenergically stimulated intact muscle strips

Intact multicellular muscle strips were preincubated with WM for 40 min. Compared to the single myocyte experiments the incubation time was doubled to

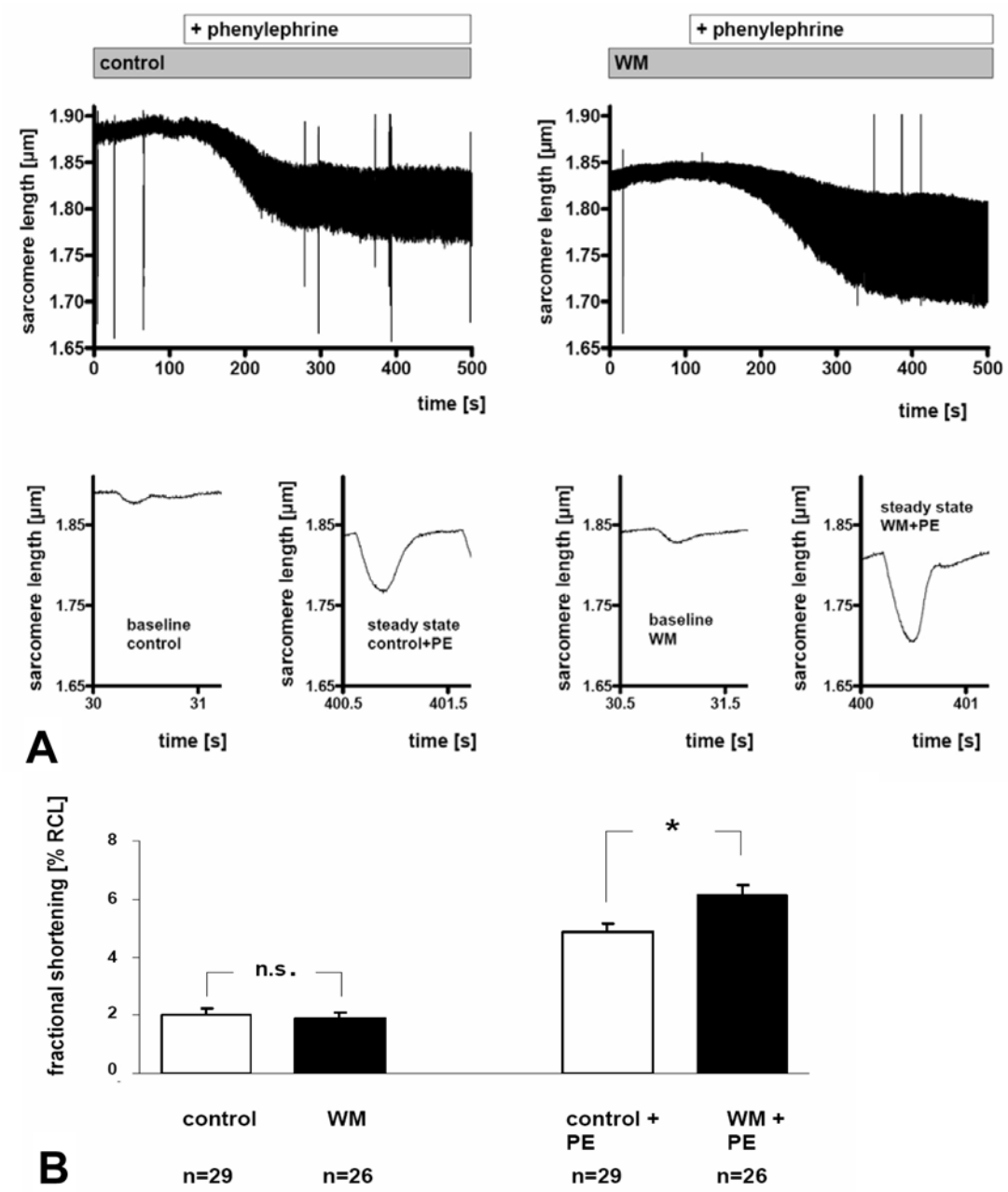

Figure 1. Effect of PI3K inhibition on fractional shortening in alpha$_1$-adrenergically stimulated single myocytes. After 20 min of incubation with wortmannin (WM, 0.1 µmol/L) isolated cardiac myocytes were stimulated with phenylephrine (PE, 10 µmol/L). A. Original registrations of myocyte shortening in representative experiments with (right panels) and without (left panels) WM. Fractional shortening was measured by sarcomere-length detection (in µm). The upper panels are original recordings demonstrating one whole experiment, respectively. The lower panels show representative single twitches under baseline conditions and after reaching a shortening steady state with PE-stimulation. B. Corresponding summary, demonstrating the impact of PI3K inhibition under baseline conditions (left side) and with alpha$_1$-adrenergic stimulation (right side). Fractional shortening (in % of resting cell length). n: as indicated. **P*<0.05, unpaired *t*-test.

account for the higher tissue thickness. Alpha$_1$-adrenergic stimulation by PE (10 µmol/L) induced a significant increase in contractility in both groups. Consistent with the findings in single myocytes inhibition of PI3K with WM significantly increased the positive inotropic effect of PE. Relative developed tension (F_{dev}/F_0) was 1.69+/-0.21 in WM-treated muscle strips versus 1.52+/-0.20 in control muscle strips (n=5 each, *P*<0.05; Figure 2).

4.3. Effect of PI3K inhibition on Ca^{2+} transients

To elucidate the mechanism of the increased contractility in PI3K-inhibited myocardium we analyzed

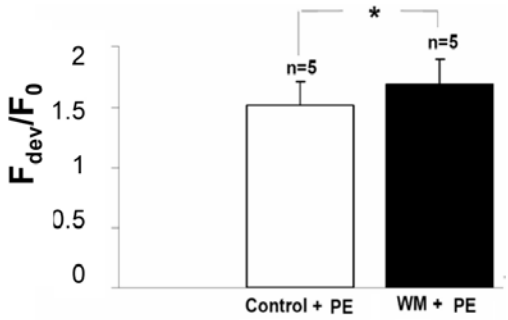

Figure 2. Effect of PI3K inhibition on alpha$_1$-adrenergically stimulated intact muscle strips. (right ventricular trabeculae and thin papillary muscles). After 40 min of incubation with WM (0.1 µmol/L), both groups of muscle strips (WM and control) were stimulated with PE (10 µmol/L). Reaching a steady-state, developed forces were measured and normalized to the baseline forces before PE-stimulation (relative developed tension, F_{dev}/F_0). n=5, each, **P*<0.05, paired *t*-test.

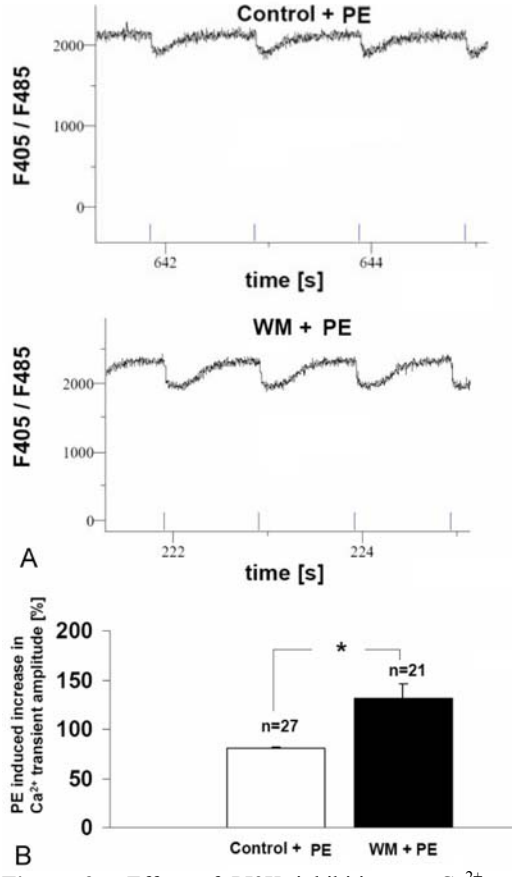

Figure 3. Effect of PI3K inhibition on Ca^{2+} transient amplitudes (indo-1). Isolated cardiac myocytes were incubated with WM (0.1 µmol/L) for 20 min. Ca^{2+} transient amplitudes of WM-treated and control myocytes were compared after alpha$_1$-adrenergic stimulation with PE (10 µmol/L). A. Original registrations of representative experiments. Upper panel: PE-stimulation of a control myocyte. Lower panel: PE-stimulation of a WM-treated myocyte. B. Relative increase in Ca^{2+} transient amplitudes in WM-treated and control myocytes after PE stimulation (in %). n: as indicated. **P*<0.05, unpaired *t*-test.

Ca^{2+} transient amplitudes (indo-1) in PE-stimulated myocytes with and without WM pretreatment. In control myocytes, PE increased the Ca^{2+} transient amplitude by 80.8+/-1.2% (n=27), whereas in WM treated myocytes PE raised the Ca^{2+} transient amplitude by 131.5%+/-14.8% (n=21, *P*<0.05). Figure 3 demonstrates the significantly higher Ca^{2+} transient amplitudes in WM-treated versus control myocytes.

4.4. Effect of PI3K inhibition on Ca^{2+} sensitivity in semi-skinned fibres

To analyze the influence of PI3K inhibition on Ca^{2+} sensitivity in alpha$_1$-adrenergically stimulated myocardium, muscle strips were semi-permeabilized with beta-escin. Figure 4A demonstrates that in alpha$_1$-adrenergically stimulated muscle strips the Ca^{2+} sensitivity was not affected by inhibition of PI3K. In PE-stimulated muscle strips the EC_{50} was 3.37+/-0.22 µmol/L following WM pretreatment versus 3.62+/-0.18 in control fibers (n=5, n.s.). The maximal calcium-activated force was slightly but not significantly increased in WM-treated versus control semi-skinned fibres (Figure 4B). Thus, increased contractility by PI3K inhibition is not caused by changes in myofilament Ca^{2+} sensitivity.

4.5. Effect of PI3K inhibition on protein expression and phosphorylation level

Since inhibition of PI3K increased Ca^{2+} transients in alpha$_1$-adrenergically stimulated myocytes we investigated its effect on sarcoplasmic reticulum Ca^{2+} ATPase (SERCA) and phospholamban (PLN). Incubation with WM (0.1 µmol/L, 20 min) had no effect on protein expression of SERCA and PLN when normalized to GAPDH in PE-stimulated cardiac myocytes (10 µmol/L, 2 min, Figure 5). However, Western blot analysis with phosphospecific antibodies revealed a significantly increased phosphorylation level of PLN in WM treated myocytes. PLN-Ser16/PLN and PLN-Thr17/PLN ratio increased by 115+/-19% (n=5, *P*<0.05) and 53+/-19% (n=5, *P*<0.05), respectively (Figure 5), indicating higher SERCA activity.

5. DISCUSSION

In the present study we demonstrate for the first time that inhibition of PI3Ks significantly improves contractility in alpha$_1$-adrenergically stimulated myocardium of rabbit hearts and that this effect is mediated by increasing PLN phosphorylation leading to higher intracellular Ca^{2+} transients.

These findings are contradictory to studies demonstrating that activation of PI3Ks by overexpression of IGF-1 receptor or IGF-1 infusion has positive inotropic effects and that decreasing PI3K activity by knockout of insulin receptor reduces myocardial contractility (5,11,16).

Differentiating the effects of the distinct isoforms of PI3Ks may help to explain these conflicting results. Four different type I PI3Ks have been described. They are characterized by a 110 kDa catalytic subunit (p110) and are divided into the subclasses IA (PI3Kalpha, PI3Kbeta,

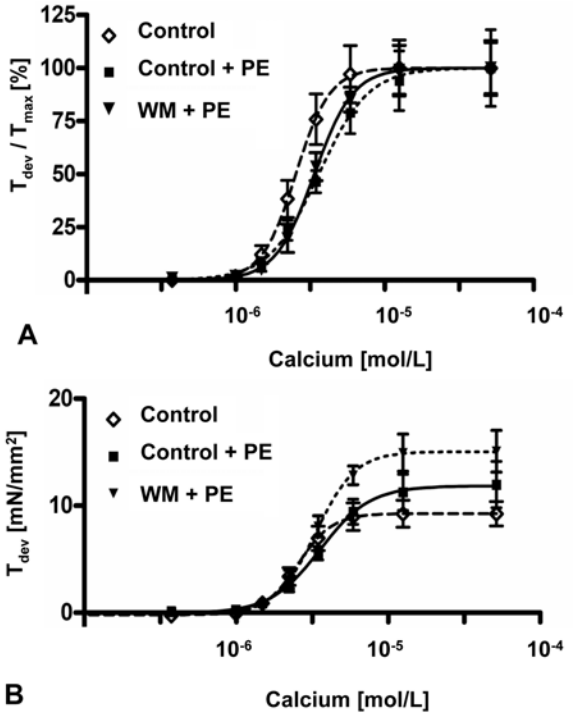

Figure 4. Effect of PI3K inhibition on Ca^{2+} sensitivity in $alpha_1$-adrenergically stimulated semi-skinned fibres. Intact muscle strips were semi-permeabilized with beta-escin to control the intracellular Ca^{2+} concentration but maintain sarcolemmal receptors and subcellular signaling mechanisms. Semi-skinned fibres were stimulated with PE (10 µmol/L) in the presence and absence of WM (0.1 µml/L). n=5. A. Concentration-response curves for Ca^{2+} (tension- Ca^{2+} relationship). Tensions (mN/mm^2) were normalized to maximal tension that could be activated by saturating Ca^{2+} concentration. Differences in EC_{50} were statistically analyzed by extra sum-of-square F test. B. Concentration-response curves for Ca^{2+} (tension- Ca^{2+} relationship). Absolute tensions (mN/mm^2).

PI3Kdelta) and IB (PI3Kgamma). The isoforms PI3Kalpha and PI3Kgamma are of particular importance for the heart (2,17). Stimulation of receptor tyrosine kinases (RTK) by IGF-1 or insulin activates the PI3K isoform alpha that is a crucial regulator of cardiac cell size, but also exerts positive inotropic effects due to enhanced Ca^{2+} cycling (18-20).

On the other hand, PI3K isoform gamma is activated by beta/gamma subunits of G-Proteins and acts downstream of G protein-coupled receptors (GPCRs) as a negative regulator of cardiac contractility (2,17). PI3Kgamma-knockout mice display a marked enhancement in contractility as assessed by increased fractional shortening and peak aortic outflow velocity (17).
Mechanistically, Crackower, Oudit *et al.* were the first demonstrating in an elegant study that PI3Kgamma decreases cardiac contractility by reducing cAMP levels (17). PI3Kgamma independently of its kinase activity controls PDE3B phosphodiesterase-mediated cAMP destruction (26). Since cAMP activates PKA which in turn mediates positive chronotropic, inotropic and lusitropic effects via phosphorylation of a variety of target proteins involved in excitation-contraction coupling, reduced cAMP levels result in reduced contractility (27-29).

In beta-adrenergically stimulated myocardium, it has been shown that PI3Kgamma modulates particularly $beta_2$-signaling. $Beta_2$-adrenergic receptors (AR) are coupled to both $G_{alpha/s}$ and $G_{alpha/I}$ G-proteins and thereby either increase (via $G_{alpha/s}$) or decrease (via $G_{alpha/i}$) cAMP levels and contractility. The opposing effects of these 2 G-protein subunits neutralize each other, resulting in no global net effect on cAMP levels and cardiac contractility upon receptor stimulation. However, when cardiac myocytes of PI3Kgamma knockout mice are stimulated with a specific $beta_2$-AR agonist, increases in cAMP levels and contractility are observed. In contrast, selective $beta_1$-AR stimulation does not differentially regulate cAMP levels and contractility in PI3Kgamma null hearts (17).

Regarding other GPCRs, it has been shown that activation of purinergic receptors (P2 receptor) diminishes Ca^{2+} oscillations via PI3Kgamma in neonatal cardiac myocytes while the depression of cardiac function observed in response to platelet-activating factor (PAF), a GPCR agonist released during ischemia, is prevented in PI3Kgamma-null hearts (30-31).

In the present study, we demonstrate for the first time that also $alpha_1$-AR signaling is modulated by PI3K. Since $alpha_1$-AR are GPCRs and inhibition of PI3K increases contractility in $alpha_1$-adrenergically stimulated myocardium, the observed effect might be caused by inhibition of PI3K isoform gamma. As a limitation of the present study, wortmannin, the PI3K inhibitor used here, is an unspecific PI3K inhibitor and does not allow us to discriminate between the different isoforms of PI3K.

Under baseline conditions, we found no effect of PI3K inhibition on cardiac contractility. However, with $alpha_1$-AR stimulation inhibition of PI3K exerts positive inotropic effects. This could be explained by higher PI3Kgamma activity under $alpha_1$-AR stimulation. Comparable to the effects on $beta_2$-signaling described above, PI3K activation by $alpha_1$-GPCR stimulation counteracts the dominating positive inotropic effect of $alpha_1$-signaling. This positive inotropic effect is enhanced by inhibition of PI3K. Mechanistically, we show that in $alpha_1$-adrenergically stimulated myocardium PI3K inhibition leads to increased phosphorylation of PLN and higher Ca^{2+} transient amplitudes whereas Ca^{2+} sensitivity of the myofilaments is not influenced. Since PI3Kgamma is known to reduce cAMP levels, inhibition of PI3K by wortmannin might increase contractility in $alpha_1$-stimulated myocardium by increasing cAMP levels with subsequent higher PKA activity. PKA enhances Ca^{2+} transients by phosphorylation of L-type Ca^{2+}-channels and PLN (at Ser16) thereby increasing $I_{Ca,L}$ and SERCA activity, effectively enhancing contractility (27-29). Interestingly, phosphorylation of PLN-Thr17 (CaMKII site) also seems to be increased slightly which may be explained

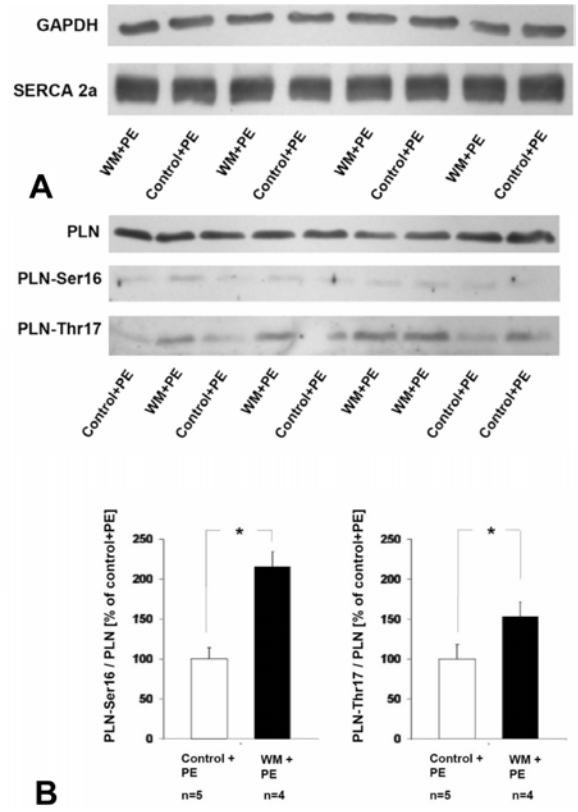

Figure 5. Effect of PI3K inhibition on expression and phosphorylation level of Ca^{2+} handling proteins. Isolated cardiac myocytes were incubated with and without WM (0.1 µmol/L) for 20 min, followed by PE stimulation for 2 min (10 µmol/L). A. Western blot analysis of SERCA protein expression. B. Western blot analysis of PLN expression and phosphorylation level (PLN-Ser16; PLN-Thr17). n: as indicated. *P<0.05, unpaired t-test.

by a secondary effect of increased intracellular Ca^{2+} cycling and hence increased CaMKII activation.

Since in human heart failure both the PI3K and the adrenergic signaling system are highly activated, it might be speculated whether inhibition of PI3Kgamma could improve cardiac function in heart-failure patients by modulating $alpha_1$-adrenergic signaling (32-34). In this regard, it must be critically marked that inhibition of PI3Kgamma increases cAMP signaling as seen with beta-AR agonists and PDE III inhibitors which have proven to be unsuccessful long-term therapies in patients with heart failure (35). Intriguingly, PI3Kgamma knockout mice are protected from hypertrophy, fibrosis and cardiac dysfunction caused by long-term exposure to the beta-AR agonist isoproterenol, suggesting that inhibition of PI3Kgamma may represent a therapeutic option for the treatment of decreased cardiac function in heart failure (36).

Although wortmannin, the PI3K inhibitor used in our study, has proven to be cardioprotective in a myocardial ischemia-reperfusion model (37), it is not an ideal compound for *in vivo* studies or for application in humans since it is also known to be toxic to rats, affecting mainly hepatocytes and the epithelial lining of the gastrointestinal tract (1). Furthermore, PI3Kalpha inhibitors have been considered as anti-cancer agents, but such inhibitors may have adverse effects in the heart (38). Therefore, a more compatible, isoform- and tissue-specific PI3K inhibitor is required for in vivo studies addressing treatment of heart failure.

In conclusion, this is the first study to demonstrate that inhibition of PI3K improves contractility in $alpha_1$-adrenergically stimulated myocardium by increasing PLN phosphorylation and Ca^{2+}-transients. This may have clinical implications for the treatment of decreased cardiac function in heart failure.

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Abbreviations: PI3K: phosphoinositide 3-kinase, Ca^{2+}: Calcium, WM: wortmannin, PE: phenylephrine, PLN: phospholamban, IGF-1: insuline-like growth factor, cAMP: cyclic adenosine monophosphate, PKA: protein kinase A, BDM: 2,3-butanedione monoxime, DMSO: dimethyl sulfoxide, PMSF: phenylmethyl-sulfonyl-fluoride, SERCA: sarcoplasmic reticulum calcium ATPase, GAPDH: glycerol aldehyde phosphate dehydrogenase, RTK: receptor tyrosine kinase, GPRC: G protein coupled receptor, PAF: platelet-activating factor

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Send correspondence to: Lars S. Maier, Dept of Cardiology and Pneumology, Heart Center, Georg-August-University Goettingen, Robert-Koch-Strasse 40, 37075 Goettingen, Germany, Tel: 49-551-39-9481, Fax: 49-551-39-8941, E-mail: lmaier@med.uni-goettingen.de

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