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AKT-PKA INTERACTION IN THE MODULATION OF CARDIAC EXCITATION-CONTRACTION COUPLING

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"Diciamo, pronunziamo, sentenziamo e dichiaramo che tu, Galileo Galilei, per le cose dedotte in processo e da te confessate come sopra, ti sei reso a questo S.o Off.o veementemente sospetto d'eresia, cioè d'aver tenuto e creduto dottrina falsa e contraria alle Sacre e divine Scritture, ch'il sole sia centro della terra e che non si muova da oriente ad occidente, e che la terra si muova e non sia centro del mondo (...). Ti condaniamo al carcere formale in questo S.o Off.o ad arbitrio nostro (...). E così diciamo, pronunziamo, sentenziamo, dichiariamo, ordiniamo e reserviamo in questo e in ogni altro meglior modo e forma che di ragione potemo e dovemo".

Estratto della sentenza di condanna per eresia, sottoscritta nei confronti di Galileo Galilei, nel 1615, dai sette inquisitori del Santo Offizio.

TABLE OF CONTENTS

INTRODUCTION

It is twenty years since the publication of the first three papers describing the cloning of a new proto-oncogene serine/threonine kinase termed protein kinase B (PKB)/Akt. Key roles for this protein kinase in cellular processes such as glucose metabolism, cell proliferation, apoptosis, transcription and cell migration are now well established. Recently a novel role was attributed to PKB/Akt such as a modulator of cardiac physiology. However, there are several and contradictory hypothesis about the role of Akt pathway on the modulation of cardiac function.

The PI3K/Akt pathway

PH-Domain. The pleckstrin homology (PH) domain is a small proteic motif of 100-120 aminoacids discovered at first in 1993 in pleckstrin and later on several proteins involved in different cellular processes (Haslam *et al.*, 1993; Mayer *et al.*, 1993). It was originally proposed that PH domains, like Src homology domains 2 and 3 (SH2 and SH3), might be involved in protein-protein interactions in cellular signalling. Subsequent work has shown that many PH domains direct

membrane targeting of their host proteins by binding to phosphoinositides rather than to proteins in cellular membranes (Vanhaesebroeck & Alessi, 2000). At present numerous proteins have been shown to contein a PH-domain, but for only a few number of them the exact function was convincingly demonstrated. In these cases the PH-domain binds with high affinity and specificity phosphoinositols. The ligands could be the phosphatidilinositol-4,5-bi-phosphate (PIP2) or the phosphatidilinositol-3,4,5-triphosphate (PIP3) whose generation could be catalyzed by the enzyme phosphatidilinositol-3-kinase (PI3K) (Lemmon & Ferguson, 1998; Rameh & Cantley, 1999). Although some PH domains bind their ligands with high affinity, the majority bind PIP weakly and in an aspecific manner; it is now unclear if these differences have some physiolgical relevances. Moreover, some studies show that some PH-domain of various proteins are able to bind only to PIP3 and not to PIP2; this is the case of serine/threonine kinease Akt (or PKB) (Lemmon & Ferguson, 2000).

All the PH domains have essentially the same structure, which is remarkable given that their pairwise sequence identities range from just 7% to a maximum of only around 23% (Lemmon & Ferguson, 2000). NMR analysis and crystal structure describe a core represented

by a β-sandwich of two nearly orthogonal β-sheets. One sheet consists of four β-strands (β1 through β4), and the other of just three (β5-β7). Loops present between β1/β2, β3/β4 and β6/β7, were found to be the most variable in length and sequence suggesting (by analogy with immunoglobulin-like domains) that they may constitute the ligand-binding site (Haslam *et al.*, 1993; Lemmon & Ferguson, 1998; Mayer *et al.*, 1993). The C-terminal is characterized by an amphipathic α-helix. PH-domains seem to be positive polarized, and the positive side agree with the position of the three variable loops (Lemmon & Ferguson, 2000). These characteristics are precisely those expected for a binding site that interacts with negatively charged membrane surfaces

Akt: a PH-domain dependent kinease. Akt, also known as protein kinease B (PKB), is a threonine/serine kinease belongs to the superfamily of proteins kinease cAMP and cGMP dependent and protein kinease C (ACG family); these proteins have in common a structure homology at the catalytic site level and are activated in an analogous manner (Song *et al.*, 2005). Akt/PKB controls a variety of regulatory responses in mammalian cells, including the inhibition of apoptosis as well as the regulation of cellular proliferation, metabolism and hypertrophy. Activated Akt/PKB phosphorylates

several downstream targets including glycogen synthase kinase-3b (GSK-3b), mammalian target of rapamycin (mTOR), endothelial nitric oxide synthase (eNOS or NOS3), 6-phospho-fructo-2-kinase (PFK2), c-Raf (a serine/threonine kinase) and several antiapoptotic effectors within cytoplasmic, mitochondrial and nuclear compartments. Multiple isoforms of Akt/PKB are expressed in the heart (Brazil & Hemmings, 2001; Oudit *et al.*, 2004). Generally there are three isoforms (Akt1-3) and knockout analysis has revealed distinct roles for each of the three Akt isoforms. Akt1 is essential for a normal growth, Akt3 is essential for the development of a normal brain size, whereas Akt2 is specifically involved in the maintenace of glucose homeostasis (Sakamoto *et al.*, 2006). Experiments from Staal and co-workers demonstrated that amplification of PKBα/Akt1 was detected in gastric adenocarcinoma (Staal, 1987).Thanks to cloning of the genes encoding PKB/Akt, the role of these genes in human cancers began to be examined more closely. Cheng and colleagues showed that the gene encoding Akt2 was amplified in two ovarian carcinoma cell lines and, similar to Staal and co-workers, the authors concluded that amplification of Akt2 contributed to the pathogenesis of the disease (Cheng *et al.*, 1992). For these reasons Akt pathway is considered the most crucial for the development of cancer. In

mammalian Akt is encoded by three genes: Akt 1 (PKBα), Akt 2 (PKBβ) and Akt 3(PKBγ) localized on chromosomes 14q32, 19q13 and 1q44 respectively. In cardiac tissue only the first two isoforms are expressed (DeBosch *et al.*, 2006). All the three isoforms keep the same structure: the N-terminal (about 100 aminoacids) is characterizes by the presence of the PH-domain (see above); the catalytic domain (KD) is localized in the central region of the molecule where a conserved residue of threonine allows a first activation of Akt. In the end, a C-terminal of 40 aminoacids is characterized by a hydrophobic motiv, an odd portion of the ACG kinease family (Fig. 1) (Song *et al.*, 2005).

In unstimulated cells, Akt resides in the cytosol and its kinase domain is thought to be masked by the C-terminal hydrophobic motif. In response to growth factor stimulation, Akt is recruited to the plasma membrane via its N-terminal PH domain. Membrane-associated Akt is sequentially phosphorylated at two regulatory phosphorylation sites, Thr-308 and Ser-473, by upstream kinases called phosphoinositide-dependent protein kinase-1 (PDK1) and the "hydrophobic motif kinase," respectively (Fig 1) (Altomare & Testa, 2005; Brazil & Hemmings, 2001; Woodgett, 2005). Cytosolic PIP3 levels depend on the action of several phosphatases among which the most important

appears to be PTEN (Phosphatase and Tensin Homolog) that reverts PIP3 phosphorilation to produce PIP2 acting as a PI3K reaction antagonist (Shiojima & Walsh, 2006). Akt is able to associate to PIP3 and PIP2 with the same. Specifically, a PH-domain that recognises only PIP3 should be recruited to mebrane level transitorily, while a protein with an affinity for both lipids, as Akt, should be associated to the membrane for a longer period with a consequent prolonged activation (Vanhaesebroeck & Alessi, 2000). It was demonstrated that a stable membrane localized variant of Akt, obtained with a myristoylation/palmitylation of its N-terminal, shows a maximal activation (Andjelkovic *et al.*, 1997). This constant activation is not influenced from other events as cellular over-stimulation or the presence of a negative dominant of PI3K. These results support that membrane localization is fundamental for serine/threonine activation and PIP3/PH-Domain interaction is relevant exclusively for membrane translocation. So, Akt activation needs membrane recruitment due to the interaction between PH-Domain and lipids generated by PI3Ks of class I (Oudit *et al.*, 2004). The kinases of this class, involved in Akt activation, are heterodimeric enzymes constituted by a regulatory subunit and a catalytic portion (p110), and they are branched in two sub-classes: class Ia (PI3Kα, PI3Kβ and

PI3Kδ) and class Ib (PI3Kγ). This distinction concern with their different activation: class Ia PI3K is stimulated by RTK agonists (Insulin; Insulin Growth Factor, IGF; Growth Factor, GH) and by receptor associate to cytokines, while class Ib kinases are activated by GPCR downstream signalling trough $G_{\beta\gamma}$ subunit (Oudit *et al.*, 2004).

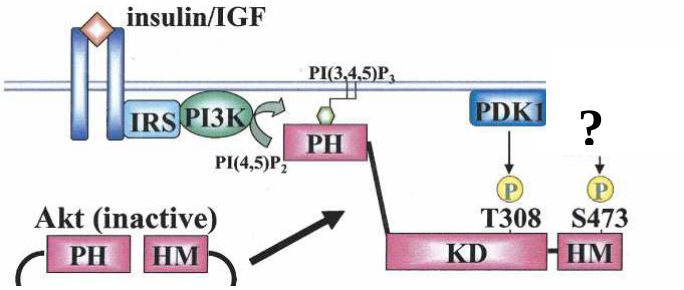


Figure 1 Schematic representation of Akt structure and activation model (modified from Shiojima *et al.*, 2006)

Once Akt has translocated at the membrane level, its regulation counts the phosphorilation of two specific sites: Thr-308, on the T-loop of the catalytic domain, and Ser-473 on the regulatory domain on the C-terminal. In detail, Threonine phosphorilation partially activates Akt, while, for a full activation, the kinease protein requires the phosphorilation of both sites. There are some evidences showing that PDK1 is required for Thr-308 phosphorilation. This enzyme has a

kinesic domain on the N-terminal and a PH-domain at the C-terminal that allows to interact with high affinity with PIP3, PIP2(4,5) and PIP2(3,4); PDK1 is associated to the membrane as Akt and when it is translocated phosphorilation occurs (Song *et al.*, 2005). PDK1 interaction with its ligands appears stronger then the one of Akt and this allows to PDK1 to bind them also in basal condition (Currie *et al.*, 1999).

At the present, the phosphorilation mechanism on Ser-473 is still quite unclear. Initially, the idea was that this residue was a second target of PDK1; however in KO embryonic stem cells, silenced for PDK1 gene, revealed that Ser-473 was phosphorilated as wild-type, while Thr-308 phosphorilation was totally abolished (Williams *et al.*, 2000). There are also some studies that hypothesize an auto-phosphorilative activity of Akt on this site, but this issue has not been further investigated (Shiojima & Walsh, 2006; Toker, 2000). Other studies show that Serine phosphorilation is due to other proteins as PDK2 (Toker & Newton, 2000) or Integrin-Linked Kinase (ILK), but it is not clear if this one is directly involved on the phosphorilative process (Lynch *et al.*, 1999). Recently, another mechanism involved in Akt activation has been discovered and seems to be PI3K-independent. Cellular stress (as heat-shock or iper-osmolarity), β-AR

agonists or agents that promote cAMP increment (as Forskoline and Prostaglandins) with PKA involvement could stimulate Akt pathway (Filippa *et al.*, 1999). Rise of cAMP levels shows an increment in Akt activity, and PKA inhibitor blunts this effect. This suggests a role of PKA in Akt activation but it seems not be directly involved in Akt phosphorylation (Filippa *et al.*, 1999). However, this mechanism needs to be clarified to better understand the correlation between cAMP elevation, PKA and Akt.

Role of PI3K/Akt pathway on the cardiac Excitation-Contraction Coupling (ECC) modulation

Excitation-Contraction Coupling. Excitation-contraction coupling (ECC) is a term coined in 1952 to describe the physiological process of converting an electrical stimulus to mechanical response (Sandow, 1952). This process is fundamental to muscle physiology, whereby the electrical stimulus is usually an action potential and the mechanical response is contraction. The general scheme is that an action potential arrives to depolarize the cell membrane. By mechanisms specific to the muscle type, this depolarization results in an increase in cytosolic calcium (Ca^{2+}) that is called calcium transient. This increase in

calcium activates calcium-sensitive contractile proteins that then use ATP to cause cell shortening.

In cardiac muscle the method is dependent on a phenomenon called Calcium-induced calcium release (CICR) (Fig. 2), which involves the conduction of calcium ions into the cell triggering further release of ions into the cytoplasm from sarcoplasmatic reticulum (SR). Action potential triggers L-type calcium channels (LTCC) causing a net flux of calcium ions into the cardiac myocyte (L-type calcium current – I_{CaL}). The increase in intracellular calcium concentration is detected by ryanodine receptors (RyR) in the membrane of the sarcoplasmic reticulum that transport calcium out into the cytosol from the SR in a positive feedback physiological response. Than cytoplasmic calcium binds to Troponin C, moving the tropomyosin complex off the actin binding site allowing the myosin head to bind to the actin filament. Using ATP hydrolysis the myosin head pulls the actin filament to the centre of the sarcomere. At the end intracellular calcium is taken up by the Sarcoplasmic Reticulum ATPase pump (SERCA) into the SR, or ejected from the cell by the Sodium-Calcium Exchanger (NCX) or the plasma membrane Calcium ATPase.

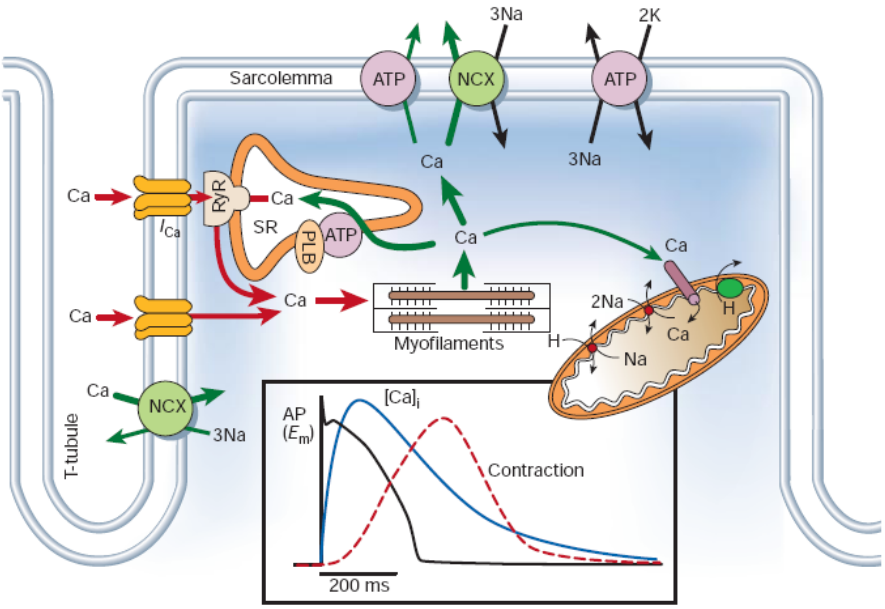


Figure 2 Schematic representation of ECC in which is show the Ca^{2+}. Inset shows a representative time course of an action potential, Ca^{2+} transient and contraction. (from Bers, 2002a)

Physiological sympathetic stimulation of the heart through β-adrenergic receptors increases developed contractions (inotropy) and accelerates relaxation (lusitropy) (Bers, 2002a) (Fig. 3). β-Adrenergic receptor stimulation activates the GTP-binding proteins, which stimulates adenylyl cyclase to produce cAMP, which in turn activates PKA. This kinase phosphorylates several proteins related to excitation–contraction coupling. At sarcolemmal level, PKA phosphorylates LTCC while at sarcoreticulum level phosphorylates

phospholamban (PLN) that in the un-phosphorilated form inhibits SERCA, and RyR. These are the more relevant proteins affected by PKA activity, but also others substrates are phosphorilated such as Troponin I and myosin bindin protein C. Generally, the lusitropic effect of PKA is mediated by phosphorylation of phospholamban and troponin I, which speed up SR calcium re-uptake and dissociation of calcium from the myofilaments, respectively. Inotropy is due to the phosphorylation of LTCC and RyR that increase the open probability of both, enhancing the calcium entry and release, consequently

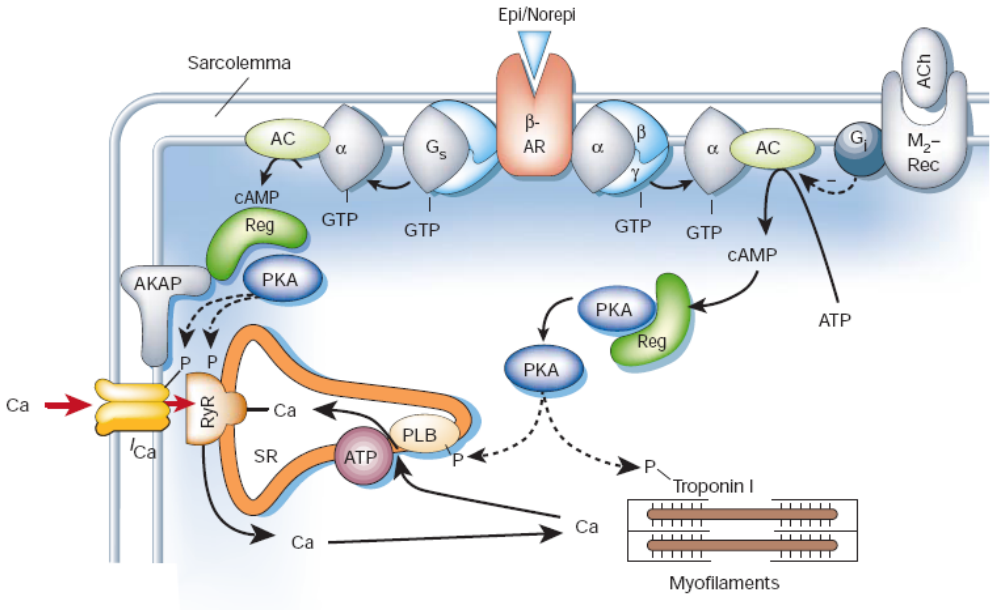


Figure 3 β-Adrenergic receptor activation and phosphorylation targets relevant to excitation–contraction coupling. (from Bers, 2002a)

How PI3K/Akt pathway affects the ECC. At the present there are several and contradictory hypothesis about the role of PI3K/Akt pathway on the modulation of cardiac function. Many data suggest an inhibitory role of this signalling pathway as many others affirm the opposite, assigning instead a positive function. Some of these studies focus the attention on the hypothetical interaction of PI3K/Akt pathway with the pathway activated by β-adrenergic receptor. It has been hypothesized that β2-AR generates a localized signalling able to modulate substrates usually close to each other in specialized membrane microdomains (lipid-rafts or caveolae). This kind of organization would limit the downstream signalling of the β2-AR to the sarcolemmal level, thus avoiding its action on SR domain. According to this hypothesis β2-AR stimulation leads inotropy through an increase in calcium entry, without affecting the SR store. This compartmentalized effect is probably caused by the activity of the Gi protein associated with β2-AR (Xiao *et al.*, 1999b). Some studies report that PI3K is involved in this process, in particular PI3Kγ couples to $G_{\beta\gamma}$ subunit of Gi (Zheng *et al.*, 2004). Zinterol, a specific synthetic β2-AR agonist, increases the contractility and the calcium transient amplitude of rat ventricular myocytes (Jo *et al.*, 2002). The PI3K inhibitor (LY294002) enhances Zinterol effects. In the same

work, LY294002 dose-dependently enhances phosphorilated PLN levels above those observed with zinterol only. In a similar way, myocytes, in which a constitutively activated PI3K was over-expressed, showed basal phosphorilation of PLN decreases and rise in cAMP concentration. In this case, LY294002 treatment failed to restore PLN phosphorilation (Jo *et al.*, 2002). These data accord with the hypothesis of PI3K involvement on the functional compartmentalization mediated by the β2-AR signalling (Fig. 4).

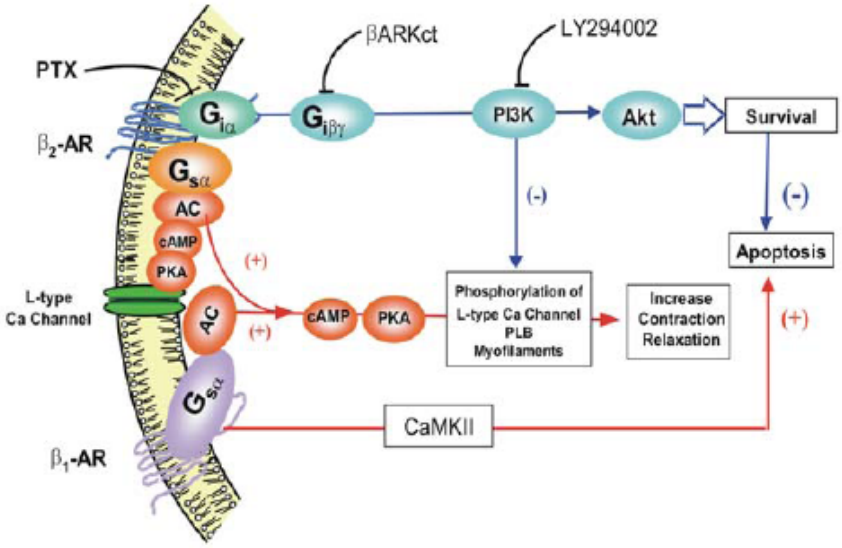


Figure 4 Dual coupling of β2AR to G_i coupling activates the Gi-Gβγ-PI3K-Akt pathway, which not only compartmentalizes and negates the concurrent Gs-AC-cAMP-PKA signaling, but also exerts an antiapoptotic effect in cardiomyocytes. In contrast, β1AR couples exclusively to G_s, which activates the Gs-AC-cAMP-PKA pathway. (from Zheng *et al.*, 2004).

A further demonstration of that is provided by experiments carried out, by the same group, in the presence of PTX (Gi inhibitor)

and βARK-ct ($G_{\beta\gamma}$ inhibitor): both studies did showed suppression of LY294002 effects (Jo *et al.*, 2002).

Otherwise, PI3K seems to be also important on the modulation of the cAMP/PKA signalling pathway stimulated by β1-AR. Specific β1 activation, induced with Norepinefrine in the presence of β2 and α1 specific antagonists (ICI and Prazosin respectively), shows an increment of PI3K activity (Leblais *et al.*, 2004). As just been observed for β2-AR, LY294002 augments the response obtained with β1-AR agonist. Thus, PI3K is also involved on the modulation of inotropism β1-AR dependent. β1-AR specific stimulation in the presence of Forskolin (FSK, Adenilate Cyclase activator) and LY294002 shows an incremented calcium current without changes in the PLN phosphorylation levels. This finding suggest that this pathway requires the activation of cAMP/PKA signalling (Leblais *et al.*, 2004). These works taken together suggest that the two distinct β-AR pathways are able to activate two different isoforms of PI3K, but there are no more evidences to assert this hypothesis that with absolute certainty. However, some evidences demonstrate the presence of two distinct PI3K activated pathways.

$PTEN^{-/-}$ and $p110\gamma^{-/-}$ ($PI3K\gamma^{-/-}$) double knock out mice are been used to demonstrate that PI3Kα/PTEN is involved in adaptative

cardiac ipertrophy, while PI3Kγ/PTEN affect cardiac muscle contraction and maladaptative ipertrophy (Crackower *et al.*, 2002; Oudit *et al.*, 2004; Oudit *et al.*, 2008). Some studies report that PI3Kγ$^{-/-}$ cardiac myocytes are characterized by higher calcium transients amplitude then the wild type ones and a more efficient SR calcium reuptake. Moreover, in this model there are not changes on calcium current amplitude, neither modifications occur on action potential duration. According with these data, PI3Kγ$^{-/-}$ cells show higher level of cytoplasmatic cAMP and phosphorilated PLN (Kerfant *et al.*, 2005). Rise of cAMP close to the SR system is really responsible for the local increase in SERCA activity and this mechanism justify the enhanced contractility observed in KO mice (Alloatti *et al.*, 2005). Moreover, Kerfant and co-workers demonstrate that in PI3Kγ$^{-/-}$ rats contractility rise without any change on cardiac size, hypothesizing a negative role of this kinase on ECC (Kerfant *et al.*, 2005). So, they conclude that this effect is due to a regulation of cAMP levels by phosphodiesterase (PDE) activity controlled by PI3Kγ (Kerfant *et al.*, 2007; Kerfant *et al.*, 2006).

These data suggest that modifications occurred after PI3K pathway alterations do not affect calcium current; however, some studies show different results. Gαq subunit of the G-protein coupled

receptors is able to reduce I_{CaL} through the inhibition of PI3K p110α/p85α (Lu *et al.*, 2005b; Lu *et al.*, 2005a). In these cells, PIP3 levels affect I_{CaL} suggesting and supporting the hypothesis of a PI3Kα-dependent current modulation. Sun and colleagues described that PTEN gene ablation leads to an increase of I_{CaL}. Specific PI3K inhibitors or Akt blockers reverse this effect, suggesting an involvement of PI3K/Akt pathway on I_{CaL} modulation (Kamp & Chiamvimonvat, 2006; Sun *et al.*, 2006). Using different mutant mice ($PI3K\gamma^{-/-}/PTEN^{-/-}$; $PI3K\alpha^{-/-}/PTEN^{-/-}$) these authors supposed a direct involvement of isoform α on LTCC regulation, a finding supported also by Akt consensus site on the channel $PTEN^{-/-}$ mice show an augmented I_{CaL}, double mutants $PI3K\gamma^{-/-}/PTEN^{-/-}$ do not change this increment while $PI3K\alpha^{-/-}/PTEN^{-/-}$ mutants blunt PTEN deletion effect. However, there are not biochemical evidences to confirm this theory. Data recently proposed by Catalucci's group suppose that an Akt-dependent phosphorylation of Ca(v)beta2, the LTCC chaperone for Ca(v)alpha1, antagonizes Ca(v)alpha1 protein degradation by preventing Ca(v)alpha1 PEST sequence recognition (Catalucci *et al.*, 2009b). These findings on calcium current suggest a positive role of PI3K/Akt pathway on ECC, in contrast with which described above. On the other hand, others studies demonstrate a positive role of

PI3K/Akt signalling on cardiac function. Over-expressed constitutively activated Akt in transgenic mice show a progressive ipertrophy and augmented contractility *in vivo* (Condorelli *et al.*, 2002). Further, *in vitro* studies on isolated rat ventricular cardiomyocytes confirmed a positive inotropic effect and an increase of SERCA activity consequent to Akt over-expression (Kim *et al.*, 2003). Taken together these data confirm again the complexity of cardiac function scenario.

PI3K/Akt pathway and heart disease. PI3K/Akt and several pathways are activated in heart disease including myocardial ischemia/reperfusion, diabetic-associated cardiomyopathy, adriamycin-induced cardiomyopathy, chronic β-AR stimulation, pressure-overload-induced hypertrophy and in advanced human heart failure (Duan *et al.*, 2003; Matsui *et al.*, 2001; Matsui *et al.*, 2002; Oudit *et al.*, 2003). Class IA and IB PI3K isoforms appear to have distinct roles in the pathogenesis of heart disease. Animal models with increased class IA PI3K and/or Akt/PKB signaling have enhanced cell survival and smaller infarct size in ischemia/reperfusion condition (Oudit *et al.*, 2004). Cardiac-specific overexpression of dominant negative of PI3Kα and knockout of the insulin receptor are associated to accelerated progression to dilated cardiomyopathy in response to

pressure-overload, which may be related to reduced phospho-Akt/PKB and cell survival and/or altered mechanotransduction (Oudit *et al.*, 2004). In addition, enhanced nuclear phospho-Akt/PKB is associated with delayed cellular aging and death in cardiomyocytes. Interestingly, phospho-Akt/PKB is higher in cardiomyocytes from adult premenopausal women compared to men or postmenopausal women which may explain the age-related decline in cardiac function as well as gender-dependent differences in susceptibility to cardiovascular diseases (Camper-Kirby *et al.*, 2001). On the other hand, redox inactivation of PTEN increased PtdIns(3,4,5)P3 levels and the consequent upregulation of PI3K signaling may contribute to the impaired myocardial contractility in oxidative stress-induced cardiomyopathies (Oudit *et al.*, 2004).

PI3Kγ activation is linked to left ventricle enlargement and decompensation in pressure-overload induced heart disease, while impaired PI3Kγ signaling is associated with limited development of hypertrophy and minimal deterioration in cardiac function. Although the mechanism for this remains to be elucidated, sustained β-AR stimulation and PI3Kγ activation are also implicated in the development of pathologic hypertrophy and progression of heart failure in animal models and humans (Lefkowitz *et al.*, 2000). Indeed,

stimulation of β-ARs activates ERK1/2, which is involved in growth factors stimulated pathway, leading to hypertrophy in neonatal and adult cardiomyocytes. While loss of PI3Kγ prevents isoproterenol-induced increases in cardiac Akt/PKB activity and ERK1/2 phosphorylation resulting in reduced hypertrophy, protection of heart function (Oudit *et al.*, 2003).

PI3Kγ is also pivotal in mediating negative inotropic effects of Gαi signaling associated with heart failure (El Armouche *et al.*, 2003). The maladaptive effects of PI3Kγ may be related to its effects on cAMP, calcium handling and myocardial contractility, suggesting that inhibiting PI3Kγ may provide an important means to negate the increased Gαi in heart failure. Indeed, altered PKA-mediated regulation of PLN and SERCA2 function is associated with cardiomyopathy in animal models and human; PI3Kγ inhibition may allow a selective upregulation of PLN/SERCA2 function while preventing a non-discrete upregulation of cAMP signaling as seen with β-AR agonists and PDE III inhibitiors that has proven to be unsuccessful therapies for heart failure (Movsesian, 1999).

In conclusion, it appears that PI3K activation in the heart can either be beneficial or harmful depending on the specific PI3K isoforms activated, suggesting that an optimal therapeutic approach

for heart disease may require selective enhancement of PI3Kα activity and/or reduced PI3Kγ activity.

AIM

The diverse effects mediated by PI3K/Akt signalling in the heart clearly support an important biological and pathophysiological role for this signalling cascade. The PI3K/Akt signalling pathways are involved in a wide variety of diseases including myocardial hypertrophy and contractility, heart failure, and preconditioning.

The aim of this study was to further investigate the role of PI3K/Akt pathway on the modulation of the cardiac excitation-contraction coupling (ECC). We focused our attention on Akt, because the role of this kinase results still unclear.

To pursue this purpose we inhibited Akt through two experimental approaches: first, we used two chemical unrelated compounds (Compound A and B) able to interact with kineases PH-domain to prevent the recognition of the PIP3 inositol ring and subsequent activation; second, Akt gene silencing through RNA intereference technique. With these two methods, we were able to rule out compound-dependent effects due to their potential aspecificity and to assert the effective consequence of Akt activity ablation on the modulation of cardiac function.

MATERIALS AND METHODS

The investigation conforms to the Guide of the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH publication 85-23, revised 1996); all experiments conforms to the Guide of the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH publication No. 85-23, revised 1996) and to the guidelines for animal care endorsed by the hosting institution.

Myocyte Isolation and culture

Male rats, weighting 100 to 125 g, were anesthetized by inhalation of ethyl ether and sacrificed by cervical dislocation, followed by exsanguinations. The extracted heart was maintained in a physiological solution (Solution 1, Tab 1) and scrap tissues were eliminated; following, the organ was mounted in a *Laghendorff* system that allows through a retro-perfusion way to wash out the blood from the ventricular and atrial wall. The temperature was kept costant at 35°C through thermostated tubes containning different solutions. When the heart appeared completely washed the perfusion switched to a calcium free solution (Solution 2, Tab 1) to stop the heart beat and to relax the cellular gap-junction and promote the

matrix desegregations. After 5 minutes an enzymatic solution was applied (Solution 3, Tab 1) to disrupt the cardiac tissue and after about 10-15 minutes the solution was switched to a KB maintaining solution (Tab 1) for about 5 minutes. At the end of this enzymatic dissociation procedure the atria were cut off and the ventricles were snipped in a 2-3 mm^2 squared fragments. The obtained tissue pieces were collected and mechanical dissociated in KB solution. Collected cells obtained by this method were centrifuged and after a calcium readjustment only rod-shaped, calcium tolerant myocytes were used.

Primary cultures were prepared by myocyte pellets obtained as described above. The pellets were resuspended in (mM), 2 L-Carnitine hydro-chloride (Sigma), 5 Creatine (Sigma), 5 Taurine (Sigma), 2mg/ml bovine serum albumin (Sigma), 0.1x Insulin-Transferrin-Selenium (Invitrogen), 10 2,3-Butanedione monoxime (Sigma), 100U/ml PEN-STREP, 2 Glutamine, in 199 Medium (Sigma). Cell suspensions were plated in 10-cm Laminin-coated Petri dishes (10ng/ml, Sigma). 1-(β-D-Arabino-furanosyl) cytosine hydrochloride (0.001 mM) was added to the culture medium to inhibit fibroblast growth. Cultures were incubated at 37°C and 5% CO_2 and subjected to measurements within 12 hours from plating.

BASE	
	mM
NaCl	143
KCl	5.4
$MgCl_2$	0.5
NaH_2PO_4	0.25
HEPES NaOH	5
D-glucose	5.5
pH 7.4 (NaOH)	

SOLUTION I	
	mM
BASE	
$CaCl_2$	1.8

SOLUTION II
BASE

SOLUTION III	
BASE	
$CaCl_2$	0.03 mM
BSA	1 mg/ml
protease	0.17 U/ml
collagenase	140 U/ml

KB	
	mM
KOH	70
Glutammic Acid	50
KCl	40
Taurin	20
KH_2PO_4	20
$MgCl_2$	3
D-glucose	10
HEPES	10
EGTA	0.5
pH 7.4 (KOH)	

Table 1 Solutions used to isolate caardiac myocytes isolation

Western Blot Analysis

Cardiomyocytes were incubated with isoproterenol 1 μM (Sigma) for 30 minutes and, subsequently, with compounds A (5 and 10 μM) or B (50 μM) for 30 seconds. Immediately after treatment, the medium was removed, cells were rinsed twice and harvested using a scraper in PBS solution supplemented with 1:25 Cocktail Complete EDTA-free (Roche), 1 mM NaF, 1 mM Na^+ orthovanadate at 4°C (pH 7.4). The suspensions were centrifuged and pellets were homogenized

by mechanical disruption using styrene beads in a Mixer Mill 300 (Retch) in ice-cold lysis buffer containing 40 mM KCl, 20 mM Imidazole (pH 7), 5 mM EDTA, 1% TRITON X-100, 1:100 Phosphatase inhibitors cocktail II and I (Sigma), 1:25 cocktail Complete EDTA-free (Roche), 1% NP-40. Cell homogenates were loaded onto a gel and proteins were detected by Western Blotting using the following antibodies: total PLN (Upstate cat. no. 05-205), Phospho-PLN (Ser16) (Santa Cruz cat. no. sc-12963), total Akt, Phospho-Akt (Ser473) and Akt1 (Cell Signaling Technologies cat. no. 9272, 9271L and 2967 respectively). A horseradish peroxidase-conjugated antibody (Pierce) was used as secondary reagent and signal was detected using the Western blotting Lightening ECL detection system (Pierce).

Small interference RNA (siRNA)

Isolated myocytes were transfected by incubation for 48 hours with 100 nM double-strand RNA oligos against Akt1 sequences 5'AGC-ACC-GUG-UGA-CCA-UGA-AdTdT3' and 3' dTdTU-CGU-GGC-ACA-CUG-GUA-CUU5' (Invitrogen) and Lipofectamin 2000 (Invitrogen). The procedure was carried out according to manufacturer's instructions provided with the BLOCK-iT Transfection Kit (Invitrogen).

Real-Time PCR

Total RNA was extracted from cells with the RNeasy mini Kit (Qiagen) according to the manufacturer's protocol. DNase treatment was performed during the extraction procedure. RNA was reverse transcribed using random hexamers and Superscript II RT enzyme (Invitrogen). After a first step of denaturation of the RNA and primers at 65°C for 5 minutes, the RT mix was incubated at 25°C for 10 minutes, at 42°C for 1 hour and at 72°C for 15 minutes for RT inactivation.

18S ribosomal RNA gene (Applied Biosystems) was used as reference gene and the delta-delta Ct method was applied for the relative quantification (User Bulletin #2: Rev B, Applied Biosystems). The required calibrator consisted of a pool of cDNAs from rat normal tissues. Akt1 primers and FAM-probe sequences were as following: AAT-GGA-GGC-GAG-CTC-TTC-TTC, GCC-CGG-TCC-TCT-GAA-AAC-A, and ACC-TGT-CTC-GTG-AGC-G.

Recording Solutions

During functional measurements, myocytes were superfused at 0.5 ml/min with Tyorode's solution containing (mM) 154 NaCl, 4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 5 HEPES-NaOH, 5.5 D-glucose, adjusted to pH 7.35. The patch pipette solution contained (mM) 110 K^+-aspartate,

23 KCl, 0.2 $CaCl_2$ (calculated free-Ca^{2+} = 10^{-7} M), 3 $MgCl_2$, 5 HEPES KOH, 0.5 EGTA KOH, 0.4 GTP-Na salt, 5 ATP-Na salt, 5 creatine phosphate Na^+ salt, pH 7.3. The composition of superfusing and pipette solutions was modified in specific protocols, as described in the respective sections (see below). All experiments were performed at 35±0.5°C; a thermostated manifold, allowing for fast (electronically timed) solution switch, was used for cell superfusion. Only the leakage protocol was performed at room temperature.

Cell shortening (twitch) measurement

Myocytes were excited by field stimulation trough a pair of platinum electrodes, with a current amplitude adequate to induce full-fledged contractions (80-120 mA, pulse duration 0.1 ms), at a frequency of 2Hz. Myocyte shortening (twitch) was measured by a Video Edge Detection System (VED-105) on images acquired at a rate of 200 frames/sec (Philips 800 Camera System).

Electrophysiological measurements

Current-clamp: action potentials were recorded from isolated myocytes by the perforated-patch technique (Amphotericin B 260μM; Sigma-Aldrich). Myocytes were stimulated through the recording pipette (3 ms current pulses). Action potential waveforms were acquired (sampling rate of 10 kHz) during steady-state stimulation.

Voltage-clamp: isolated myocytes were patch-clamped in the whole-cell configuration. Membrane capacitance (C_m) and series resistance were measured and compensated to at least 80% of their initial value during calcium current recordings. Current signals were filtered at 2 kHz and digitized at 5 kHz.

Trace acquisition (Axon Digidata 1200) was controlled by dedicated software (Axon pClamp 8.0), also used for offline analysis.

Cytosolic Ca^{2+} Measurement

Isolated myocytes were incubated with the membrane permeable Ca^{2+}-sensitive dye FLUO4-AM (10μM; Molecular Probes) for 30 minutes. The fluorescent emission was collected by a 40X oil immersion objective, its 535nm component (F_{535}) was amplified by a phototube (Electron Tubes Inc. UK) and converted in voltage. The signal was lowpass filtered (200 Hz, Analog Devices) and digitized at 2 kHz.. During offline analysis, the background was digitally subtracted and the F_{535} signal was smoothed by lowpass digital filtering (100Hz, FFT filter, Microcal Origin). Cytosolic Ca^{2+} measurements were performed under V-clamp, according to the protocols described below. Absolute values of Ca^{2+} activity were not required for the purpose of the study; thus, Ca^{2+} activity is reported in F_{535} arbitrary units (A.U.).

Experimental protocols

Protocolo 1 (I_{CaL} measurement): test pulses (450 ms) were applied from a holding potential of -40mV (cell cycle = 500ms). Contamination by K^+ currents was minimized by replacing pipette and extracellular K^+ with Cs^+. I-V relationships were obtained by measuring peak I_{CaL} during test pulses from -40 mV to 50mV.

Protocol 2 (SR Ca^{2+} uptake function): this protocol, named "reloading protocol", aimed to test SR Ca^{2+} uptake in the presence of various luminal Ca^{2+} contents, including low ones. To rule out its contribution to cytosolic Ca^{2+} transients, the Na/Ca-exchanger (NCX) was inhibited by incubation of the cells for 30 min in a Na^+ and Ca^{2+} free solution (replaced by equimolar Li^+ and 1 mM EGTA; Tab 2). The pipette solution was Na^+-free (replaced by K- or Tris- salts) and contains EGTA (0,5 mM). The SR was initially depleted by a pulse (15 s) of caffeine (10 mM) added to 154 mM Na^+ solution, to allow extrusion of the released Ca^{2+} through NCX (membrane potential held at – 40 mV) (Tab. 2). The SR was then progressively reloaded, under Na^+ -free conditions, by a train of depolarizing steps (-40 to 0 mV, 200 ms, 0.25 Hz) delivered in the presence of 1 mM Ca^2 (Tab. 2) (protocol outlined in the inset of Fig. 7A). Voltage-induced Ca^{2+} transients and transmembrane current (I_m, reflecting I_{CaL}) were

recorded during each step. Contamination of I_m by the transient outward current (I_{to}) was minimized by 4-aminopyridine (2mM). The SR Ca^{2+}-uptake function (SERCA2 uptake flux minus leak flux) was evaluated by the following parameters: 1) the time constant of Ca^{2+} decay (τ_{decay}) within each step (by monoexponential fitting); 2) the increment of Ca^{2+} transient amplitude along the stimulation train; 3) the increment of the ratio between peak Ca^{2+} transient and I_{CaL} amplitude, a rough measure of excitation-release coupling gain (Bers, 2002b), along the stimulation train.

0 Na^+ 0 Ca^{2+}	
	mM
LiCl	154
$MgCl_2$	1
HEPES KOH	5
D-glucose	5.5
KCl	5
EGTA	1
pH 7.35 with KOH	

154 Na^+ 0 Ca^{2+}	
	mM
NaCl	154
$MgCl_2$	1
HEPES NaOH	5
D-glucose	5.5
KCl	5
Caffeine	10
pH 7.35 with NaOH	

0 Na^+ 1 Ca^{2+}	
LiCl	154
$MgCl_2$	1
HEPES KOH	5
D-glucose	5.5
KCl	4
$CaCl_2$	1
pH 7.35 with KOH	

Pipette	
KAsp	110
KCl	23
$MgCl_2$	3
HEPES KOH	5
EGTA	0,5
$CaCl_2$	1
GTP Tris-salt	0.4
ATP Tris-salt	5
CP Tris-salt	5
pH 7.3 with KOH	

Table 2 Solutions used in the "reloading protocol"

Protocol 3 (SR Ca^{2+} content -$[Ca]_{SRT}$-): This protocol aimed to measure total SR Ca^{2+} content at steady-state during cyclic membrane depolarization and under physiological ionic conditions. I_m at a holding potential -40 mV and cytosolic Ca^{2+} were simultaneously recorded (in normal Tyrode's solution, 1 mM EGTA in the pipette) during a 10 s caffeine pulse (10 mM) applied 15 s after a "loading" train of voltage-steps (-40 to 0 mV, 200 ms, 0.37 Hz) (Fig 1). $[Ca]_{SRT}$ (in μmoles/liter of cytosol) was estimated by integrating I_m elicited by the caffeine pulse (reflecting I_{NCX} – see Table 3) (Trafford *et al.*, 1999) and dividing the result by the estimated cell volume (C_m * 9.94)(Bers, 2002b).

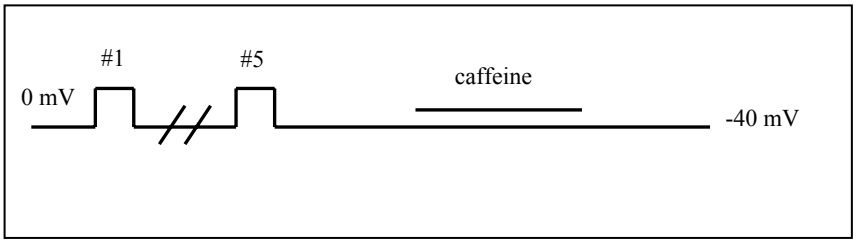


Figure 1 Protocol used to assess the SR calcium content.

$\int I_{NCX}=Q$	INCX = Na/Ca exchanger current Q = charge quantity
Moles number = Q/e/N	e = elemntar charge N = Avogadro number
Cytosolic volume = capacity/cytosolic factor	Cytosolic factor = 9.94 (Bers, 2002b)
$[Ca^{2+}]_{SRT}$ = n° moles/cytosolic volume	

Table 3 Functions used to estimate the SR calcium content.

Protocol 4 (SR Ca^{2+} leak/load relation): this protocol aimed to test the relation between unstimulated Ca^{2+} efflux through RyR channels (SR Ca^{2+} leak) and SR Ca^{2+} load, according to the method previously described (Curran *et al.*, 2007). Measurements were performed on FLUO-4 (10µM) loaded intact myocytes, activated by field stimulation. Myocytes were stimulated at different frequency in normal Tyrode solution to reach the SR loading steady state. Superfusion was then rapidly switched to a Na^{+}- and Ca^{+}-free solution (to block NCX) containing the reversible RyR blocker tetracaine (1mM). After achievement of stable cytosolic Ca^{2+} signal during quiescence, tetracaine wash-out led to an increase in cytosolic Ca^{2+}, completed within 30 sec, from which tetracaine-sensitive Ca^{2+} efflux from the SR was estimated. $[Ca]_{SRT}$ was then measured, within the same myocyte, from the peak amplitude of the Ca^{2+} transient elicited by a 10 mM caffeine pulse (15 s) (protocol was elucidated in figure 2).

Direct quenching of the FLUO-4 signal by 1 mM tetracaine was preliminarily measured with the following protocol: previously myocytes were incubated with 30μM Ryanodine, to block the RyR channel opening, and 10μM FLUO-4; fluorescence was recorded during superfusion with Ca^{2+}- and Na^{+}-free Tyrode solution plus Ryanodine (30μM) and CpA (Cyclopiazonic acid, 50μM – Sigma) to inhibit SERCA function. When fluorescence reach steady state, 1mM Tetracaine was applied. The fluorescence step generated from Tetracaine superfusion was calculated as quenching, estimated to be 16% F535 A.U. at steady state fluorescence signal.

The difference between the F535 signal just before tetracaine wash-out and at steady-state after it, corrected for the quench value, was used as a measure of tetracaine-sensitive SR Ca^{2+} leak. Leak/load relationships were obtained, under each experimental condition, by plotting Ca^{2+} leak vs $\underline{[Ca]}_{SRT}$ pairs, for each stimulation frequency.

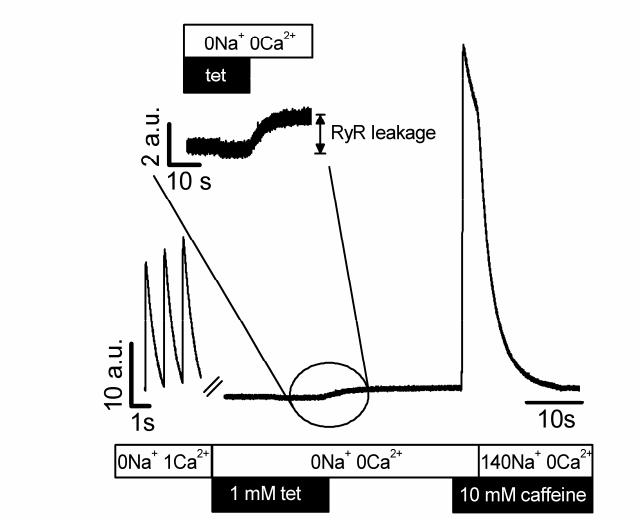


Figure 2 Schematic representation of leak/load protocol

Normal Tyrode (NT)	
	mM
NaCl	140
KCl	4
$MgCl_2$	1
$CaCl_2$	2
HEPES NaOH	5
D-glucose	10
pH 7.4 (NaOH)	

0 Na^+ 0 Ca^{2+}	
	mM
LiCl	140
KCl	4
EGTA	10
$MgCl_2$	1
D-Glucose	10
$MgCl_2$	3
HEPES KOH	5
pH 7.4 (LiOH)	

140 Na^+ 0 Ca^{2+}	
	mM
NaCl	140
KCl	4
$MgCl_2$	1
HEPES NaOH	5
D-glucose	10
pH 7.4 (NaOH)	3

Tabella 4 Solutions used in the leak/load protocol.

Substances

1,3-Dihydro-1-(1-((4-(6-phenyl-1H-imidazo[4,5-g]quinoxalin-7-yl)phenyl)methyl)-4-piperidinyl)-2H-benzimidazol-2-one (Cat. No. 124018, CAS 612847-09-3, Merck Catalogue) was synthetized by Nerviano Medical Sciences (NMS94.65) and will be referred to as CpdA. CpdA was used at the concentration of 5 μM, the maximal which could be stably dissolved in Tyrode. The biological activity (on Akt phosphorylation) of this concentration is reported in the results.

A glucuronic derivative with a phosporamidate group at the anomeric position, which mimicks the natural ligand phosphatidyl inositol, was sinthetized by us (Dr. L Cipolla, see also (Cipolla *et al.*, 2009) and will be referred to as CpD B. CpdB was used at concentration of 50μM , representing the EC_{25} of the concentration-response curve on Akt phosphorylation measured by Western Blot in preliminary experiments (Cipolla *et al.*, 2009). Higher concentrations, tested on myocytes in preliminary experiments, invariably caused cell death by contracture.

Isoproterenol (ISO) was used as a non isoform-selective β-AR agonist. To discriminate the effect of β1-AR and β2-AR selective stimulation, ISO (10 nM) was applied in the presence of the subtype antagonists ICI-118.551 (ICI, β2-selective, 10μM) and CGP-20712A

(CGP, β1-selective, 300nM) respectively. Selective stimulation of β_2-AR was also pursued by using the specific agonist Salbutamol (50 μM). All β-AR ligands were dissolved in water.

Stock FLUO4-AM solution (1 mM) was prepared by dissolving the substance in anidrous DMSO. Final FLUO4-AM solution, used for myocyte loading, was prepared by stock 1:100 dilution in Tyrode (final concentration = 10μM).

FLUO4-AM was purchased from Molecular Probes, all other chemicals from Sigma-Aldrich.

Statistical analysis

Means were compared by paired or unpaired t- test and, wherever appropriate, by one-way ANOVA. Linear and non linear fitting was performed by the least-square method (Microcal Origin). Data are expressed and plotted as the mean ± S.E value obtained from a number of independent determinations. Absolute values of the variables and sample size (n) for each experimental condition are specified in the tables, figures and the respective legends.

RESULTS

Effects of PH-domain antagonists on twitch amplitude

Intact myocytes were activated by field-stimulation at a cycle length (CL) of 1 sec. Under basal conditions, challenge with 5 μM CpdA consistently increased twich amplitude by 26% ($p<0.05$); a similar effect was observed with CpdB at the concentration of 50 μM (Fig.3). Washout of effects was very slow and could be partially observed within the recording time in only 50% of myocytes.

In a further set of experiments, the effects of Cpd A (5 μM) and Cpd B (50 μM) were tested in the presence of β-AR stimulation (Fig 4). To ensure detection of both positive and negative Cpd interactions with βAR-induced effects, ISO was used at 10 nM, a concentration found in preliminary experiments to exert about 60% of maximal enhancement of twitch amplitude. CpdA, applied after ISO inotropic effect had achieved a steady state, further increased twitch amplitude. The percent increment in twitch amplitude induced by CpdA was significantly larger in the presence of ISO than under basal conditions (Fig 5). Similar results were obtained with CpdB (Figs 3-5). From these results we conclude that Cpd A and B have a positive inotropic effect and interact in a cooperative way with β-AR stimulation.

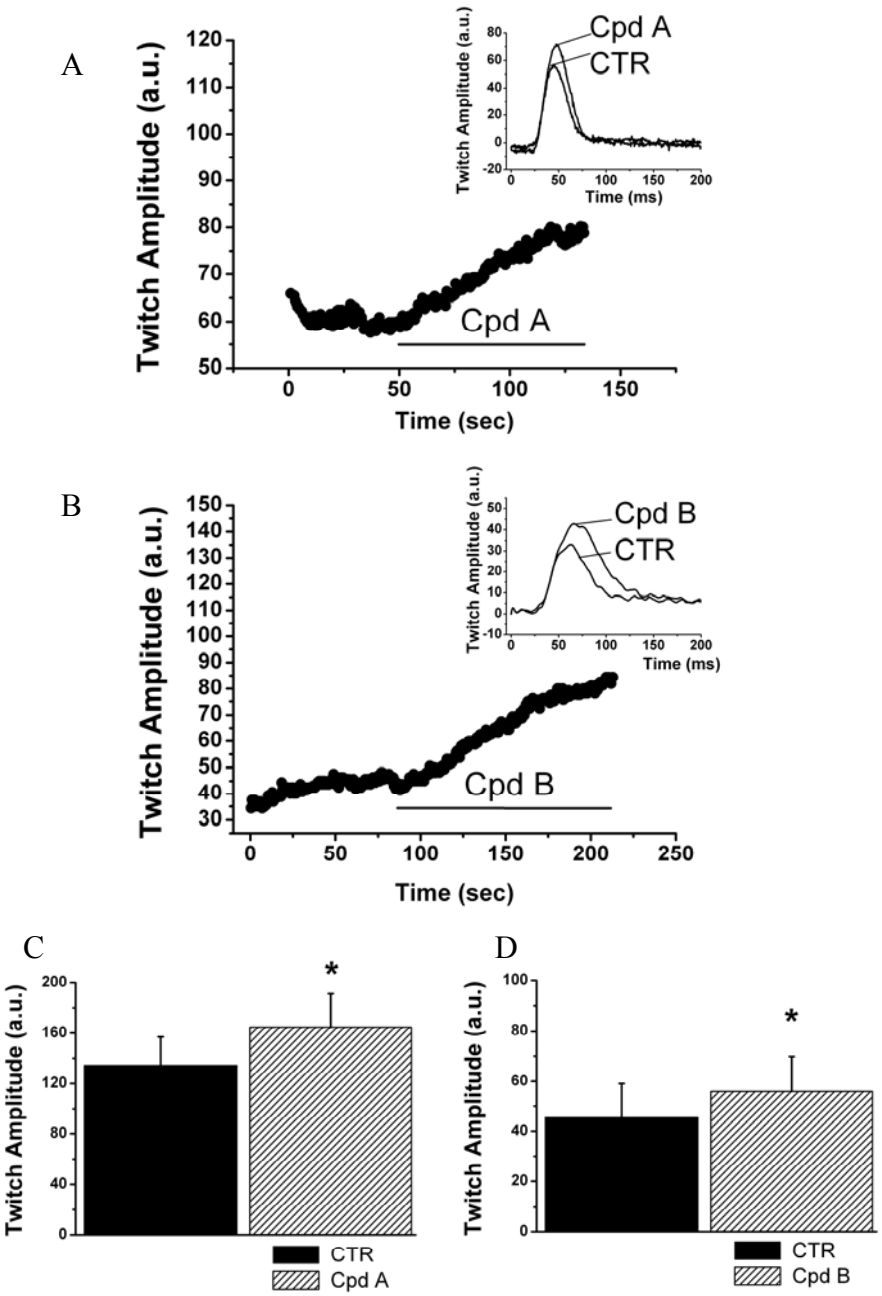


FIGURE 3: *Effects of PH-domain inhibitors on twitch amplitude in basal condition.* Time course representation of twitch peak amplitude measurements at each cycle (cl = 1s) in field stimulated cells during superfusion of Cpd A (5μM) (A) and B (50μM) (B). The insets show twitch sample traces recorded in control condition (CTR) and during superfusion of Cpds. C), D) Avarage results for Cpd A (n=12) and B (n=6) respectively (* $p<0.05$ vs CTR).

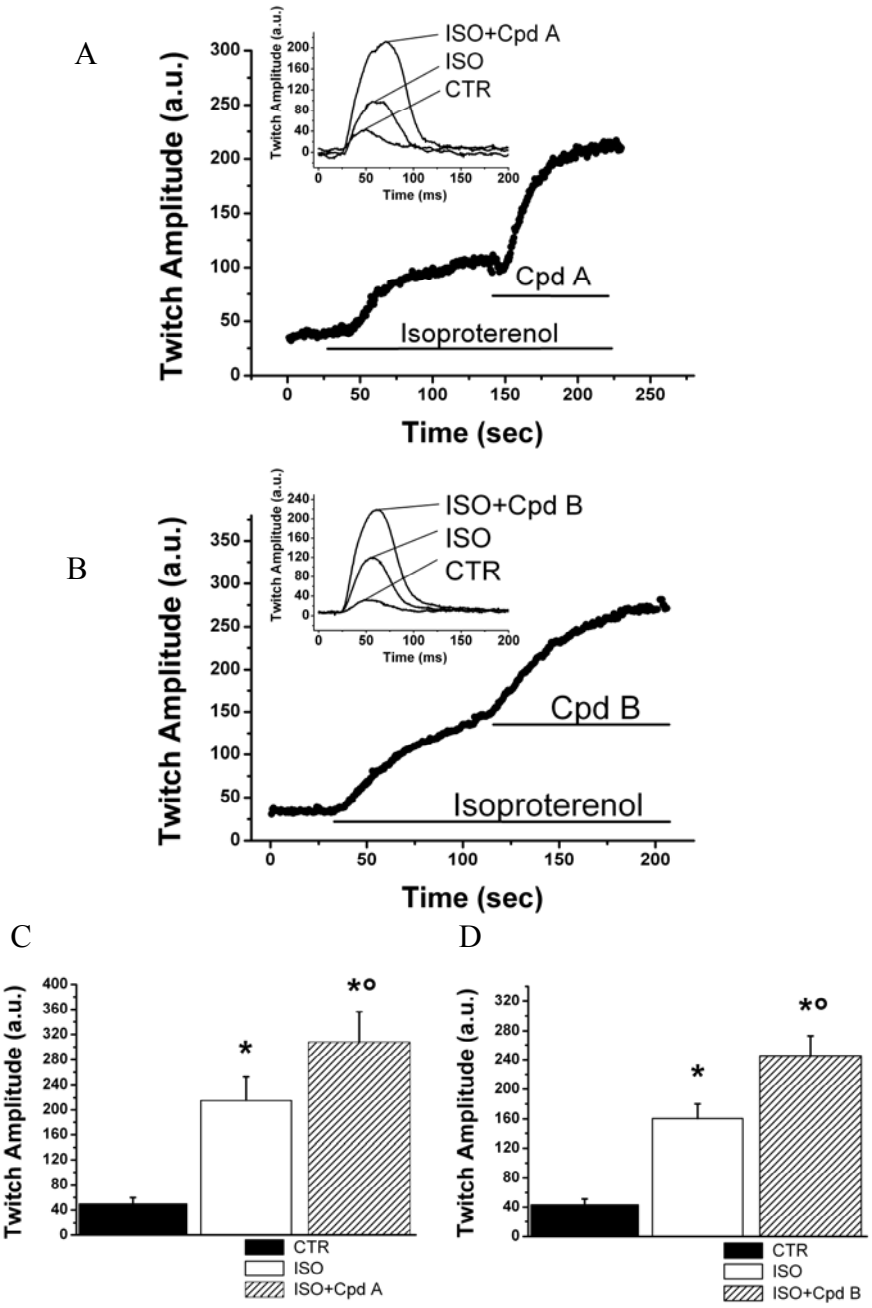


FIGURE 4: *Effect of PH-domain inhibitors on twitch amplitude under low β-AR stimulation.* Non saturating concentration of Isoproterenol (ISO, 10nM) was superfused in field stimulated cells. Cpd A (A, 5μM) and B (B, 50μM) further enhanced the effect of ISO on twitch peak amplitude C) D) Avarage results for Cpd A (n=13) and B (n=5) respectively. Insets: sample contraction traces recorded during each experimental condition (* $p<0.05$ vs CTR; ° $p<0.05$ vs ISO).

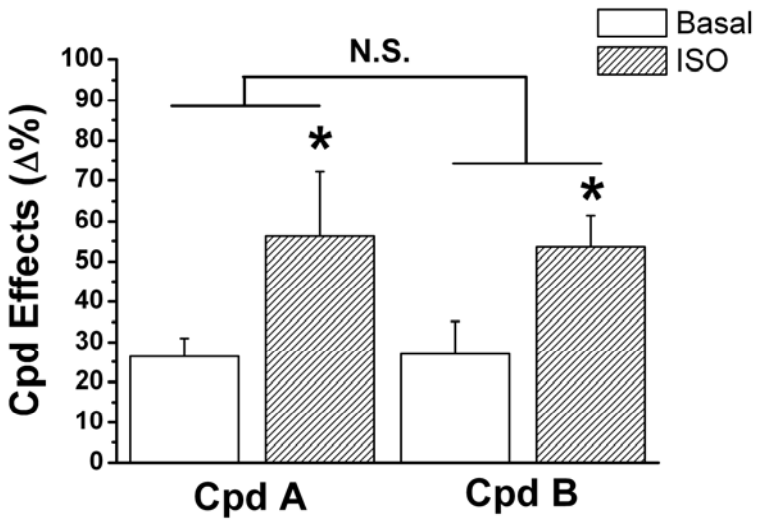


FIGURE 5: *Effects of PH-domain inhibitors on twitch amplitude.* Summary of drugs-induced twitch amplitude modulation (Δ%) in basal condition (Basal) and in the presence of β-AR stimulation (ISO). (* $p<0.05$ vs Basal).

Mechanism of the inotropic effect of PH-domain antagonists

This set of experiments aimed to identify the components of the EC-coupling machinery whose modulation is involved in the inotropic effect of PH-domain antagonists.

Modulation of action potential duration. Action potentials were recorded by patch electrodes in the whole-cell configuration during pacing at a cycle length (CL) of 500 ms. The perforated-patch technique was used in a subset of myocytes (n=6) to rule out interference by cell dialysis; no difference was observed between ruptured- and perfored-patch measurements; therefore, results were pooled.

Under basal conditions CpdA and CpdB prolonged APD_{50} by 48.3 % and 19.2 % respectively (Fig 6; $p<0.05$ vs control for both). Similar effects were observed for APD_{90} (not shown).

ISO (10nM) prolonged APD_{50} by an average of 114%. When applied in the presence of ISO CpdA and CpdB further increased APD_{50} by 57.0% and 34.9% (as % of APD_{50} achieved at steady state ISO; $p<0.05$ for both) (Fig 7). Cpd effects observed under basal and ISO conditions were not significantly different.

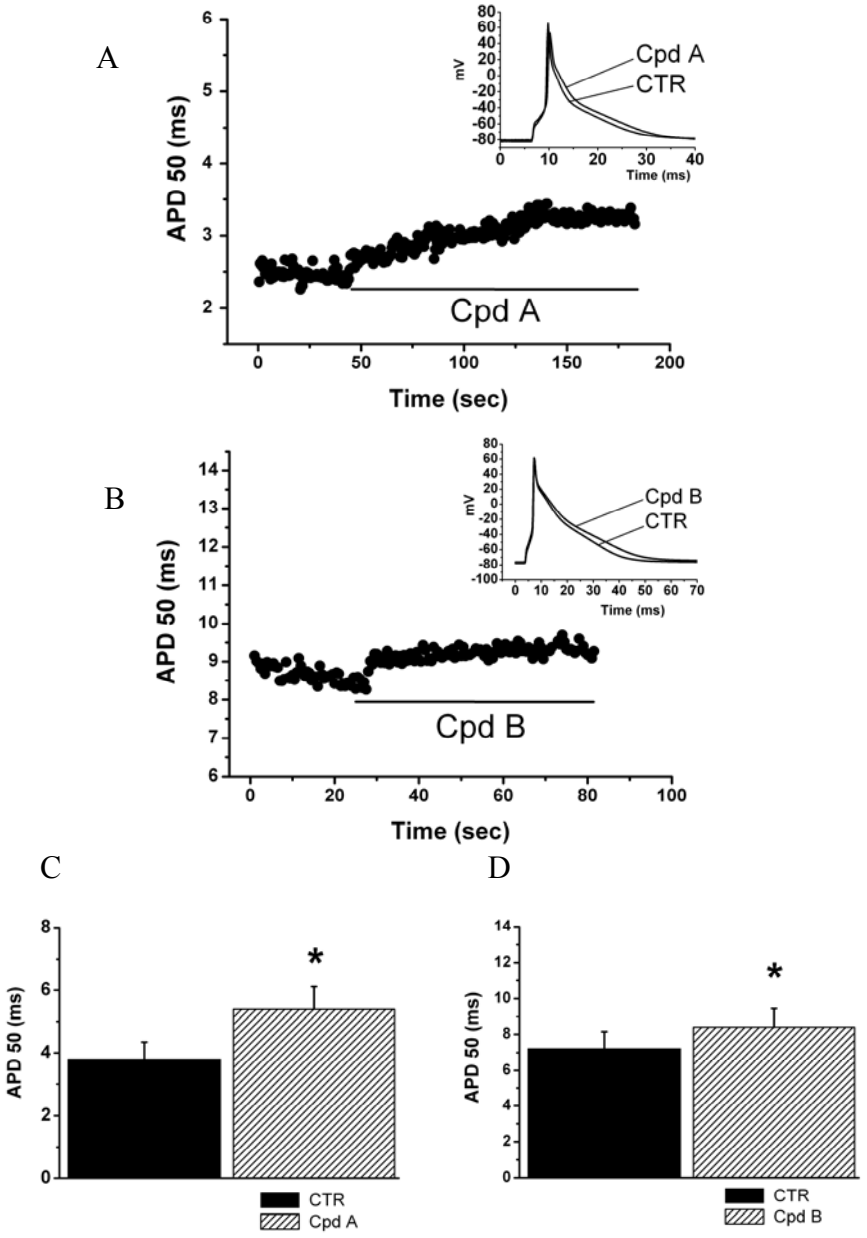


FIGURE 6: *Effects of PH-domain inhibitors on action potential (AP) under basal condition.* Time course representation of APD-50 measurements at each cycles (cl=0,5s) during perfusion with Cpd A (A, 5µM) and B (B, 50µM) in path-clamped myocytes. Insets show AP sample traces recorded in control condition (CTR) and in the presence of inhibitors. C) D) Avarage results for CPD A (n=7) and B (n=11) respectively. (* $p<0.05$ vs CTR).

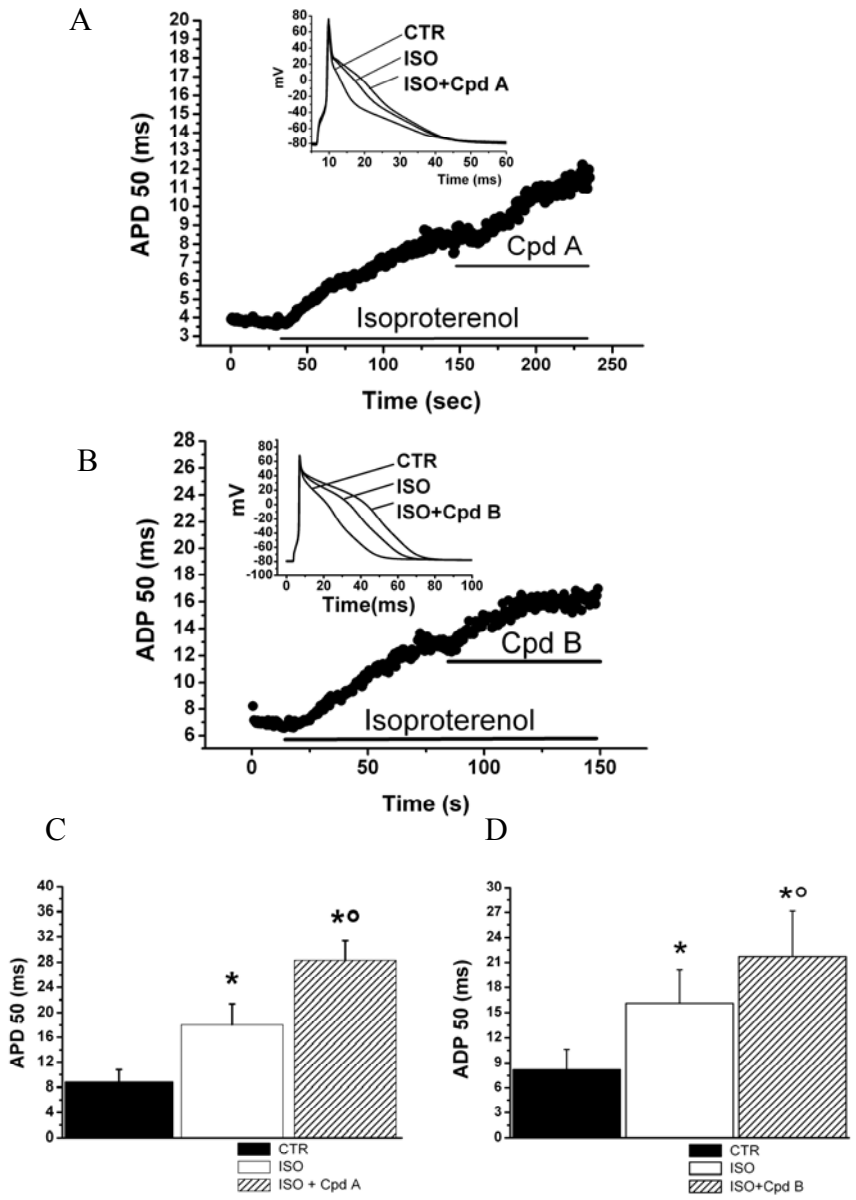


FIGURE 7: *Effects of PH-domain inhibitors on AP under low β-AR stimulation.* Time course representation of APD-50 measurements at each cycles (cl=0,5s) during perfusion with Cpd A (A, 5µM) and B (B, 50µM) in path-clamped myocytes in presence of Iso 10nM. Insets show AP sample traces recorded in control condition (CTR) and in the presence of Iso and inhibitors (Cpd and ISO respectively). C) D) Avarage results for CPD A (n=5) and B (n=5) respectively. (* $p<0.05$ vs CTR).

Modulation of I_{CaL}. Under basal conditions, CpdA decreased peak I_{CaL} density (at +10mV) by 32.0 % ($p<0.05$) (Fig 8). CpdB showed similar results with a decreased peak of 27% (data not shown). The compounds also slightly slowed I_{CaL} inactivation (data not shown). As Cpd effect on I_{CaL} was opposite to that suitable to account for positive cooperativity with ISO on contractility modulation, effects on I_{CaL} in the presence of ISO were not assessed.

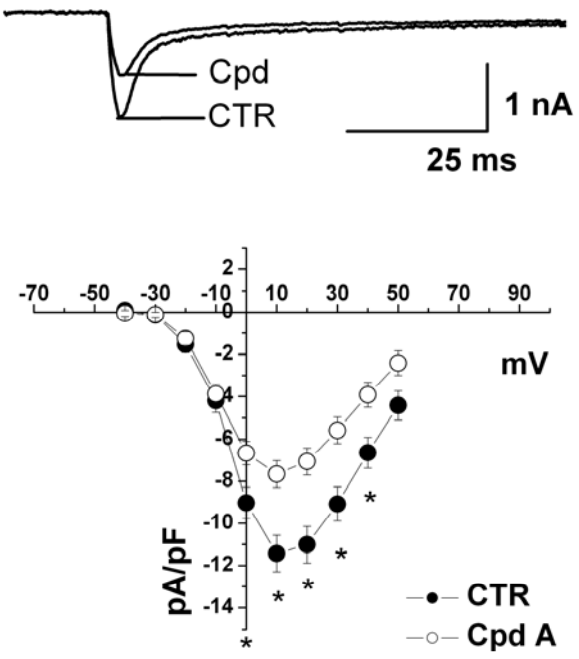


FIGURE 8: *ICaL recording.* Current/Voltage relationship of mean peak ICaL density recorded in presence of Cpd A (C; n=9) (* $p<0.05$). Inset: sample traces recorded under protocol shown below in control condition and under superfusion of compound.

Modulation of SR Ca^{2+} uptake function. The aim of this and the following sets of experiments were to assess whether modulation of SR function could account for the inotropic effect of PH-domain antagonism under basal conditions. To evaluate SR ability to sequester Ca^{2+} we used an experimental protocol (Protocol 2 in *Materials and Methods*) devised to assess SR-dependent (i.e. NCX-independent) Ca^{2+} fluxes over a wide range of SR loading conditions; in previous studies (Rocchetti *et al.*, 2008) the parameters obtained by such a protocol proved to be exquisitely sensitive in disclosing modulation of SR function. In consideration of the striking similarity between the effects of CpdA and CpdB reported above and of the complexity of Ca^{2+} measurement, the latter were applied only to CpdA (Fig 9).

CpdA effects included the following: 1) a decrease in τ_{decay} of single Ca^{2+} transients (Fig 9B), reflecting faster SR Ca^{2+} uptake within each activation; the effect increased as SR loading progressed (compare pulse 1 to 6); 2) faster progression of transient amplitudes during SR reloading (Fig 9C), standing for an increased rate of Ca^{2+} accumulation in the SR over the stimulation train; 3) faster progression of EC-gain during SR reloading (Fig 9D), which allows to

attribute the previous effects to enhancement of SR function, rather than to I_{CaL} modulation.

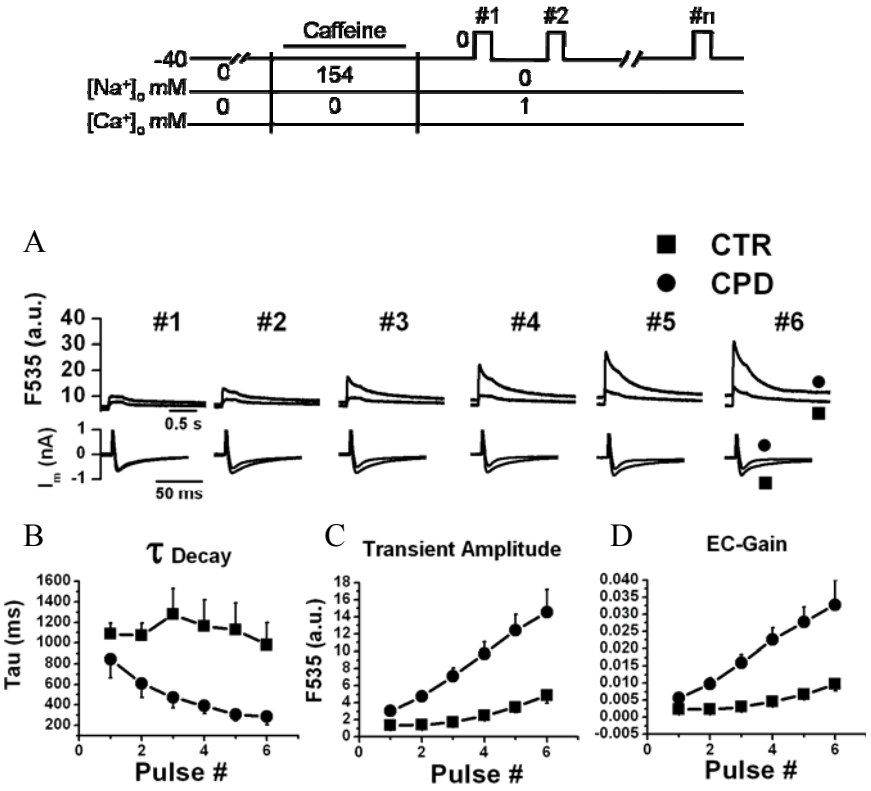


FIGURE 9: *Compound A effects during SR loading with blocked Na^+/Ca^+ exchanger.* A) Examples of Ca2+ transient expressed as unit of fluorescence (F_{535}) and membrane current (I_m) recorded concurrently during SR reload in control (■,n=11)and in presence of Cpd A (5μM) (●,n=9). The position of the pulse in the stimulation train was shown above (#1-6). B-D) Average results of Ca^{2+} transient parameters measured during each of the 6 pulse of the stimulation train. Transient amplitude was expressed as arbitrary unit of fluorescence (F_{353}). CICR gain was estimated by the method described and expressed in arbitrary unit (A.U.) Inset: outline of the experimental protocol. Significance of Cpd effects was detected by two-way ANOVA ($p<0.05$ for all variables).

Modulation of PLN phosphorylation. Ser16 on PLN is a PKA specific phosphorylation site (Sande *et al.*, 2002). As shown in Figure 10A, levels of phosphorylation status of PLN on Ser16, was increased when treating with both compounds suggesting an increased activity of PKA.

The phosphorylated form of PLN on Ser16 was increased after β-adrenergic stimulation by Isoproterenol treatment as expected, but it was even more induced when treating with 10 μM Akt inhibitor (Fig 10B). These data taken together demonstrate that after β-adrenergic stimulation when the Akt pathway is inhibited there is an increase of the PKA activity on the phosphorylation of PLN.

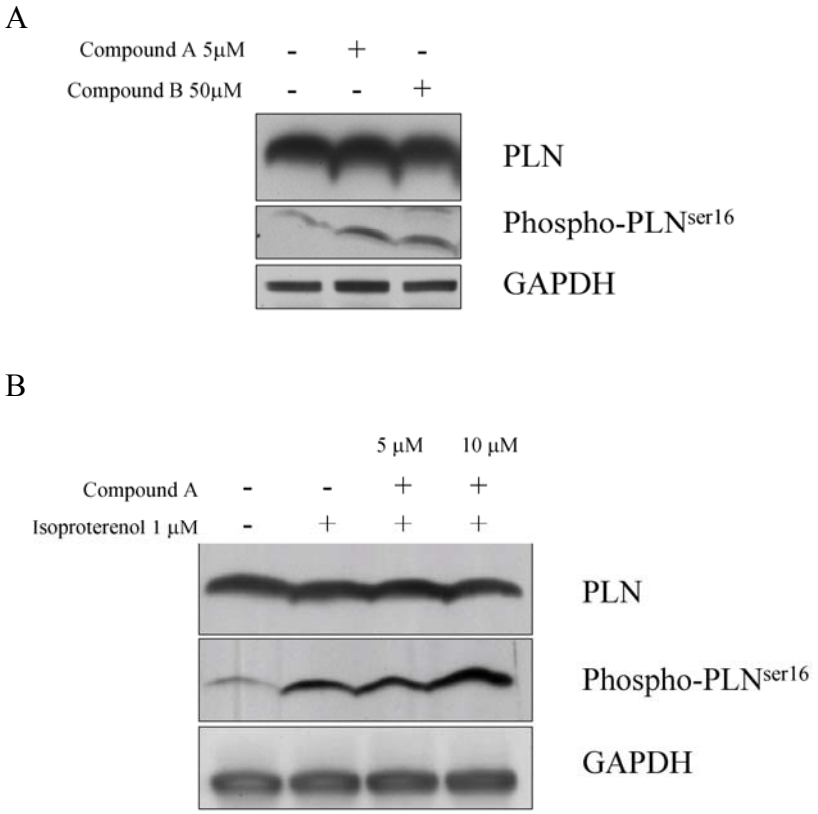


FIGURE 10: *Western Blot analysis of total PLN and Phospho-PLNser16 in basal condition and after stimulation with Iso.* A) Normal cardiomyocytes treated with 5 μM Cpd A or 50 μM Cpd B for 30 seconds. An increase in Phospho-PLNser16 was observed when treating with both compounds (lane 2 and 3) compared to the not treated cells (lane 1). B) Phosphorylation of Phospho-PLNser16 was also increased by Isoproterenol stimulation (lane 2) and it became higher when the Cpd A was added to the medium in a dose dependent manner (lanes 3 and 4). Total PLN levels were not affected by the treatments. GAPDH was measured as loading control.

Modulation of total SR Ca^{2+} content at steady-state. CpdA effect on total SR Ca^{2+} content ($[Ca]_{SRT}$), measured at steady-state during cyclic activation in physiological solutions (active NCX), was evaluated by protocol 3 (in *Materials and Methods*). As shown in figure 11D, in the average CpdA did not affect $[Ca]_{SRT}$ and even a small reduction was seen in some myocytes. A possible way to reconcile this unexpected observation with the robust stimulation of SR uptake function (observed with the previous protocol) is to consider that the presence of full (steady-state) SR Ca^{2+} load and a functioning NCX, may unveil the influence of Ca^{2+} leakage from the SR. Therefore we proceeded to assess CpdA effect on the relationship between SR Ca^{2+} leakage and SR Ca^{2+} load (leak/load relation).

Modulation of fractional Ca^{2+} release. A possible way to enhance contractility without increasing $[Ca]_{SRT}$ is through positive modulation of the fraction of $[Ca]_{SRT}$ released by excitation (fractional Ca^{2+} release). Fractional Ca^{2+} release was estimated as the ratio between the amplitudes of the V-induced Ca^{2+} transient (recorded during the loading phase of protocol 4) and of the caffeine-induced transient of the same myocyte. As shown in figure 12, CpdA increased the

amplitude of V-induced Ca^{2+} transients by 41% (A) and fractional Ca^{2+} release by 51% (B) ($p<0.05$ for both).

Modulation of the SR Ca^{2+} leak/load relation. Facilitation of RyR opening by CpdA was suggested by increased fractional release and was required to reconcile unchanged $[Ca]_{SRT}$ with the robust stimulation of Ca^{2+} uptake exerted by the agent. Modulation of RyR open probability by CpdA was evaluated by testing its effect on the relation between tetracaine-sensitive Ca^{2+} leak from the SR and $[Ca]_{SRT}$ according to protocol 4 (in *Materials and Methods*). As shown in figure 13, CpdA markedly increased the steepness of the leak/load relationship, to indicate an increase of RyR open probability at each SR Ca^{2+} load. Statistical comparison of the leak/load ratio was performed by pooling measurements with similar value of each variable (leak or load) and comparing the other variable between control and CpdA superfusion. In measurements reporting similar load between control and CpdA, leak was significantly larger in the presence of CpdA (Fig 13C); symmetrically, in measurements with similar leak between control and CpdA, load was significantly lower for CpdA (Fig. 13B). These results indicate that CpdA may enhance the probability of spontaneous RyR opening primarily, i.e. independently of its effect on SR Ca^{2+} content. Consistent with this

finding, diastolic Ca^{2+} (i.e. cytosolic Ca^{2+} measured between pulses during the loading train, Fig 12C) was significantly increased by CpdA.

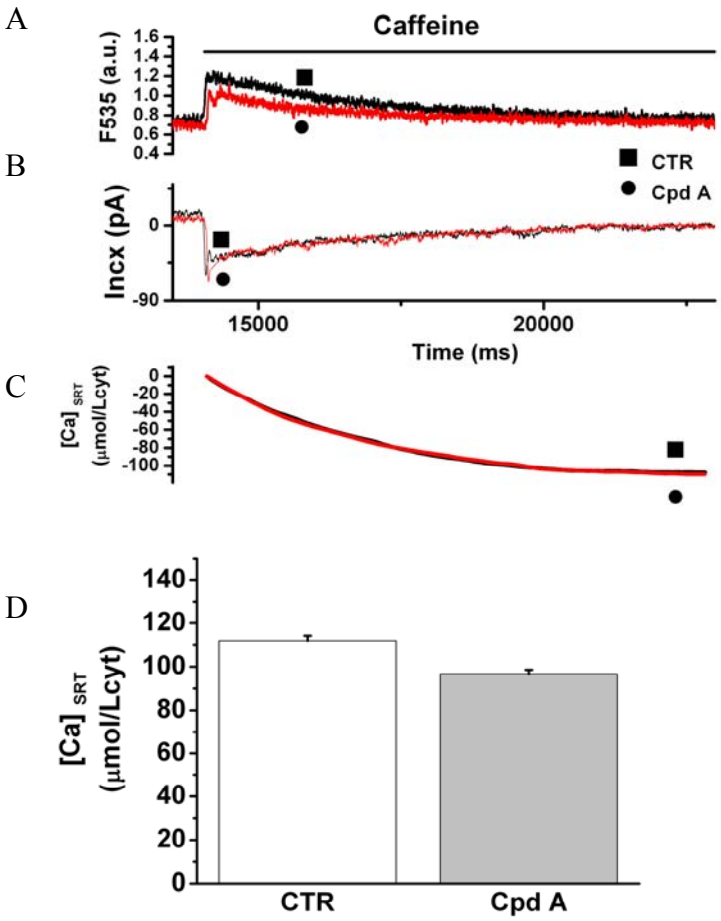


FIGURE 11: *Compound A effect on Ca_{SRT}.* Representative intracellular Ca^{2+} fluorescence, expressed in arbitrary unit of fluorescence F535, during 10mM caffeine pulse (A) and respective induced I_{NCX} (B) (holding potential -80mV) and its corresponding cumulative I_{NCX} integrals (C) in control condition (■) and following Cpd A superfusion (●). D) average results of Ca_{SRT} (n=9).

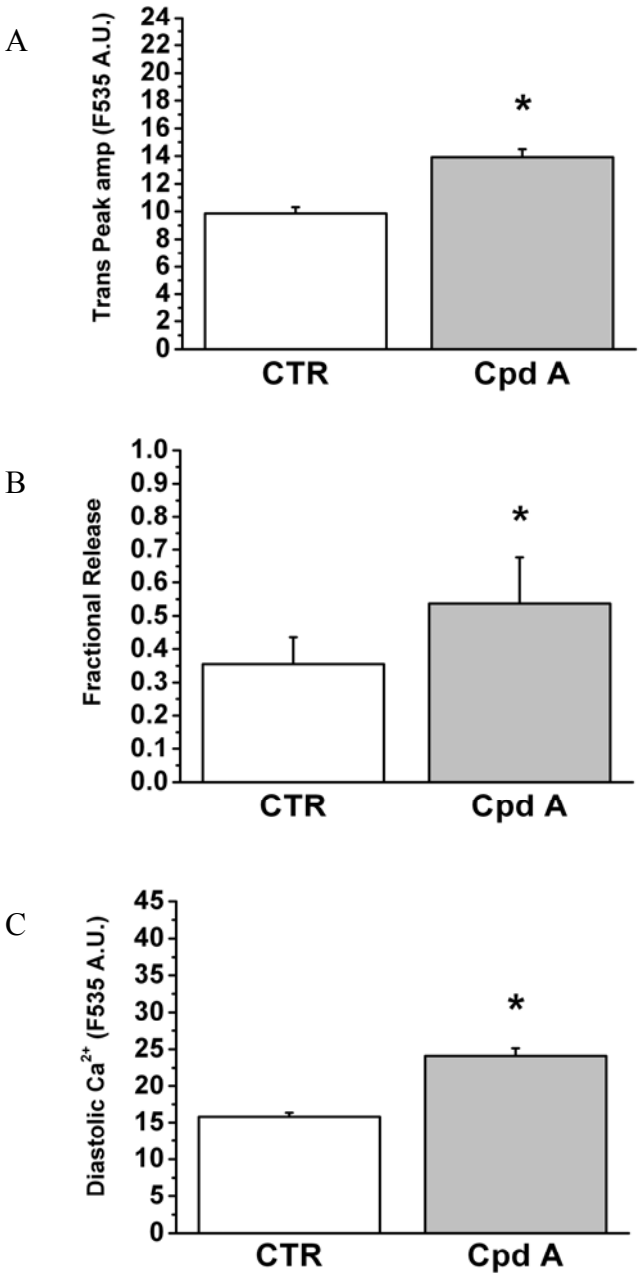
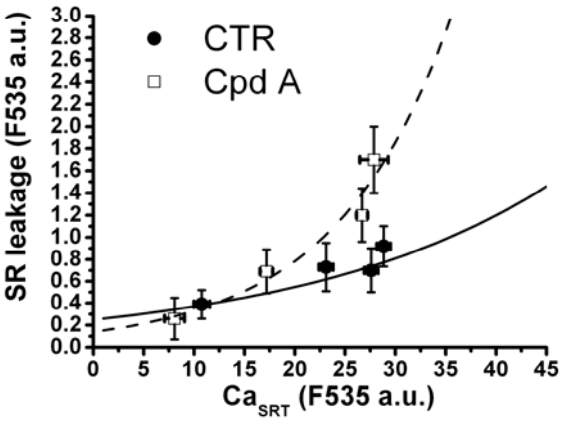
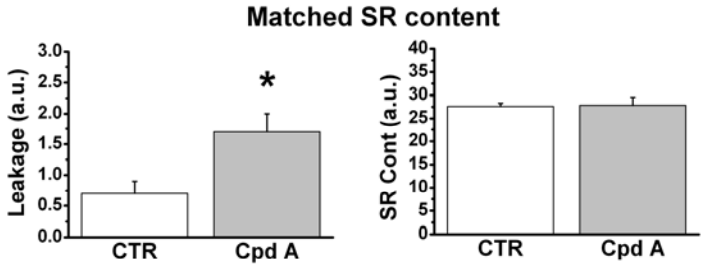


FIGURE 12: *Ca^{2+} transient analysis at loading steady state.* A) Comparison of transient peak amplitude (A), fractional release (B) and diastolic Ca^{2+} (C) calculated for each cells in presence of Cpd A (CTR n=12; Cpd A n=14) (* $p<0,05$).

A



B



C

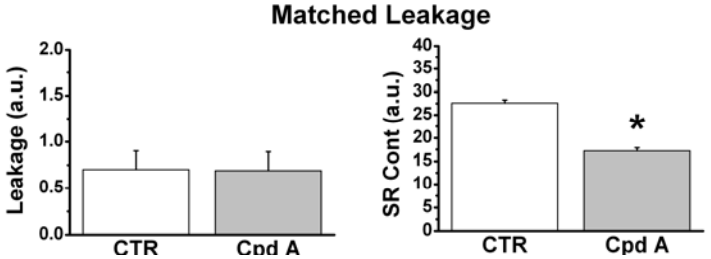


FIGURE 13: *Compound A effect on reticular Ca^{2+} leakage.* A) Leak/Load relationship changes during superfusion of Cpd A (●=CTR; □=Cpd A, n=12 to 14). B) Statistical analysis of grouped cells for same reticular Ca^{2+} leakage (left; CTR n=12, Cpd A n=12) that present a lower $[Ca]_{SRT}$ in presence of Cpd A (right; CTR n=12; Cpd A n=12;). C) Statistical comparison for matched cells for the same $[Ca]_{SRT}$ (left; CTR n=12; Cpd A n=14) that show higher leakage during Cpd A superfusion (right; CTR n=12; Cpd A n=14) (* $p<0.05$ vs CTR)..

Akt involvement in the effects of PH-domain antagonists

Effects of PH-domain antagonists on Akt phosphorylation. A previous work has shown that β-AR agonists enhance PI3Kγ activity and it has been generally assumed that this would result in increased Akt phosphorylation (Chandrasekar *et al.*, 2004). The purpose of this set of experiments was to test whether β-AR activation may enhance Akt phosphorylation and whether this can be prevented by PH-domain antagonism. Because phosphorylation activates Akt, phosphorylated-Akt level may also be considered as an indirect measure of Akt activation (Blume-Jensen & Hunter, 2001). Myocytes were collected after 30 minutes exposures to ISO (1μM) and 30 sec ISO plus Cpd A (5 μM) or Cpd B (50 μM); the levels of phosphorylation at Akt residue Ser473 (Phospho-Akt^{ser473}) were detected by Western Blot. Figure 14 shows that ISO markedly increased Phospho-Akt^{ser473} and that this effect was completely reversed by CpdA at the concentrations found to affect myocyte contractility. None of the interventions changed the unphosphorylated Akt fraction.

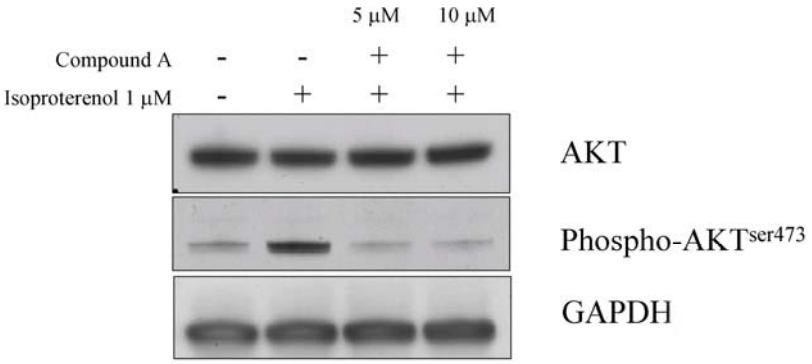


FIGURE 14: *Western Blot analysis of total Akt and Phospho-Akt-ser473 in cardiomyocytes after β-adrenergic stimulation*. Akt phosphorylation on Ser473 was increased when stimulating with Isoproterenol (lane 2) compared to the control (lane 1) and decreased when Cpd A was added to the medium in a dose dependent manner (lanes 3 and 4). Total Akt levels were not changed. GAPDH was measured as loading control.

Effect of PH-domain antagonists after Akt silencing. The involvement of Akt was addressed by contractility measurements under field stimulation, which required minimal cell manipulation. The effect of CpdA and B were compared between myocytes subjected to Akt1 silencing and those not transfected considered as controls. Significant silencing of Akt1 transcription and expression was achieved, as shown by the decrease in Akt mRNA (Fig 15B) and protein levels (Figure 16) 24 hrs after transfection.

Twitch amplitude (in the absence of any Cpd) was larger in Akt1-silenced myocytes than in control ones (Fig 17). Such a difference (87,6%) was similar to the increase of twitch amplitude induced by

CpdA and B in non-silenced myocytes (26,3% and 26,9% respectively).

The effects of Cpd A and CpdB on contractility were suppressed in Akt1 silenced myocytes, both under basal condition (Fig 18) and during exposure to ISO (Fig 19). On the other hand, ISO-induced increase in contractility was preserved in Akt1-silenced myocytes (Fig.19).

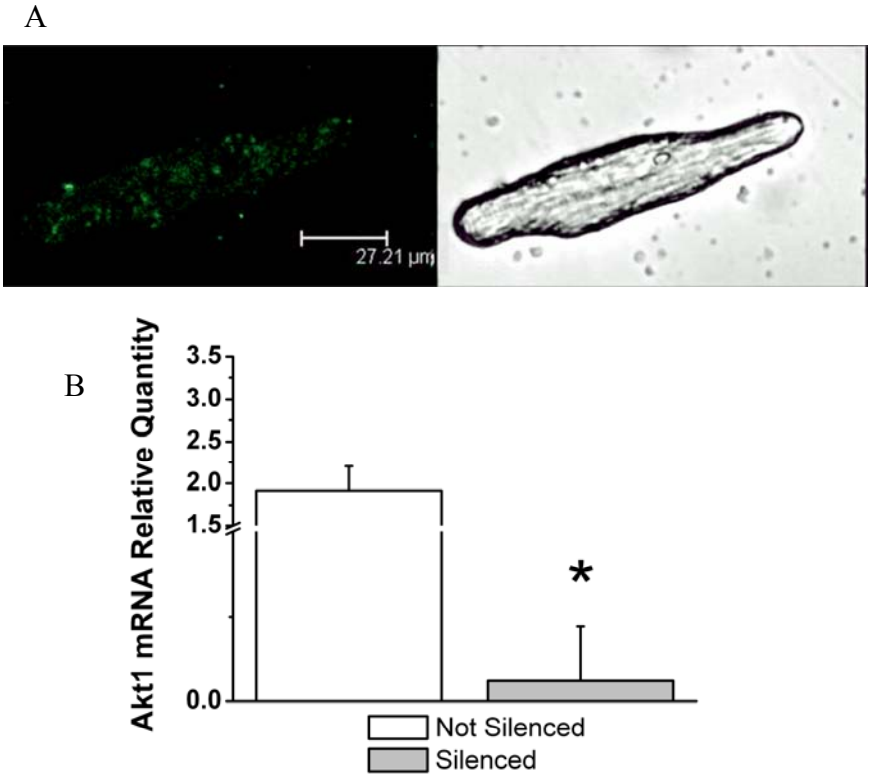


FIGURE 15: *Akt-1 silencing.* Confocal image of a representative transfected myocyte (A) and quantitative analysis of Akt1 mRNA expression on the same pool of cells used for experiments (B) (Not Silenced n=25; Silenced n=19)(* $p<0.05$).

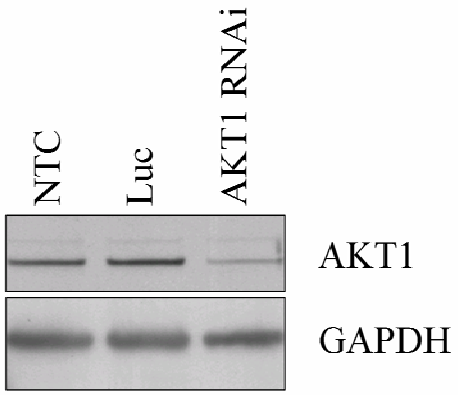


FIGURE 16: *Western Blot analysis of Akt1 in normal cardiomyocytes transfected with Akt1 dsRNA oligo for RNA interference*. In lane 1 the not treated control cells, in lane 2 cells transfected with a Luciferase control dsRNA oligo and in lane 3 cells transfected with a specific Akt1 dsRNA oligo. Akt1 was decreased when cells were transfected with the specific dsRNA (lane 3). GAPDH was measured as loading control.

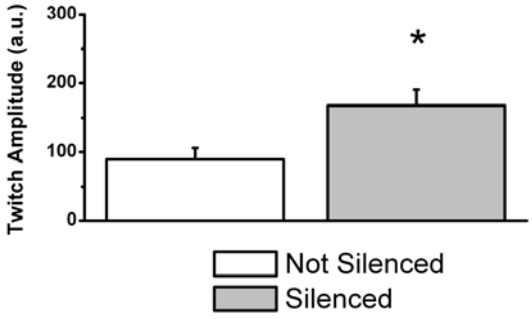


FIGURE 17: *Effects of Akt-1 silencing on basal twitch amplitude*. Statistical comparison between not-silenced cells versus silenced basal twitch amplitude (n=25 and n=23, respectively).

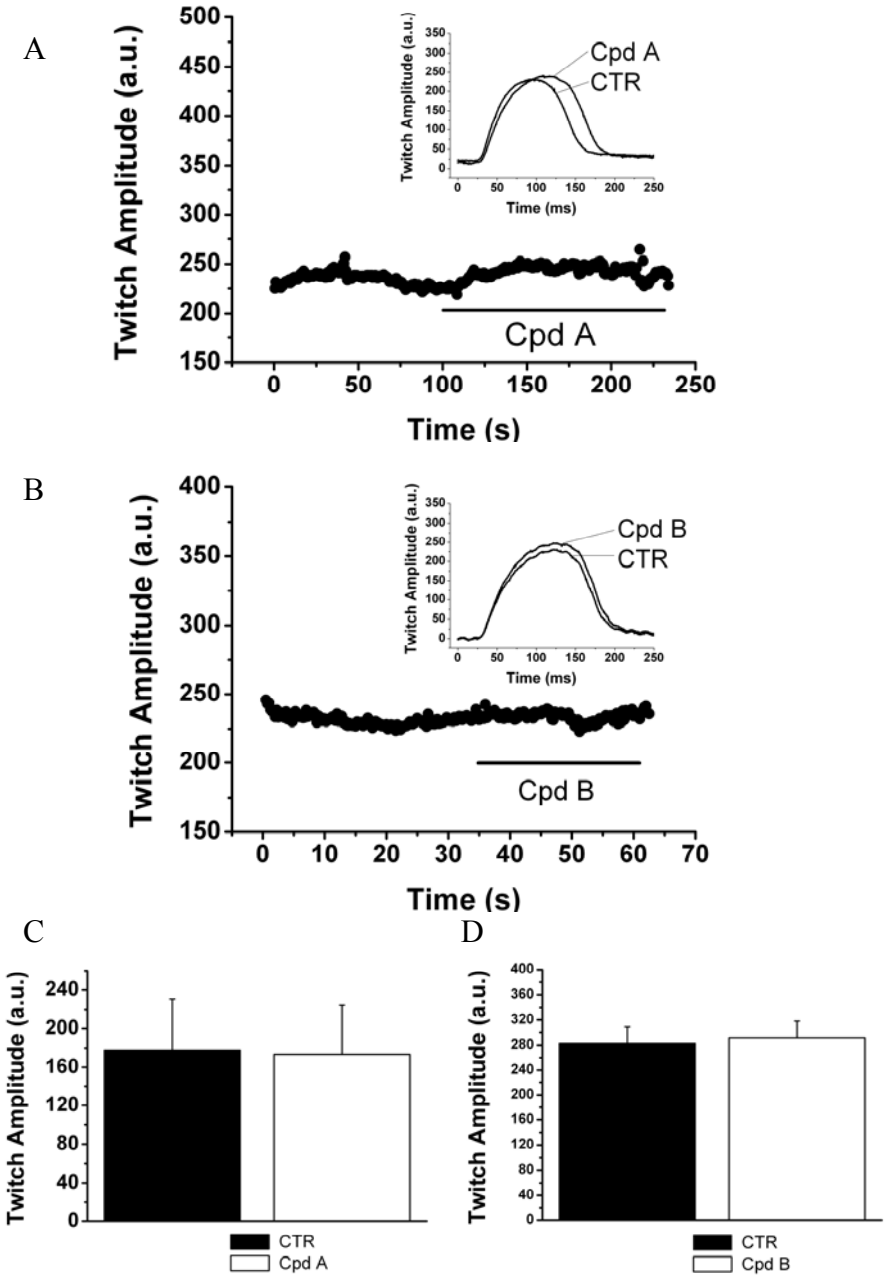


FIGURE 18: *Effects of the two PH-domain inhibitors on twitch amplitude in Akt-1 silenced myocytes in basal condition.* Representative time-courses of 5µM Cpd A (A) (n=11) and 50µM Cpd B (B) (n=8) tested on transfected myocytes with iRNA technique versus Akt-1, and respective sample recorded traces (insets). Average results for Cpd A (C) and Cpd B effects (D).

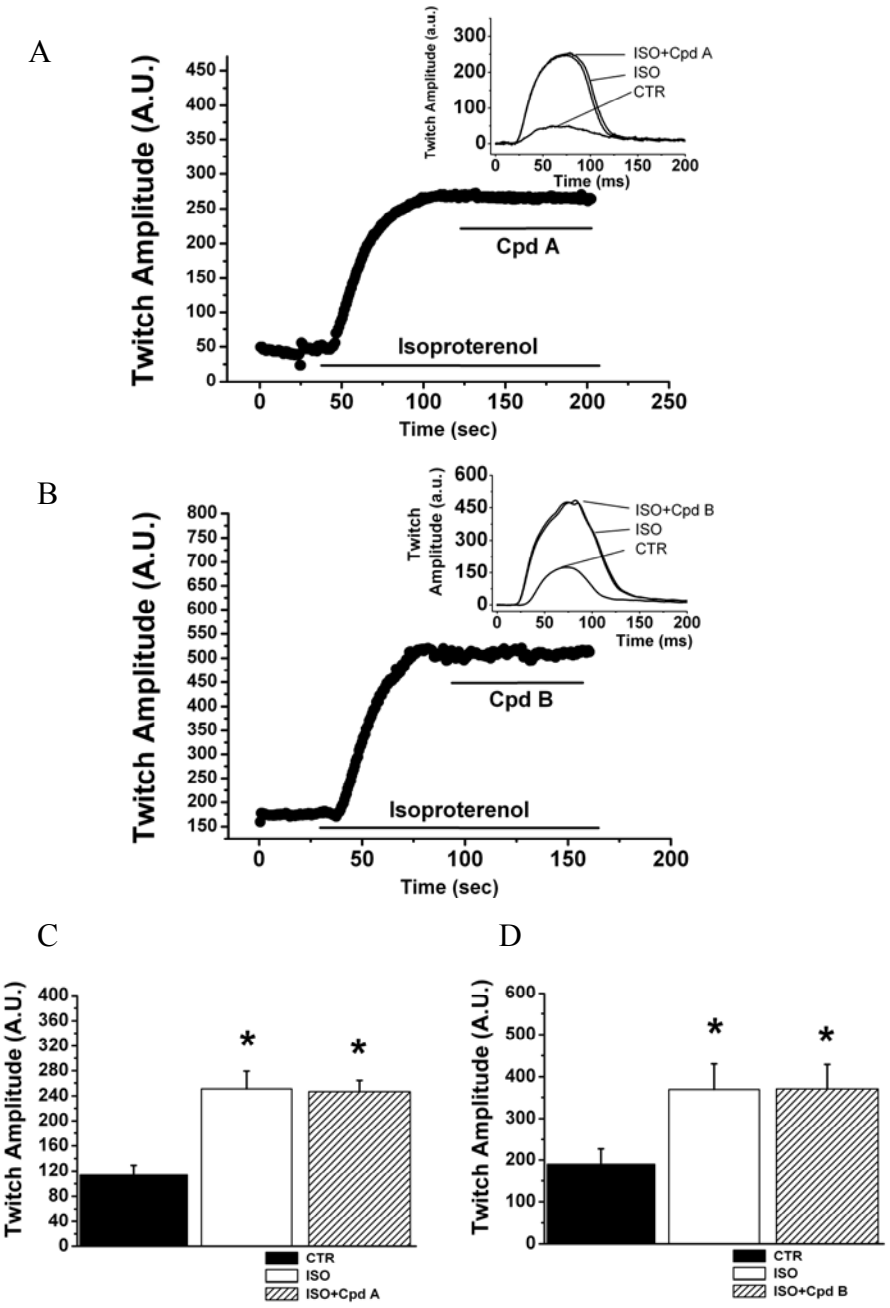


FIGURE 19: *Effects of the two PH-domain inhibitors on twitch amplitude in Akt-1 silenced myocytes under β-AR stimulation.* Representative time-courses of 5μM Cpd A (A) (n=9) and 50μM Cpd B (B) (n=11) tested on transfected myocytes with iRNA technique versus Akt-1, and respective sample recorded traces (insets). Average results for Cpd A (C) and Cpd B effects (D).

β-AR subtypes involved in the interaction with PH-domain antagonists.

The involvement of each β-AR subtype was evaluated by testing the effect of CpdA and CpdB on twitch amplitude of field-stimulated myocytes in the presence of subtype-specific antagonists (see *Methods*) under basal and ISO-stimulated conditions (Fig 20). The rationale for testing receptor blockade also in the absence of ISO is to assess the contribution of intrinsic (agonist-independent) receptor activity, which is also inhibited by receptor antagonists (Milano *et al.*, 1994). Simultaneous blockade of both β1- and β2-AR (β-block, ICI+CGP) abolished the inotropic response to CpdA (Fig 20 A) during ISO superfusion, as expected, but also under basal conditions. Thus, activity of β-AR activated signaling, either intrinsic or stimulated, is a prerequisite for the observed modulation of inotropy by PH-domain antagonism.

In the presence of ISO, response to CpdA was enhanced and was only partially blunted by selective blockade of either β2- (ISO+ICI) or β1- (ISO+CGP) AR. This suggests that both β1- and β2-AR subtypes contribute to activate the pathway modulated by PH-domain antagonism.

The results obtained, under the same set of conditions, with CpdB (Fig 20 B) almost overlap those of CpdA, thus confirming that Cpd interaction with the β-AR pathway depended on PH-domain antagonism (shared feature), rather than on the specific chemical structure of the PH-domain antagonist (structurally unrelated).

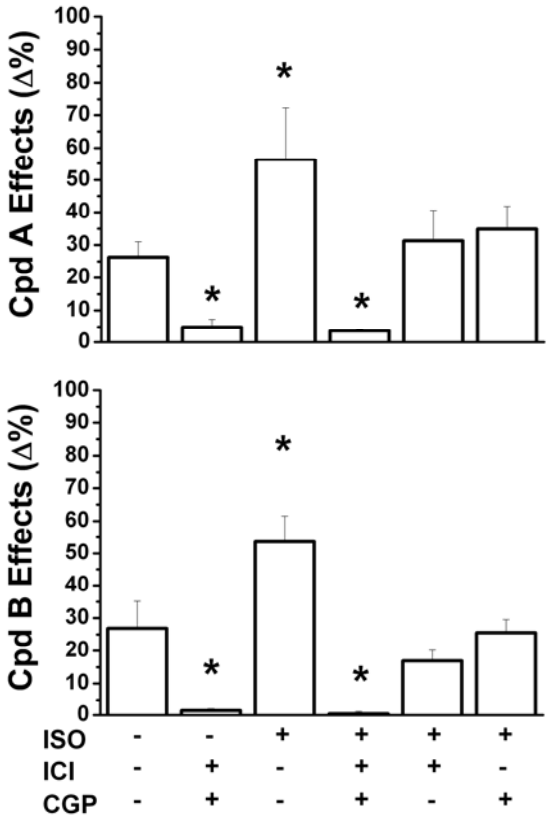


FIGURE 20: *Statistical comparison of compounds effects during different β-AR stimulation.* Each panel represents the comparison of Δ% of compound A (A) and B (B) effects in different β-AR blockade protocols. (Cpd A: ICI+CGP n=15; ICI+CGP+ISO n=7; ISO+ICI n=14; ISO+CGP n=11) (Cpd B: ICI+CGP n=17; ICI+CGP+ISO n=6; ISO+ICI n=19; ISO+CGP n=24) (*$p<0.05$ vs Basal).

DISCUSSION

To assert that a compound inhibits a specific intracellular pathway it requires to demonstrate that the pathway is effectively affected and the effect is completely ascribed to the compound at issue. In this study we showed that our compounds are able to inhibit Akt-phosphorilation (Fig. 14) and its consequent activation, and these effects were completely abolished when Akt was silenced by iRNA technique (Fig. 18-19). Thus, we can assert that the effects of both compounds are mainly due to the PI3K/Akt pathway inhibition. This conclusion is also supported by the evidence that we obtained the same qualitative and quantitative data with two completely chemical unrelated compounds unlike to share common ancillary actions. The PI3K/Akt pathway inhibition was evaluated as modulation of on cardiac myocytes contractility and of ECC mechanisms; these aspects are discussed separately.

Modulation of myocytes contractility

Both compounds enhanced the twitch amplitude in isolated rat ventricular myocytes in basal condition and under unselective β-adrenergic stimulation (Fig. 3-5). Contractility enhancement was observed at sub-saturating Isoproterenol concentration (10nM) and

abolished in the presence of high concentration of the agonist (1μM) (data not shown). Cooperativity between Isoproterenol and PH-domain inhibitors led us to hypothesize an interaction between PKA and PI3K/Akt pathway. As widely described these two pathways seem to be closely correlated and cooperate together to modulate the mechanisms that regulate the cardiac contraction (Condorelli *et al.*, 2002; Kerfant *et al.*, 2007; Leblais *et al.*, 2004). However, how exactly these pathways work together is still unclear. Previous studies demonstrated that PI3K$\gamma^{-/-}$ cardiac myocytes have a higher basal contractility than the normal ones. Thus, suggesting a constitutive inhibitory activity of the pathway. In wild-type rat ventricular cardiomyocytes contractility was enhanced by PI3K inhibitor (LY294002). following aspecific β-AR stimulation (Condorelli *et al.*, 2002; Kerfant *et al.*, 2007; Leblais *et al.*, 2004). These findings are in agreement with our results, underlying the role of PI3K/Akt pathway as a negative modulator of cardiac contractility.

The involvement of PI3K/Akt signalling in the effect of PH domain inhibitors was directly proven by its suppression after Akt1 silencing. Noteworthy, Akt1 silencing itself enhanced twitch amplitude to an extent similar to that observed with PH-domain inhibitors (Fig. 17).

The positive inotropic effect of PH-domain inhibitors was considerably enhanced in the presence of β-AR activation by isoproterenol, thus confirming an interaction between PI3K/Ak and PKA pathways.

Activation of the PI3K/Akt pathway by β-AR is considerably complex and may involve specific receptor subtypes and signal compartmentalization. The inotropic response to catecholamines was abolished in β1-AR KO mice (Rohrer *et al.*, 1996), whereas it was preserved in β2-AR KO ones (Chruscinski *et al.*, 1999). In normal myocytes selective β2-AR stimulation (by Zinterol) slightly enhances contractility, and this effect is markedly enhanced by pretreatment with LY294002, a PI3K inhibitor (Alloatti *et al.*, 2005; Jo *et al.*, 2002). Interestingly, in intact myocytes, the inotropic effect of β2-AR activation was not associated with a lusitropic effect, thus implying lack of SERCA stimulation by this receptor subtype. On the other hand, a lusitropic effect of β2-AR agonists was unveiled by pretreatment with Metil-β-Cyclodestrine, a compound promoting caveolae disruption (Calaghan & White, 2006; Calaghan *et al.*, 2008). These observations can be interpreted to suggest that β2-AR pathway self-restrains its downstream effects, preventing them to reach SR targets, and that PI3K is required for this to occur. Thus, the positive

inotropic effect of PH-domain antagonists, observed in the present study, can be interpreted as removal of a PI3K-mediated restrain on the stimulatory β-AR pathway. Our experiments showed that positive inotropy of PH domain antagonism resulted from increased SR Ca^{2+} uptake and PLN phosphorylation, whereas I_{CaL} changes were unsuitable to account for it. Therefore, our observations confirm that the PI3K-mediated mechanism may prevent propagation of β-AR signal to the SR.

Our results show that PH-domain inhibition enhanced contractility even in the absence of β-AR stimulation, but its effect disappeared in the presence of unselective β-AR blockade. This suggests the presence of a constitutive component of β-AR signaling, which can be enhanced by PH-antagonists and eliminated by binding of receptor antagonists. Indeed, it has been shown that activation of the PI3K/Akt pathway may be supported, in the absence of agonists, by a sub-population of constitutively activated β2-AR (Milano *et al.*, 1994; Xiao *et al.*, 1999a; Milano *et al.*, 1994).

In the presence of isoproterenol, selective blockade of either β1 or β2 AR receptor subtypes partially inhibited the inotropic effect of PH domain antagonists, thus suggesting that both β-AR subtypes may be

required for full activation of the pathway restrained by PI3K/Akt (Fig.20).

In apparent contrast with our findings, sevral studies show that activation of the PI3K pathway exerts a positive effect on cardiac inotropy. An increment of cardiac contractility was observed during IGF1 stimulation, an activator of PI3Kα signalling (Kinugawa *et al.*, 1999) and in mice overexpressing a constitutively active Akt mutant (E40K) (Condorelli *et al.*, 2002; Kim *et al.*, 2003; Rota *et al.*, 2005). Whereas PH-domain antagonists enhanced PKA dependent PLN phosphorylation (on Ser-16), the E40K transgenic mouse showed an enhancement of PLN phopsholamban on the CaMKII target (Threonine-17) instead (Catalucci *et al.*, 2009a). Moreover, the positive inotropic effect the E40K construct was not shared by expression of other constitutively activated Akt, such as myr-Akt (Matsui *et al.*, 2002). The effect of PI3K/Akt in experiments showing a positive inotropy can be interpreted as a prevailing activation of PI3Kα-dependent signaling. Whereas stimulation of such a pathway was absent in our experiments, PI3Kγ signaling was activated by β-AR activity (constitutive or stimulated), thus explaining the different sign of the inotropic effect.

Functional mechanisms involved in twitch amplitude enhancement

Contraction is the final result of the excitation-contraction coupling; the steps of this process that can be modified by PI3K/Akt inhibition were also investigated. Three main mechanisms can explain an enhanced contractility: changes in electrical activity (action potential shape), intracellular Ca^{2+} handling, or sarcomeric response to Ca^{2+} (Bers, 2002b). Only action potential and Ca^{2+} handling modulation were addressed in the present study, without excluding possible effects at the sarcomeric level.

Action potential analysis. Both compounds induced a small, but significant, action potential prolongation at 50% of the ripolarization (APD50), in basal condition and after aspecific β-AR stimulation (Fig. 6-7). We have to consider, at first, if the observed prolongation might justify the positive inotropic effects described above. Action potential prolongation enhances twitch amplitude, a mechanism of positive inotropy observed in physiological and pathological conditions (Richard *et al.*, 1998; Sah *et al.*, 2001). A longer *plateu* phase prolongs the time for Ca^{2+} entry (trough I_{CaL}) and curtails Ca^{2+} extrusion time (by the Na^{+}/Ca^{2+} exchanger) (Sah *et al.*, 2001). These events result in augmented cytosolic Ca^{2+} concentration and more

availability of this ion for the contraction. The prolongation of action potential induced by both compounds was mild, but qualitatively directed to justify an increment of cell contraction. Nevertheless Ca^{2+} transient amplitude was increased by PH domain antagonists also in experiments in which membrane potential was controlled by V-clamp (SR loading experiments, see below). Therefore modulation of APD may not be required to explain the positive inoptropic effect.

The mechanisms by which Cpd A and B modulated repolarization might reflect either Akt inhibition or an ancillary effect on membrane currents. The *plateu* phase of the action potential is generated by a balance between inward depolarizing currents (I_{NCX} and I_{CaL}) and outward repolarizing currents (I_{to}, I_K, I_{K1}, and I_{NaK}) (Bers, 2002b) and simple action potential recordings do not allow to discriminate which set of conductances is affected. The decrease of I_{CaL}, induced by PH-domain antagonists, would actually cause APD shortening instead. Interpretation of previous reports that AP may be prolonged by PI3K inhibition (LY294002), is complicated by the fact that LY294002 also inhibits $I_{K,slow}$ (Sun *et al.*, 2004). Thus, our results do not allow to discriminate between PI3K/Akt dependent and unrelated uncillary actions as the mechanism of APD modulation by PH-domain antagonists

L-type calcium current analysis. The results obtained from voltage-clamp experiments showed a reduction of I_{CaL} density in the presence of the PH-domain inhibitors (Fig. 8). This result is opposite to that expected based on our previous data. I_{CaL} reduction observed in the presence of PH-domain antagonists might be ascribed to either channel modulation by the PI3K/Akt pathway, or to diret blockade by the compounds used. The literature is quite confused about PI3K/Akt modulation of I_{CaL}. It has been demonstrated that L-Type calcium channel have two consensus sites for Akt mediated phosphorylation, thus suggesting that Akt may enhance the open probability of the channel (Sun *et al.*, 2006). Moreover, PI3K was required to sustain a normal activity of the channel, as IP_3 reduction or PTEN infusion (trough patch clamp pipette) turned down I_{CaL} (Lu *et al.*, 2005b). Moreover, records from $PTEN^{-/-}$ mice showed enhanced I_{CaL}, an increment blunted by inhibitors of PI3K or Akt (Sun *et al.*, 2006). In the same work, such effect was ascribed to the PI3K-α pathway, the one involved in the positive modulation of I_{CaL} by IGF-1 (Vanhaesebroeck & Waterfield, 1999). On the other hand, PI3Kγ activation by β2-AR stimulation seems to limit I_{CaL} enhancement by concomitant PKA activation (Zheng *et al.*, 2004). Moreover, Catalucci and co-workers recently reported that Akt may regulates I_{CaL} density

by preventing channel protein degradation by ubiquitination (Catalucci *et al.*, 2009b).

Although our experiments do not allow to conclude on the mechanism of the observed I_{CaL} inhibition, the latter is opposite to what would be required to account for the positive inotropic effect and, therefore, not related with it.

Effects on sarcoplasmatic reticulum function. Data obtained from the calcium current records do not explain how PH-domain inhibition is able to enhance the cardiac contractility. As already described, besides Ca^{2+} entry, another mechanism allows to increment contractility: the improvement of sarcoplasmatic reticulum (SR) functions (see Introduction). This hypothesis was addressed by analysing calcium transients during the re-loading protocol (Fig. 9): Cpd A enhanced SR Ca^{2+} uptake as shown by the increased SR reloading and Ca^{2+} decay rates. Cpd A also increased the efficency by which Ca^{2+} influx triggers SR Ca^{2+} release (CICR gain). An action of SR Ca^{2+} uptake function is also consistent with the enhancement by PH-domain inhibitors of the levels of PLN phosphorilated on Serine 16 (Fig. 10). Increased PLN phosphorylation positively modulates SERCA pump.

Enhanced phospholamban phosphorilation was previously observed in PI3K$\gamma^{-/-}$ myocytes, which showed elevated contractility levels compared to WT. In this study enhancerment of PLN phosphorylation was associated to an increment of cAMP levels (Crackower *et al.*, 2002; Kerfant *et al.*, 2005; Marcantoni *et al.*, 2006). A reduced phospholamban phosphorilation occured when a constitutively activated PI3Kγ was expressed (Jo *et al.*, 2002).

An incremented SERCA2 activity should be coupled to a higher sarcoplasmatic reticulum Ca^{2+} content ($[Ca^{2+}]_{SRT}$) (Bers, 2002a); however, in the present study $[Ca^{2+}]_{SRT}$ analysis showed no difference between control and PH-domain inhibition (Fig. 9). To explain this apparently paradoxical finding, we have to consider that SR Ca^{2+} content is the result of a Ca^{2+} flux balance between Ca^{2+} uptake by SERCA2 and unstimulated Ca^{2+} efflux through RyRs, defined as Ca^{2+} leakage (Bers, 2002a). For this reason we hypothesized that our compounds not only increased Ca^{2+} uptake by SERCA, but also affected Ca^{2+} leakage through RyR. Consistent with this hypothesis, myocytes with comparable SR load showed higher SR leakage after Cpd A than in control (Fig. 12). RyR open probability is known to be enhanced by phosphorylation, but the kinase mainly involved seems to be CaMKII, rather than PKA. However, PI3K is able to co-precipitate

with phosphodiesterase (PDE) and allows a fine tuning on PDE3 or PDE4 activity, thus modulating cAMP levels in the proximity of the SR (Kerfant *et al.*, 2006; Patrucco *et al.*, 2004). It is possible to hypothesize that cAMP dependent phosphorylation of RyR may explain the observed increase in SR Ca^{2+} leakage.

Study limitations

Before to conclude we have to consider two points that represent the main limitations of this study. First, RNA intereference experiments shows the specificity of the PH-domain inhibitors on contractility measurements. Nevertheless, we can not exclude at all that, in the other experiments, possible side effects occur. Akt is not the only protein that conteins PH-domain, also PDK1, or substrates at the end of this signalling, such as GSKβ, show the presence of this domain (Lemmon & Ferguson, 1998). Finally, we can not perform a dose-dependent analysis of Cpd A because its solubility it's limited and we must carry out all the experiments at the concentration cited. This is probably due to its chemical structure common to other PH-domain inhibitors that limits their solubility (Kumar & Madison, 2005).

Conclusions

We can conclude that PH-domain inhibitors unmask the inhibitory interaction of PI3K/Akt pathway with PKA signalling in modulation of myocardial contractility. The pathawy involved includes Akt, as its effect was suppressed by silancing of this protein. Our results also indicate that the end targets of PI3K/Akt-dependent modulation are represented by SR effector proteins as PLN/SERCA2 and RyR channels. In sight of the previous literature (Zheng *et al.*, 2005), the observed effects of PH-domain antagonism are more probably linked to the β2-ARs-PI3Kγ pathway.

Finally, since similar effects were observed with chemical unrelated PH-domain inhibitors the observed effects reflect PH-domain inhibition, rather than drug-specific actions.

Practical implications

Selective stimulation of the SR function represents a hopefully approach for pharmacological induced inotropism, useful in heart failure therapy (Kerfant *et al.*, 2005). Our PH-domain inhibitors could be considered as a new therapeutic tool. Nevertheless, several PI3K/Akt system components mediated effects need further consideration. First of all, Akt was notoriously associated to a positive

regulation of cell surviving and proliferation (Brazil & Hemmings, 2001) and its inhibition could led to an increase of apoptosis phenomenon augmenting troubles on still suffering heart. However, some studies revealed a beginning hypertrophic response followed by fibrosis and tissue remodeling due to modulation of the PI3K/Akt pathway (Dorn & Force, 2005). Isoproterenol infusion in transgenic PI3K$\gamma^{-/-}$ mice resulted in an attenuated cardiac hypertrophic response and markedly reduced interstitial fibrosis (Oudit *et al.*, 2003). Intriguingly, chronic β-adrenergic receptor stimulation triggered impaired heart functions in wild-type mice, whereas PI3K$\gamma^{-/-}$-deficient mice retained their increased heart function and did not develop heart failure (Oudit *et al.*, 2003). As widely discussed, adaptative hypertrophy and cardiac contraction, associated with maladaptative hypertrophy, are uncoupled events just because they would concern different PI3K isoforms pathways (Crackower *et al.*, 2002; Oudit *et al.*, 2004). This finding is in agreement with the evidence that a chronic stimulation of β1 or β2 evoked myocytes hypertrophy and apoptosis in the first case and cell survival and stress in the last (Zheng *et al.*, 2005). In heart failure, β2-AR role increases consequenlty of β1-AR downregulation and in PI3K-deficient mice did not produce apoptosis (Lohse *et al.*, 2003; Oudit *et al.*, 2003). If

our compounds activity would be really restrain to PI3Kγ component signalling, their positive inotropic effect may be uncoupled from the negative one on cell survival. Otherwise, this hypothesis needs further studies to be confirmed and an analysis in chronic condition on animal model of heart failure is required.

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