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A Multi-Protein Complex with TRPC, PDE1C, and A_2R Plays a Critical Role in Regulating Cardiomyocyte cAMP and Survival

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Abstract

BACKGROUND—cAMP plays a critical role in regulating cardiomyocyte survival. Various cAMP signaling pathways behave distinctly or in opposition. We have previously reported that activation of cAMP hydrolysis by cyclic nucleotide phosphodiesterase 1C (PDE1C) promotes cardiomyocytes death/apoptosis, yet the underlying molecular mechanism remains unknown. In this study, we aimed to identify the specific cAMP signaling pathway modulated by PDE1C and determine the mechanism by which Ca^{2+}/calmodulin-stimulated PDE1C is activated.

METHODS—To study cardiomyocyte death/apoptosis, we used both isolated mouse adult cardiomyocytes *in vitro* and doxorubicin (DOX)-induced cardiac toxicity *in vivo*. We used a variety of pharmacological activators and inhibitors, as well as genetically engineered molecular tools to manipulate the expression and/or activity of proteins of interest.

RESULTS—We found that the protective effect of PDE1C inhibition/deficiency on Ang II or doxorubicin (DOX)-induced cardiomyocyte death/apoptosis is dependent on cAMP-generating adenosine A_2 receptors (A_2Rs), suggesting that PDE1C's cAMP-hydrolyzing activity selectively modulates A_2R-cAMP signaling in cardiomyocytes. In addition, we found that the effects of PDE1C activation on Ang II-mediated cAMP reduction and cardiomyocyte death are dependent on transient receptor potential-canonical (TRPC) channels, in particular TRPC3. We also observed synergistic protective effects on cardiomyocyte survival from the combination of A_2R stimulation together with PDE1 or TRPC inhibition. Co-immunostaining and co-immunoprecipitation studies showed that PDE1C is localized in proximity with A_2R and TRPC3 in the plasma membrane and perhaps T-tubules. More importantly, we found that DOX-induced cardiac toxicity and dysfunction in mice are attenuated by the PDE1 inhibitor IC86340 or in PDE1C knockout mice, and that this protective effect is significantly diminished by A_2R antagonism.

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DISCLOSURE

None

CONCLUSION—We have characterized a novel multi-protein complex comprised of A_2R, PDE1C, and TRPC3, in which PDE1C is activated by TRPC3-derived Ca^{2+}, thereby antagonizing A_2R-cAMP signaling and promoting cardiomyocyte death/apoptosis. Targeting these molecules individually or in combination may represent a compelling therapeutic strategy for potentiating cardiomyocyte survival.

Keywords

cAMP; Phosphodiesterase; cardiomyocyte death; cardiac toxicity adenosine signaling

INTRODUCTION

Cardiomyocyte death is associated with many cardiac diseases including myocardial infarction, ischemia-reperfusion (I/R) injury, chemotherapy-induced cardiotoxicity, myocarditis, and heart failure.[1, 2] Loss of cardiomyocytes causes contractile dysfunction, arrhythmia, inflammation, fibrosis, and ultimately heart failure.[3–5] Thus, new therapeutic strategies that target cell death and promote survival are in high demand. The second messenger cAMP plays a critical role in regulating cardiomyocyte survival and death. cAMP can be produced through stimulation of various G-protein coupled receptors (GPCRs) that couple to different membrane-associated adenylyl cyclases (ACs), or through direct stimulation of soluble AC by bicarbonate. cAMP degradation can be catalyzed by diverse cyclic nucleotide phosphodiesterases (PDEs). Different sources of cAMP can have distinct or opposing functions in cardiomyocytes. For example, beta-adrenergic receptor 1 (β1-AR)-derived cAMP promotes myocyte death,[6, 7] while adenosine type-2 receptor (A_2R)-derived cAMP does the opposite.[8, 9]

PDEs function as critical modulators in regulating the amplitude, duration, and compartmentalization of cyclic nucleotide signaling. PDEs constitute a superfamily of enzymes with 21 different genes and more than 100 variants grouped into 11 broad families (PDE1–PDE11) based on distinct structural, kinetic, regulatory, and inhibitory properties. Different PDEs play varied roles in cardiac remodeling and dysfunction.[10, 11] PDE1 represents the highest PDE activity in human myocardium.[12, 13] PDE1 family members are encoded by three genes, *PDE1A*, *1B*, and *1C*, with PDE1A and 1C expressed in the heart.[13, 14] We have previously reported that PDE1C expression is increased in failing mouse and human hearts.[15] Using PDE1C deficient mice, we previously demonstrated that PDE1C plays a causative role in pathological cardiac remodeling, involving in myocyte hypertrophy, myocyte death, fibrosis, and cardiac dysfunction induced by chronic pressure overload,[15] yet the underlying molecular mechanisms remain uncharacterized. Here, we have attempted to unveil the mechanism by which PDE1C regulates cardiomyocyte death/apoptosis. Through a variety of *in vitro* and *in vivo* approaches, we demonstrated that the activation of PDE1C by TRPC-derived Ca^{2+} negatively regulates cardioprotective A_2R-derived cAMP signaling. Furthermore, we characterized a multiprotein complex containing PDE1C, $A_{2A}R$, and TRPC3 localized proximal to the cell membrane and possibly T-tubules. Coordination of these proteins into a complex allows precise control of cAMP signaling for myocyte survival. More importantly, these mechanistic studies may provide novel insight into using combination therapy to promote synergistic effects on myocyte survival.

METHODS

All animal procedures were performed in accordance with the National Institutes of Health (NIH) and University of Rochester institutional guidelines. An expanded Methods section is available in the Online Data Supplement. The data, methods and materials will be available to others for purposes of reproducing the results or replicating procedures by contacting the corresponding author.

Animal models

Global PDE1C knockout (PDE1C-KO) mice were backcrossed to C57BL/6J mice for more than 10 generations. For experiments with genetically modified mice, age/sex/genetic background matched mice were randomly separated into indicated groups based on animal identification number. For the Ang II infusion model, PDE1C-KO and wild type (PDE1C-WT) mice from sibling mating at 10–12 weeks of age were subjected to subcutaneous infusion with vehicle saline or Ang II (1.4 mg/kg/day) for 28 days as described previously.[16] For the doxorubicin cardiac toxicity model, PDE1C-WT, PDE1C-KO, or C57BL/6J male or female mice aged 10–12 weeks were randomly separated into indicated groups. Doxorubicin (20 mg/kg in saline) was administered via intra-peritoneal injection (i.p.) in a single bolus. IC86340 (6 mg/kg/day) and/or ZM241385 (10 mg/kg/day) in 20% DMSO/buffered saline were injected via i.p. two days prior to doxorubicin treatment and continued for additional five days. Mouse cardiac function was measured by echocardiography before sacrifice. Echocardiography was monitored in anesthetized mice using a Vevo2100 echocardiography machine equipped with an MS-550D 40 MHz frequency probe (VisualSonics, Toronto, Canada) as described previously in a blinded manner.[15]

Adult mouse cardiomyocyte isolation and culture

Adult mouse cardiomyocytes were isolated by enzymatic dissociation using collagenase type II (Worthington) in a Langendorff perfusion system as previously described.[15] Cardiomyocytes were cultured in the presence of blebbistatin (a myosin II inhibitor) to block myocyte contraction and to extend their survival during the culture as described previously.[15] Cardiomyocyte death/apoptosis was induced by indicated reagents for 24–48 hours. Cell death/apoptosis was measured by trypan blue staining, TUNEL staining or caspase-3/7 activity assay.

Trypan blue viability assay

Trypan blue staining was performed as described previously.[15] At the end of treatments, cells were centrifuged at 1,000g for 3min, stained with Trypan blue solution (0.2%) for 5 minutes, and photographed. 20 fields were randomly selected and an average of 300 myocytes were counted for each dish.

Co-immunoprecipitation

Co-immunoprecipitation experiments were performed with the Immunoprecipitation kit (ThermoFisher) based on manufacturer's protocol. In order to reduce interference of IgG light or heavy chain, mouse TrueBlot® ULTRA: Anti-Mouse IgG HRP, and sheep

TrueBlot®: Anti-sheep IgG HRP (Rockland) were used as secondary antibodies for immunoblotting.

Statistics

All data are presented as mean ± SEM. Statistical analysis was performed using SPSS 20. The unpaired Student's t test was used for comparisons between two groups, while one-way ANOVA followed by Bonferroni's post-hoc test was used for comparisons between multiple groups. P values <0.05 were considered significant.

RESULTS

PDE1C but not PDE1A regulates adult mouse cardiomyocyte survival

We have previously found that PDE1C deficiency or treatment with the pan PDE1 inhibitor IC86340 attenuated Ang II and isoproterenol (ISO)-stimulated adult mouse cardiomyocyte death and apoptosis *in vitro*.[15] Because two PDE1 family members, PDE1A and PDE1C, are expressed in cardiomyocytes,[14] we examined their differential roles in cardiomyocyte death. Using the trypan blue staining method, we showed that Ang II-induced cell death was significantly induced in wild-type (WT) mouse adult cardiomyocytes, which was abolished in PDE1C-KO but not PDE1A-KO myocytes (Online Supplemental Fig. S1). Similar to Ang II, doxorubicin (DOX) and H_2O_2-stimulated cell death were also blocked in PDE1C-KO but not in PDE1A-KO myocytes (Online Supplemental Figure S1). Together, these results suggest that PDE1C exclusively regulates cardiomyocyte death in response to multiple stimuli.

PDE1C deficiency reduces apoptotic cardiomyocytes in mouse hearts with chronic Ang II-infusion

To determine the effects of PDE1C deficiency on Ang II-induced cardiomyocyte death *in vivo*, we subjected PDE1C-WT or PDE1C-KO mice to chronic vehicle or Ang II infusion and assessed cardiomyocyte apoptosis via TUNEL staining. PDE1C deficiency did not have effects on blood pressure (data not shown), which is consistent with the finding that PDE1C is not expressed in contractile smooth muscle cells in normal vessel walls.[17] We found that cardiomyocyte apoptosis was significantly increased in Ang II-treated PDE1C-WT hearts compared to vehicle, which was significantly attenuated in PDE1C-KO hearts (Online Supplemental Fig. S2). This result is consistent with our previous finding in thoracic aorta contraction (TAC)-induced heart failure,[15] suggesting that PDE1C deficiency is protective against myocardial death in multiple forms of cardiac disease models.

The protective effects of PDE1C inhibition or deficiency on cardiomyocyte death and apoptosis are dependent on A_2R activation

To further investigate the anti-apoptotic mechanism of PDE1C inhibition, we first examined whether PDE1C regulates adenosine-cAMP signaling which was previously found to mediate myocyte survival[8]. We confirmed that adenosine dose-dependently inhibited Ang II-induced myocyte death (online supplemental Fig. S3). There are 4 adenosine receptors: A_1R, $A_{2A}R$, $A_{2B}R$ and A_3R.[8] Among them, $A_{2A}R$ and $A_{2B}R$ are coupled to $G\alpha_s$, which stimulates cAMP production and therefore represent promising targets for PDE1C. To

determine whether A_2R-cAMP signaling is involved in PDE1C-mediated regulation of myocyte survival, we first use a high dose of ZM241385 (200 nmol/L), which is able to blocked both $A_{2A}R$ and $A_{2B}R$ subtypes at the high dose. When we measured myocyte death by trypan blue staining (Figure 1A), the protective effect of the PDE1 inhibitor IC86340 on Ang II-induced cell death seen in PDE1C-WT myocytes was almost completely abolished when blocking A_2R with ZM241385 (Fig. 1B). Consistently, the protective effect of PDE1C deficiency was also reversed upon A_2R blockade (Fig. 1C). These results suggest that the protective effects of PDE1C deficiency/inhibition on myocyte death are dependent on A_2R activation.

Since trypan blue allows only for nonspecific assessment of cell death and viability, we next examined myocyte apoptosis by TUNEL staining and caspase 3/7 activities. As shown in Figure 2A–C, the anti-apoptotic effects of IC86340 or PDE1C deficiency were completely abolished by treatment with A_2R antagonist ZM241385. In addition, the reduced caspase 3/7 activity seen with PDE1 inhibition (Fig. 2D) or PDE1C deficiency (Fig. 2E) was also completely abolished under A_2R blockade. These findings also confirmed that Trypan blue staining is a valid method for assessing cell death in adult cardiomyocytes *in vitro*; we therefore used it for the majority of further experiments investigating cell death.

We next determined the specific contributions of $A_{2A}R$ or $A_{2B}R$ to PDE1C-regulated protective signaling. Blockade of either $A_{2A}R$ (with a low dose of ZM241385, 5 nmol/L) or $A_{2B}R$ (with PSB603) alone each abolished the protective effects of PDE1 inhibition (Fig. 3A) or PDE1C deficiency (Fig. 3B) on myocyte death as assessed by both trypan blue and TUNEL staining (Online Supplemental Fig S4A). Knocking down either $A_{2A}R$ or $A_{2B}R$ through lentivirus-mediated expression of shRNA against either $A_{2A}R$ or $A_{2B}R$ (Online Supplemental Fig. S4B and S4C) has similar effects (Fig. 3C and 3D). These results suggest that both $A_{2A}R$ and $A_{2B}R$ are necessary for the protective effects of PDE1C deficiency, and that the two receptor subtypes may act as hetero-oligomers or in other forms of cooperativity.[18]

Stimulating cardiomyocytes with PGE_2 can increase cAMP. $G\alpha_s$-coupled EP4 represents the most dominant subtype expressed in the heart [19] and the cardiac protective effects of EP4 on I/R injury and cardiac remodeling have been documented.[20–22] Interestingly, we found that treatment with an EP4 antagonist failed to block the protective effect of PDE1C deficiency on myocyte death (Online Supplemental Fig. S4D). The effectiveness of this EP4 antagonist was confirmed in a separate myocyte hypertrophy study (data not shown). This supports the notion of selective interaction between PDE1C and a cAMP pool coupled to A_2R.

Given the functional interaction between PDE1C and adenosine-cAMP signaling, we examined if activation of A_2R and inhibition of PDE1C have synergistic protective effects. At very low doses, neither adenosine (at 3 nmol/L) nor IC86340 (at 3 nmol/L) alone had any visible effect on cell death (Fig. 4A) or apoptosis (Fig. 4B), yet in combination, they synergistically inhibited cell death and apoptosis. This synergistic protective effect was blocked by treatment with the $A_{2A}R$ blocker ZM241385 (5 nmol/L) or $A_{2B}R$ blocker PSB603 (100nmol/L) (Fig. 4C). Besides adenosine, low doses of the A_2R-selective agonist CGS21680 with IC86340 also elicited a synergistic effect, which again was abolished by

A_2R blockade (Fig. 4D), further supporting the notion of an A_2 receptor-PDE1C specific module.

TRPC is involved in PDE1C-mediated regulation of cardiomyocyte death

We next investigated how PDE1C is activated by Ang II. PDE1C is a Ca^{2+}/calmodulin-stimulated PDE. It has been shown that the canonical transient receptor potential TRPC3 is responsible for both stress-induced and Ang II type 1 receptor (AT1R)-dependent slow increases of intracellular Ca^{2+} in mouse cardiomyocytes.[23] TRPC3 also plays a critical role in cardiomyocyte apoptosis in response to I/R [24]. Therefore, we interrogated the role of TRPC3 channels in Ang II-induced cell death with the non-selective TRPC channel blocker SFK96365, as well as the TRPC3-selective blocker Pyr3. We found that both SFK96365 and Pyr3 inhibited Ang II-induced cell death similarly in WT myocytes (Fig. 5A), but had no further effects in PDE1C-KO myocytes (Fig. 5B). We then tested the effect of suppressing TRPC3 expression via lentivirus shRNA vectors, which reduced TRPC3 expression by about 70% relative to control scramble shRNA (Online Supplementary Fig. S5A). TRPC3 shRNA had very similar effects as the TRPC3 inhibitor Pyr3 on cell death in WT and PDE1C-KO myocytes (Fig. 5C and 5D). These results suggest that TRPC3 and PDE1C regulate cell death in a redundant manner, or via the same signaling pathway.

To further confirm the role of TRPC3, we overexpressed TRPC3 in WT myocytes (Online Supplemental Fig. S5B) and found that cells were sensitized to Ang II-stimulated cell death (Fig. 5E). The TRPC3 inhibitor Pyr3, PDE1 inhibitor IC86340, and extracellular Ca^{2+} chelator EGTA each completely abolished Ang II-stimulated cell death under TRPC3 overexpression (Fig. 5E). To further determine the role of TRPC3 in PDE1C-mediated regulation of myocyte death, we ectopically expressed PDE1C via an adenoviral vector in PDE1C-KO myocytes to a level roughly equivalent to that in WT myocytes, as evaluated by Western blotting and immunostaining (Online Supplementary Fig. S5C and S5D). We found that PDE1C expression re-sensitized myocytes to Ang II-stimulated cell death, which was abolished by Pyr3, IC86340, or EGTA (Fig. 5F). These results strongly suggest that PDE1C's function on myocyte death is dependent on TRPC3 activation and extracellular Ca^{2+}, which also provides an explanation for our findings that inhibition of PDE1C and TRPC3 act redundantly, with no additive effect on myocyte death. In support of this notion, we also found that neither adenosine nor SKF96365 treatment at very low doses alone had any visible effect on cell death, but in combination, they synergistically inhibited cell death (Online Supplemental Fig. S5E).

TRPC and PDE1C are involved in Ang II-mediated reduction of cAMP in cardiomyocytes

Subsequently, we investigated the role of PDE1C in regulating myocyte cAMP. We found that in WT myocytes, Ang II treatment rapidly induced a reduction in cAMP, which was abolished by PDE1 inhibition with IC86340 or TRPC3 inhibition with Pyr3 (Fig. 5G), IC86340 together with Pyr3 did not have additive effects on cAMP levels (Fig. 5G). However, Ang II failed to reduce cAMP in PDE1C-KO myocytes (Fig. 5H), and neither IC86340, Pyr3, nor combination treatment had any additional effects in PDE1C-KO cells (Fig. 5H). In addition, A_2R antagonism blocked the inhibitory effects of IC86340 on Ang II-mediated reduction of cAMP (online supplemental Fig. S6). These results support the notion

that Ang II treatment induces cardiomyocyte Ca^{2+} entry, activating PDE1C, resulting in a loss of cAMP that is coupled to A_2R activation.

PDE1C, A_2R and TRPC3 form a multi-protein complex in cardiomyocyte membrane

TRPC3 is primarily localized to T-tubules[25]. Because of the functional relationship between TRPC3 and PDE1C, we investigated the localization of PDE1C. We found that endogenous PDE1C staining largely overlapped with Na^+/Ca^{2+} exchanger (NCX) which also primarily localizes to T-tubules (Online supplemental Fig. S7), suggesting that PDE1C may as well. Having observed functional coupling among PDE1C, A_2R and TRPC3, we hypothesized that PDE1C is localized in proximity with A_2R and TRPC3. We therefore performed co-immunostaining for endogenous PDE1C, $A_{2A}R$, and TRPC3 in cardiomyocytes and in heart tissues. Triple immunostaining and three-color histogram analysis revealed significant colocalization among PDE1C, $A_{2A}R$ and TRPC3 in isolated cardiomyocytes (Fig. 6A and 6B). PDE1C/$A_{2A}R$ or PDE1C/TRPC3 double staining also revealed significant overlap between PDE1C and $A_{2A}R$ (Fig. 6C and 6D), as well as PDE1C and TRPC3 in heart tissues (Fig. 6E and 6F). These findings suggest that PDE1C is present in proximity with $A_{2A}R$ and TRPC3 in cardiomyocytes. Due to the difficulty in finding a specific $A_{2B}R$ antibody for immunostaining, we focused on $A_{2A}R$.

Next, we performed co-immunoprecipitation assays to further demonstrate the association among PDE1C, $A_{2A}R$ and TRPC3. When immunoprecipitating (IP) PDE1C in WT or PDE1C-KO heart samples with PDE1C antibody, we were able to detect $A_{2A}R$ and TRPC3 by Western blotting (IB) in WT but not PDE1C-KO heart lysates (Fig. 6G). Consistently, when pulling down TRPC3 in WT heart samples with TRPC3 antibody or IgG, we were able to detect PDE1C and $A_{2A}R$ with TRPC3 antibody but not IgG (Fig. 6H). Furthermore, when pulling down $A_{2A}R$ in WT or $A_{2A}R$-KO hearts samples, we were able to detect PDE1C and TRPC3 in WT but not $A_{2A}R$-KO heart samples (Fig. 6I). These results suggest that PDE1C, $A_{2A}R$ and TRPC3 are present in the same multi-protein complex.

We next investigated the specific interactions between these proteins. We pulled down TRPC3 in WT and PDE1C-KO heart lysates, and detected $A_{2A}R$ in both samples (Online supplemental Fig. S8A), suggesting that the association between TRPC3 and $A_{2A}R$ is independent of PDE1C. Similarly, we pulled down TRPC3 in WT and $A_{2A}R$-KO heart lysates, and detected PDE1C in both WT and $A_{2A}R$-KO samples (Online supplemental Fig. S8B), suggesting that the association between TRPC3 and PDE1C is independent of $A_{2A}R$. These results may suggest that these three proteins do not directly interact with each other, and that other scaffold protein(s) are important for the complex formation.

A_2R activation is critical for the protective effects of PDE1C inhibition or PDE1C deficiency on doxorubicin-induced cardiomyocyte death *in vitro* as well as cardiac toxicity and dysfunction *in vivo*

The anthracycline doxorubicin (DOX) is a well-known chemotherapy drug which causes cardiotoxicity, ultimately leading to heart failure[26]. DOX induces cardiomyocyte death, one of mechanisms responsible for DOX-induced cardiac atrophy and functional impairment.[27] Given our findings that PDE1C and A_2R regulate cardiomyocyte death, we investigated

whether they could also modulate DOX-induced cardiotoxicity. Similar to Ang II induced myocyte death, the PDE1 inhibitor IC86340 (Fig. 7A) or PDE1C deficiency (Fig. 7B) significantly inhibited DOX-induced myocyte death *in vitro*. This protective effect was also abolished by A_2R antagonism with ZM241385.

We next investigated the protective effects of IC86340 and the role of A_2R on DOX-induced cardiomyopathy and cardiac dysfunction *in vivo*. Consistent with previous studies, cardiac atrophy was induced by a single dose of DOX, as evidenced by significant reductions in heart size (Fig. 7C) and weight (Fig. 7D). Treatment with the PDE1 inhibitor IC86340 prevented this atrophy, yet this protective effect was reversed by treatment with A_2R antagonist ZM241385. Histological analyses revealed that myocyte apoptosis, assessed by TUNEL staining, was markedly increased by DOX treatment (Fig. 7E–F). IC86340 protected from DOX-induced myocyte apoptosis, yet failed to do so in the presence of ZM241385. Echocardiographic measurements showed that DOX caused a significant loss in cardiac contractile function, reflected by reduced fractional shortening (FS%) and ejection fraction (EF%), and an increase in left ventricular end systolic diameter (LVESD), each of which was significantly improved by IC86340 but not by IC86340 with ZM241385 (Fig. 7G and Supplemental Fig. S9A–D). Cardiac Troponin I (cTnI), a circulating marker of cardiac damage, was altered in a similar pattern (Fig. 7H). Moreover, IC86340 increased the survival rate of mice compared with both the DOX treatment group, yet this was lost in the group treated with DOX, IC86340 and ZM241385 (Online supplemental Fig. S9E).

We also investigated the protective effects of PDE1C deficiency and the role of A_2R in the DOX model *in vivo*. Similar to PDE1 inhibition, PDE1C deficiency also protected against cardiac atrophy as assessed by heart size/weight change (Fig. 8A–B), myocyte apoptosis (Fig. 8C–D), cardiac contractile dysfunction including reduced FS (Fig. 8E) or EF (online supplemental Fig. S10A–B), cardiac damage maker cTnI (Fig. 8F), LVESD (online supplemental Fig. S10C). Animal death rate was also reduced by PDE1C knockout (online supplemental Fig. S10E). The protective effects of PDE1C deficiency were significantly attenuated or largely abolished by A_2R antagonism using ZM241385. Together, these results suggest that PDE1 inhibition or PDE1C deficiency ameliorate cardiac structural and functional deterioration and improves survival of mice after treatment with DOX, which is dependent upon A_2R activation.

DISCUSSION

In this study, we have investigated the mechanism by which PDE1C protects against cardiomyocyte death/apoptosis *in vitro* and *in vivo*, primarily focusing on defining proximal positive and negative regulators of the cardio-protective cAMP-signaling module. The current study has revealed a number of novel findings, summarized as follows: (1) PDE1C activation promotes cardiomyocyte death/apoptosis by antagonizing a novel myocyte protective cAMP signaling pathway that is derived from activation of the adenosine receptors $A_{2A}R/A_{2B}R$. (2) PDE1 inhibition or PDE1C deficiency elicits protective effects on DOX-induced cardiomyopathy and dysfunction *in vivo*, consistent with its central role in regulating cardiomyocyte death. These protective effects are dependent on A_2R, consistent with the findings in isolated cardiomyocytes. (3) PDE1C, as a Ca^{2+}/CaM-stimulated PDE, is

likely activated by TRPC3-mediated extracellular Ca^{2+} influx. PDE1C, $A_{2A}R$, and TRPC3 are physically and functionally associated, and form a macromolecule complex that is localized proximate to the cell membrane and T-tubules. Our discovery of this complex has offered a novel mechanism by which Ca^{2+} influx regulates GPCR-cAMP signaling via Ca^{2+}-stimulated PDE1C activation. (4) Simultaneously activating A_2R and inhibiting PDE1C or TRPCs can induce synergistic protective effects on myocyte death. The ability of low doses of drugs targeting these proteins to elicit potent cardioprotective effects may have therapeutic significance. Taken together, our results indicate that we have identified a cardiac protective cAMP signaling cascade that is positively regulated by A_2R and negatively regulated by PDE1C through activation of TRPC3 (Fig. 8G). Coordination of these proteins into a complex allows precise control of cAMP signaling for myocyte survival. More importantly, our findings may provide novel insight into using combination therapy to promote synergistic effects on myocyte survival.

Adenosine is an important regulator of the cardiovascular system. The beneficial effects of adenosine receptor (AR) stimulation in the cardiovascular system make this an attractive therapeutic target. However, the ubiquitous distribution of ARs throughout the body has posed a challenge regarding the specificity of AR-based pharmacological therapy. Interestingly, we found that at very low doses, neither adenosine (or A_2R agonist) nor PDE1 inhibitor alone had any effect, yet in combination, they produced synergistic protective effects on cardiomyocytes. In addition, PDE1C displays a highly tissue-type and cell-type specific expression profile.[28, 29] Thus, our findings may support investigation of novel therapeutic strategies involving combination treatment of low dose A_2R agonists and PDE1C inhibitors, which could allow specific targeting exclusively in cells or tissues where both A_2R and PDE1C exist. Such combination treatment regimens deserve to be further evaluated in the intact heart and *in vivo* in the future.

TRPCs, voltage-independent cation channels, are best known for their functions related to Ca^{2+} influx. In cardiomyocytes, TRPCs have been shown to play a role in coordination of signaling in local domains through interaction with Ca^{2+}-dependent proteins, thus playing critical roles in pathological cardiac structure remodeling.[30, 31] The TRPC channel family is comprised of seven members, TRPC1-TRPC7.[32] In this study, we have primarily focused on TRPC3 because it has been shown to mediate Ang II-inducedCa^{2+} entry in cardiomyocytes,[23] and is important for cardiomyocyte apoptosis after I/R injury.[24] However, the involvement of other TRPC family members in regulating PDE1C activation cannot be excluded, particularly because TRPC channels can form heterotetramers, such as TRPC1/TRPC3,[33] TRPC1/TRPC3/TRPC7,[34] TRPC3/TRPC6,[30] TRPC3/TRPC6/TRPC7.[35] It will be interesting to investigate the relationship between PDE1C with other TRPCs in cardiomyocytes in the future. TRPCs possess a variety of regulatory and protein integrations sites, which not only allows TRPCs to form homo-/hetero-tetramers, but also associate with and be regulated by other proteins. For example, it has been shown that PKG can phosphorylate TRPC3, which negatively regulates TRPC3 activity.[36] PKA shares the same consensus phosphorylation on TRPC3 site as PKG. Therefore, it may be worthwhile to examine whether cAMP/PKA is able to negatively regulate TRPC3 through feedback signaling.

The PDE1 inhibitor IC86340 is able to inhibit all PDE1 family members. In cardiomyocytes, both PDE1A and PDE1C are expressed. Our findings from isolated cardiomyocytes indicate that PDE1C but not PDE1A regulates myocyte death, and that the protective effects of IC86340 primarily result from PDE1C inhibition (Online Supplemental Fig. S1). In support of this, we observed similar protective effects between IC86340 and PDE1C deficiency against myocyte death induced by different death stimuli, including Ang II, H_2O_2, and DOX (Online Supplemental Fig. S1) and ISO[15] *in vitro*, and Ang II-infusion (Online Supplemental Fig. S2), DOX treated mice (Fig. 7–8), and TAC[15] *in vivo*. This suggests that PDE1C may play a critical role in diverse cardiac diseases associated with myocyte death regardless of etiology. PDE1C has also been implicated in cancer cells. For example, a focal genomic amplification at the PDE1C locus has been found in more than 90 human glioblastoma multiforme (GBM) cell lines, and PDE1C is critical for GBM cell proliferation, migration and invasion.[37] PDE1C is also important for the growth of human malignant melanoma cell lines.[38] These findings could indicate that PDE1C inhibition or suppression could reduce growth in cancer cells. Thus, the protective effects of PDE1 inhibition/deficiency on DOX-induced cardiac toxicity suggest that PDE1 inhibition may represent a novel therapeutic strategy: inhibiting cancer growth while simultaneously preventing cardiac toxicity.

It is worth noting that the beneficial effects of the PDE1 inhibitor IC86340 or global PDE1C deficiency on preventing cardiac toxicity and increasing mice survival rate in DOX-treated mice may not exclusively rely on PDE1C inhibition and protection against myocyte death. In addition to cardiomyocyte apoptosis, DOX also induces cardiac atrophy, inflammation, and oxidative stress as well as toxicity in other tissues/organs, which may indirectly affect cardiac performance. Thus, the role of PDE1C or other PDE1 isozymes in other cells, tissues and organs should be also taken into the consideration. Moreover, it is still not known whether the cyclic nucleotide signaling modulated by PDE1C directly regulates cardiomyocyte contractility, which deserves to be further characterized. Adenosine exerts cardiac electrophysiological effects, which are believed to be mainly through A_1R activation.[39] The effects of A_2R on cardiac contractile function remain controversial.[39] Moreover, the vasodilatory function of A_2R action[39] may also contribute to worsening myocardial damage and function by A_2R antagonist ZM241385.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Anversa P, Kajstura J. Myocyte cell death in the diseased heart. Circ Res. 1998; 82:1231–1233. [PubMed: 9633922]

2. Konstantinidis K, Whelan RS, Kitsis RN. Mechanisms of cell death in heart disease. Arterioscler Thromb Vasc Biol. 2012; 32:1552–1562. [PubMed: 22596221]

3. Yamaguchi O, Higuchi Y, Hirotani S, Kashiwase K, Nakayama H, Hikoso S, Takeda T, Watanabe T, Asahi M, Taniike M, Matsumura Y, Tsujimoto I, Hongo K, Kusakari Y, Kurihara S, Nishida K, Ichijo H, Hori M, Otsu K. Targeted deletion of apoptosis signal-regulating kinase 1 attenuates left ventricular remodeling. Proc Natl Acad Sci U S A. 2003; 100:15883–15888. [PubMed: 14665690]

4. Diwan A, Krenz M, Syed FM, Wansapura J, Ren X, Koesters AG, Li H, Kirshenbaum LA, Hahn HS, Robbins J, Jones WK, Dorn GW. Inhibition of ischemic cardiomyocyte apoptosis through targeted ablation of Bnip3 restrains postinfarction remodeling in mice. J Clin Invest. 2007; 117:2825–2833. [PubMed: 17909626]

5. Mandl A, Huong Pham L, Toth K, Zambetti G, Erhardt P. Puma deletion delays cardiac dysfunction in murine heart failure models through attenuation of apoptosis. Circulation. 2011; 124:31–39. [PubMed: 21670227]

6. Lohse MJ, Engelhardt S, Eschenhagen T. What is the role of beta-adrenergic signaling in heart failure? Circ Res. 2003; 93:896–906. [PubMed: 14615493]

7. Feldman DS, Elton TS, Sun B, Martin MM, Ziolo MT. Mechanisms of disease: detrimental adrenergic signaling in acute decompensated heart failure. Nat Clin Pract Cardiovasc Med. 2008; 5:208–218. [PubMed: 18283305]

8. Peart JN, Headrick JP. Adenosinergic cardioprotection: multiple receptors, multiple pathways. Pharmacol Ther. 2007; 114:208–221. [PubMed: 17408751]

9. Szentmiklosi AJ, Cseppento A, Harmati G, Nanasi PP. Novel trends in the treatment of cardiovascular disorders: site- and event- selective adenosinergic drugs. Curr Med Chem. 2011; 18:1164–1187. [PubMed: 21291368]

10. Knight W, Yan C. Therapeutic potential of PDE modulation in treating heart disease. Future Med Chem. 2013; 5:1607–1620. [PubMed: 24047267]

11. Lee DI, Kass DA. Phosphodiesterases and cyclic GMP regulation in heart muscle. Physiology (Bethesda). 2012; 27:248–258. [PubMed: 22875455]

12. Wallis RM, Corbin JD, Francis SH, Ellis P. Tissue distribution of phosphodiesterase families and the effects of sildenafil on tissue cyclic nucleotides, platelet function, and the contractile responses of trabeculae carneae and aortic rings in vitro. Am J Cardiol. 1999; 83:3C–12C.

13. Vandeput F, Wolda SL, Krall J, Hambleton R, Uher L, McCaw KN, Radwanski PB, Florio V, Movsesian MA. Cyclic Nucleotide Phosphodiesterase PDE1C1 in Human Cardiac Myocytes. J Biol Chem. 2007; 282:32749–32757. [PubMed: 17726023]

14. Miller CL, Oikawa M, Cai Y, Wojtovich AP, Nagel DJ, Xu X, Xu H, Florio V, Rybalkin SD, Beavo JA, Chen YF, Li JD, Blaxall BC, Abe J, Yan C. Role of Ca2+/calmodulin-stimulated cyclic nucleotide phosphodiesterase 1 in mediating cardiomyocyte hypertrophy. Circ Res. 2009; 105:956–964. [PubMed: 19797176]

15. Knight WE, Chen S, Zhang Y, Oikawa M, Wu M, Zhou Q, Miller CL, Cai Y, Mickelsen DM, Moravec C, Small EM, Abe J, Yan C. PDE1C deficiency antagonizes pathological cardiac remodeling and dysfunction. Proc Natl Acad Sci U S A. 2016; 113:E7116–125. [PubMed: 27791092]

16. Wu MP, Zhang YS, Xu X, Zhou Q, Li JD, Yan C. Vinpocetine Attenuates Pathological Cardiac Remodeling by Inhibiting Cardiac Hypertrophy and Fibrosis. Cardiovasc Drugs Ther. 2017; 31:157–166. [PubMed: 28321644]

17. Cai Y, Nagel DJ, Zhou Q, Cygnar KD, Zhao H, Li F, Pi X, Knight PA, Yan C. Role of cAMP-phosphodiesterase 1C signaling in regulating growth factor receptor stability, vascular smooth muscle cell growth, migration, and neointimal hyperplasia. Circ Res. 2015; 116:1120–1132. [PubMed: 25608528]

18. Headrick JP, Ashton KJ, Rose'meyer RB, Peart JN. Cardiovascular adenosine receptors: expression, actions and interactions. Pharmacol Ther. 2013; 140:92–111. [PubMed: 23764371]

19. Yokoyama U, Iwatsubo K, Umemura M, Fujita T, Ishikawa Y. The prostanoid EP4 receptor and its signaling pathway. Pharmacol Rev. 2013; 65:1010–1052. [PubMed: 23776144]

20. Xiao CY, Yuhki K, Hara A, Fujino T, Kuriyama S, Yamada T, Takayama K, Takahata O, Karibe H, Taniguchi T, Narumiya S, Ushikubi F. Prostaglandin E2 protects the heart from ischemia-

reperfusion injury via its receptor subtype EP4. Circulation. 2004; 109:2462–2468. [PubMed: 15123528]

21. Qian JY, Harding P, Liu Y, Shesely E, Yang XP, LaPointe MC. Reduced cardiac remodeling and function in cardiac-specific EP4 receptor knockout mice with myocardial infarction. Hypertension. 2008; 51:560–566. [PubMed: 18180401]
22. Pang L, Cai Y, Tang EH, Irwin MG, Ma H, Xia Z. Prostaglandin E Receptor Subtype 4 Signaling in the Heart: Role in Ischemia/Reperfusion Injury and Cardiac Hypertrophy. J Diabetes Res. 2016; 2016:1324347. [PubMed: 27190998]
23. Yamaguchi Y, Iribe G, Kaneko T, Takahashi K, Numaga-Tomita T, Nishida M, Birnbaumer L, Naruse K. TRPC3 participates in angiotensin II type 1 receptor-dependent stress-induced slow increase in intracellular Ca(2+) concentration in mouse cardiomyocytes. J Physiol Sci. 2018; 68:153–164. [PubMed: 28105583]
24. Shan D, Marchase RB, Chatham JC. Overexpression of TRPC3 increases apoptosis but not necrosis in response to ischemia-reperfusion in adult mouse cardiomyocytes. Am J Physiol Cell Physiol. 2008; 294:C833–C841. [PubMed: 18184877]
25. Goel M, Zuo CD, Sinkins WG, Schilling WP. TRPC3 channels colocalize with Na+/Ca2+ exchanger and Na+ pump in axial component of transverse-axial tubular system of rat ventricle. Am J Physiol Heart Circ Physiol. 2007; 292:H874–H883. [PubMed: 17012351]
26. Singal PK, Li T, Kumar D, Danelisen I, Iliskovic N. Adriamycin-induced heart failure: mechanism and modulation. Mol Cell Biochem. 2000; 207:77–86. [PubMed: 10888230]
27. Sweeney M, Yiu A, Lyon AR. Cardiac Atrophy and Heart Failure In Cancer. Card Fail Rev. 2017; 3:62–65. [PubMed: 28785478]
28. Yan C, Zhao AZ, Bentley JK, Loughney K, Ferguson K, Beavo JA. Molecular cloning and characterization of a calmodulin-dependent phosphodiesterase enriched in olfactory sensory neurons. Proc Natl Acad Sci U S A. 1995; 92:9677–9681. [PubMed: 7568196]
29. Lakics V, Karran EH, Boess FG. Quantitative comparison of phosphodiesterase mRNA distribution in human brain and peripheral tissues. Neuropharmacology. 2010; 59:367–374. [PubMed: 20493887]
30. Eder P, Molkentin JD. TRPC channels as effectors of cardiac hypertrophy. Circ Res. 2011; 108:265–272. [PubMed: 21252153]
31. Wu X, Eder P, Chang B, Molkentin JD. TRPC channels are necessary mediators of pathologic cardiac hypertrophy. Proc Natl Acad Sci U S A. 2010; 107:7000–7005. [PubMed: 20351294]
32. Cheng KT, Ong HL, Liu X, Ambudkar IS. Contribution and regulation of TRPC channels in store-operated Ca2+ entry. Curr Top Membr. 2013; 71:149–179. [PubMed: 23890115]
33. Chen J, Crossland RF, Noorani MM, Marrelli SP. Inhibition of TRPC1/TRPC3 by PKG contributes to NO-mediated vasorelaxation. Am J Physiol Heart Circ Physiol. 2009; 297:H417–H424. [PubMed: 19502552]
34. Zagranichnaya TK, Wu X, Villereal ML. Endogenous TRPC1, TRPC3, and TRPC7 proteins combine to form native store-operated channels in HEK-293 cells. J Biol Chem. 2005; 280:29559–29569. [PubMed: 15972814]
35. Goel M, Sinkins WG, Schilling WP. Selective association of TRPC channel subunits in rat brain synaptosomes. J Biol Chem. 2002; 277:48303–48310. [PubMed: 12377790]
36. Kwan HY, Huang Y, Yao X. Regulation of canonical transient receptor potential isoform 3 (TRPC3) channel by protein kinase G. Proc Natl Acad Sci U S A. 2004; 101:2625–2630. [PubMed: 14983059]
37. Rowther FB, Wei W, Dawson TP, Ashton K, Singh A, Madiesse-Timchou MP, Thomas DG, Darling JL, Warr T. Cyclic nucleotide phosphodiesterase-1C (PDE1C) drives cell proliferation, migration and invasion in glioblastoma multiforme cells in vitro. Mol Carcinog. 2016; 55:268–279. [PubMed: 25620587]
38. Shimizu K, Murata T, Watanabe Y, Sato C, Morita H, Tagawa T. Characterization of phosphodiesterase 1 in human malignant melanoma cell lines. Anticancer Res. 2009; 29:1119–1122. [PubMed: 19414353]

39. Mustafa SJ, Morrison RR, Teng B, Pelleg A. Adenosine receptors and the heart: role in regulation of coronary blood flow and cardiac electrophysiology. Handb Exp Pharmacol. 2009:161–188. [PubMed: 19639282]

CLINICAL PERSPECTIVE

What is New?

- PDE1C functions as a key negative regulator of the adenosine-cAMP signaling in cardiomyocytes, a well-characterized cardio-protective signaling pathway.
- TRPC3, PDE1C and $A_{2A}R$ are coordinated in a novel macromolecule complex localized to cardiomyocyte membranes and perhaps T-tubules, offering a novel mechanism by which Ca^{2+} influx negatively regulates GPCR-cAMP signaling via activation of Ca^{2+}-dependent PDE1C.
- Low doses of A_2R agonist combined with PDE1 inhibitor or TRPC3 inhibitor provide synergistic effects on myocyte survival.
- PDE1 inhibition or PDE1C deficiency protects against doxorubicin-induced cardiac toxicity and dysfunction *in vivo,* which is dependent on A_2R activation.

What Are the Clinical Implications?

- Our findings suggest that PDE1C may represent a novel therapeutic target for various cardiac diseases associated with cardiomyocyte death.
- PDE1C inhibition may represent a novel therapeutic strategy in cancer therapy by which PDE1C inhibition blocks cancer cell growth while simultaneously preventing cardiac toxicity.
- It may be possible to achieve synergistic protective effects against cardiomyocyte death by simultaneously activating the positive cAMP regulator A_2R and inhibiting the negative cAMP regulators PDE1C or TRPC3.

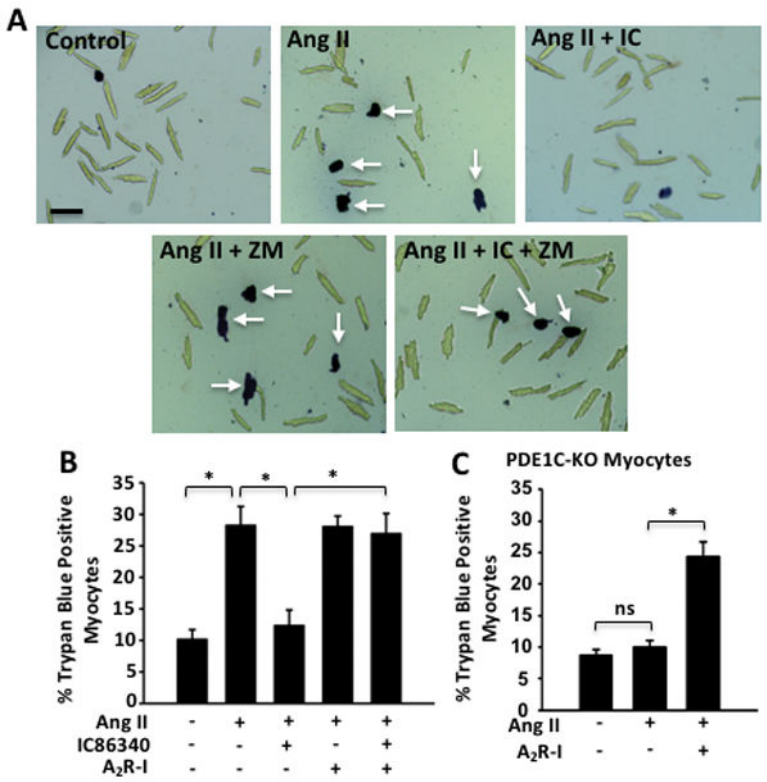

Figure 1. A_2R mediates the protective effect of PDE1C inhibition or deficiency on Ang II-induced cardiomyocyte death

(A–C) Cardiomyocytes isolated from PDE1C-WT or -KO mice were stimulated with 200 nmol/L Ang II for 24 hours in the presence of IC86340 (15 μmol/L), A_2R antagonist (A_2R-I, ZM241385, 200 nmol/L) or vehicle; (A) Trypan blue staining. White arrows indicate trypan blue-positive myocytes. The scale bar is 50 μm. (B–C) Quantification of trypan blue staining results. 20 random fields and approximate average of 300 myocytes were counted per dish. Data were presented as mean ± SEM. *p<0.05, ns: not significant by one-way ANOVA with Bonferroni's correction. B: n=6 replicates from 3 mice. C: n=5 replicates from 3 mice.

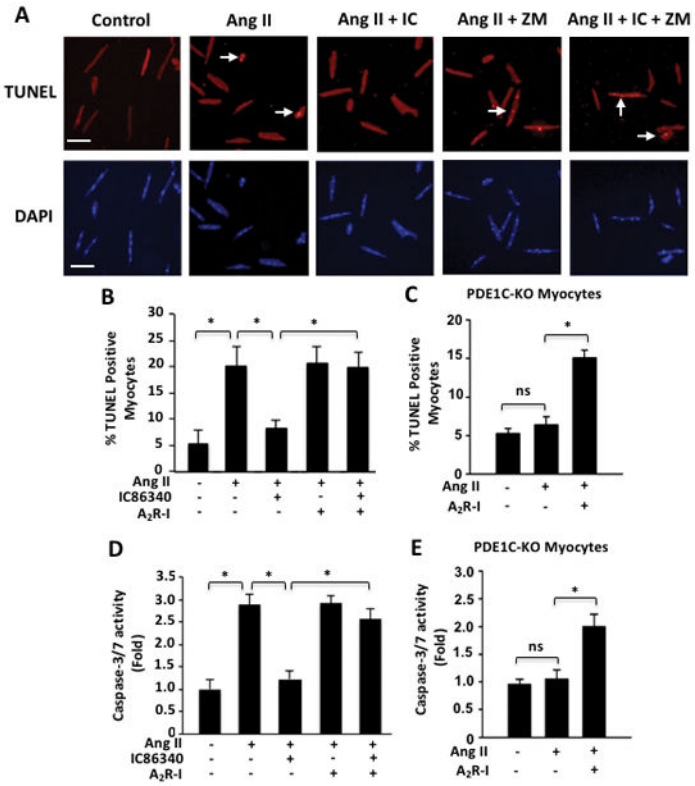

Figure 2. A_2R mediates the protective effect of PDE1C inhibition or deficiency on Ang II-induced cardiomyocyte apoptosis

(A) Representative images of TUNEL staining (Red) with DAPI (blue) staining for nuclei. White arrows point to apoptotic cells with TUNEL positive nuclei. The scale bar is 50 μm. Myocytes were treated with 200 nmol/L Ang II in the presence of IC86340 (15 μmol/L), A_2R antagonist (A_2R-I, ZM241385, 200 nmol/L) or vehicle for 48 hours. (B–C) Quantification of TUNEL-positive myocytes. (D–E) Quantification of results of caspase-3/7 activity assay. Data are presented as mean ± SEM *p<0.05, ns, not significant by one-way ANOVA with Bonferroni's correction. B and D: n=6 replicates from 3 mice. C and E: n=5 replicates from 3 mice.

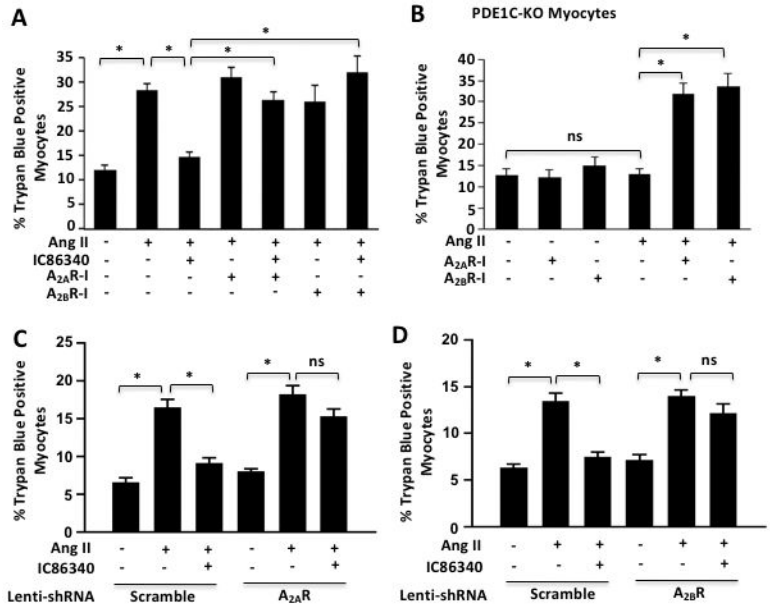

Figure 3. $A_{2A}R$ and $A_{2B}R$ activation are necessary for the protective effect of PDE1C inhibition and deficiency on cardiomyocyte death induced by Ang II

(A–B) Effects of $A_{2A}R$-I ($A_{2A}R$ antagonist ZM241385, 5 nmol/L) or $A_{2B}R$-I ($A_{2B}R$ antagonist, PSB603, 100 nmol/L) on PDE1C inhibition or deficiency against cardiomyocyte death. (C–D) Effects of depleting $A_{2A}R$ or $A_{2B}R$ via lentivirus-mediated shRNA expression combined with PDE1C inhibition against cardiomyocyte death. Cell death was detected by trypan blue staining. Data are presented as mean ± SEM. *p<0.05, ns, not significant by one-way ANOVA with Bonferroni's correction. A and B: n=6 replicates from 3 mice. C and D: n=5 replicates from 3 mice.

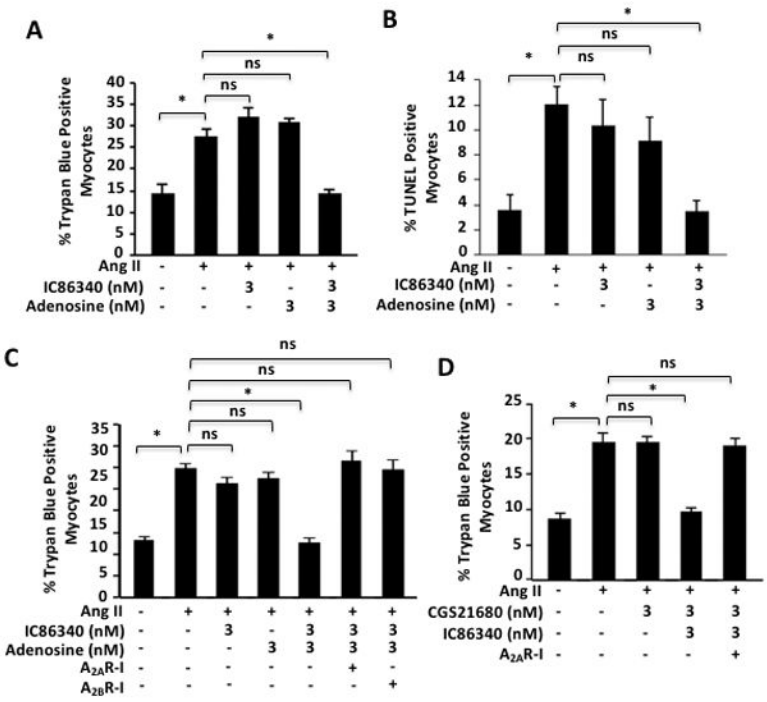

Figure 4. The synergistic effect of low dose PDE1 inhibitor and adenosine/A_{2A}R agonist on Ang II-induced cardiomyocyte death

(A–B) Cardiomyocytes from PDE1C-WT mice were stimulated with Ang II (200 nmol/L) in the presence of low dose of PDE1 inhibitor (IC86340, 3 nmol/L) and adenosine (3 nmol/L). Cell death was assessed 24 hours after treatment by Trypan blue staining (A), or 48 hours after treatment by TUNEL staining (B). (C) Cardiomyocytes from PDE1C-WT mice were stimulated with Ang II (200 nmol/L) in the presence of low dose of PDE1 inhibitor (IC86340, 3 nmol/L), adenosine (3 nmol/L), A_{2A}R-I (A_{2A}R antagonist, ZM241385, 5 nmol/L), A_{2B}R-I (A_{2B}R antagonist, PSB603, 100 nmol/L), or vehicle as indicated. (D) Cardiomyocytes from PDE1C-WT mice were stimulated with Ang II (200 nmol/L) in the presence of low dose of PDE1 inhibitor (IC86340, 3 nmol/L), CGS21680 (A_{2A}R agonist, 3 nmol/L), A_{2A}R-I (A_{2A}R antagonist, ZM241385, 5 nmol/L), or vehicle as indicated. Cell death was assessed 24 hours after treatment by Trypan blue staining. Data are presented as mean ± SEM. *$p<0.05$, ns, not significant by one-way ANOVA with Bonferroni's correction. n=6 replicates from 3 mice.

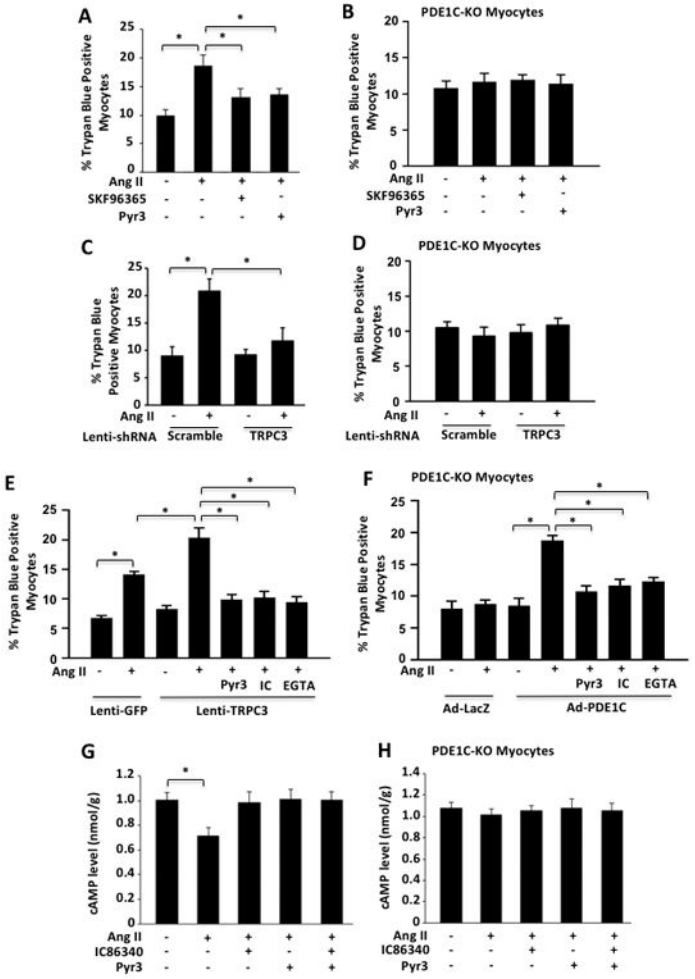

Figure 5. Role of TRPC3 in PDE1C-mediated regulation of cardiomyocyte death and cAMP reduction

(A–B) Cardiomyocytes from PDE1C-WT or PDE1C-KO mice were stimulated by Ang II (200 nmol/L) in the presence of TRPC pan inhibitor (SKF96365, 100 nmol/L), TRPC3 inhibitor (Pyr3, 100 nmol/L) or vehicle for 24 hours; (C–D) or treated with lentivirus expressing TRPC3 shRNA or scramble shRNA for 48 hours and followed by Ang II treatment for an additional 24 hours. Cell death was detected by trypan blue staining. (E–F) PDE1C-WT cardiomyocytes were treated with lentivirus expressing GFP or TRPC3 for 48 hours, or PDE1C-KO cardiomyocytes were treated with adenovirus expressing LacZ or PDE1C1 for 48 hours. Cells were then treated with Ang II (200 nmol/L), Pyr3 (100 nmol/L), IC86340 (15 μmol/L) or EGTA (1 μmol/L) as indicated for 24 hours. Cell death was detected by trypan blue staining. (G–H) Cardiomyocytes isolated from PDE1C-WT or PDE1C-KO mice were pretreated with IC86340, Pyr3, or vehicle for 30 min followed by Ang II stimulation for 15 min. Cells were then lysed and cAMP levels were measured via AlphaScreen assay. Data are presented as mean ± SEM. *$p<0.05$ by one-way ANOVA with Bonferroni's correction. A and C: n=6 replicates from 3 mice. B, D, E and F: n=5 replicates from 3 mice. G and H: n=3 replicates from 3 mice.

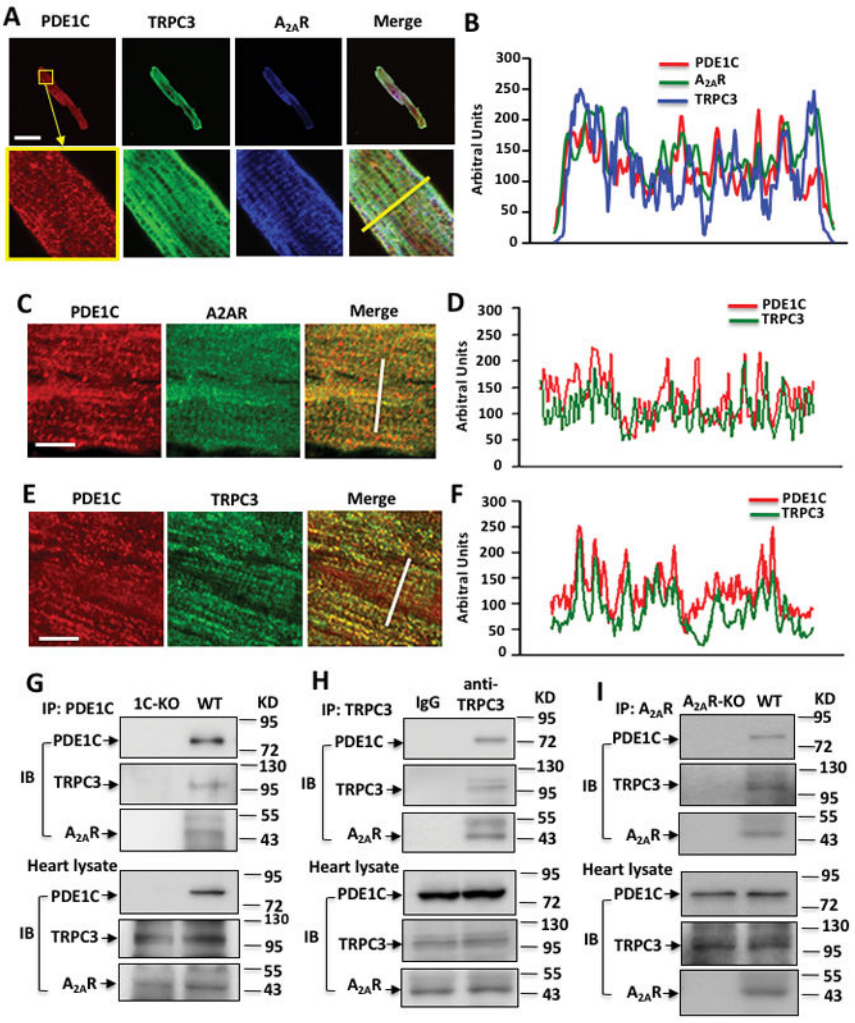

Figure 6. PDE1C, TRPC3, and $A_{2A}R$ form a complex in cardiomyocyte

(A) Representative images showing PDE1C, TRPC3 and $A_{2A}R$ colocalization in cardiomyocytes (red: PDE1C, green: TRPC3, blue: $A_{2A}R$). Scale bar 25 μm. (B) Line scanning analysis of PDE1C, TRPC3 and $A_{2A}R$ colocalization. (C, E) Representative image showing PDE1C and TRPC3 or PDE1C and $A_{2A}R$ colocalization mouse heart sections (red: PDE1C, green: TRPC3 or $A_{2A}R$). Scale bar 10μm. (D, F) Line scanning analysis of PDE1C/TRPC3 or PDE1C/$A_{2A}R$ colocalization in heart. (G–I) Co-immunoprecipitation assays with anti-PDE1C antibody in WT or PDE1C-KO heart lysates (G), with IgG or anti-TRPC3 antibody in WT heart lysates (H), or with anti-$A_{2A}R$ antibody in WT or $A_{2A}R$-KO heart lysates (I). The experiments were repeated for three times and similar results were obtained.

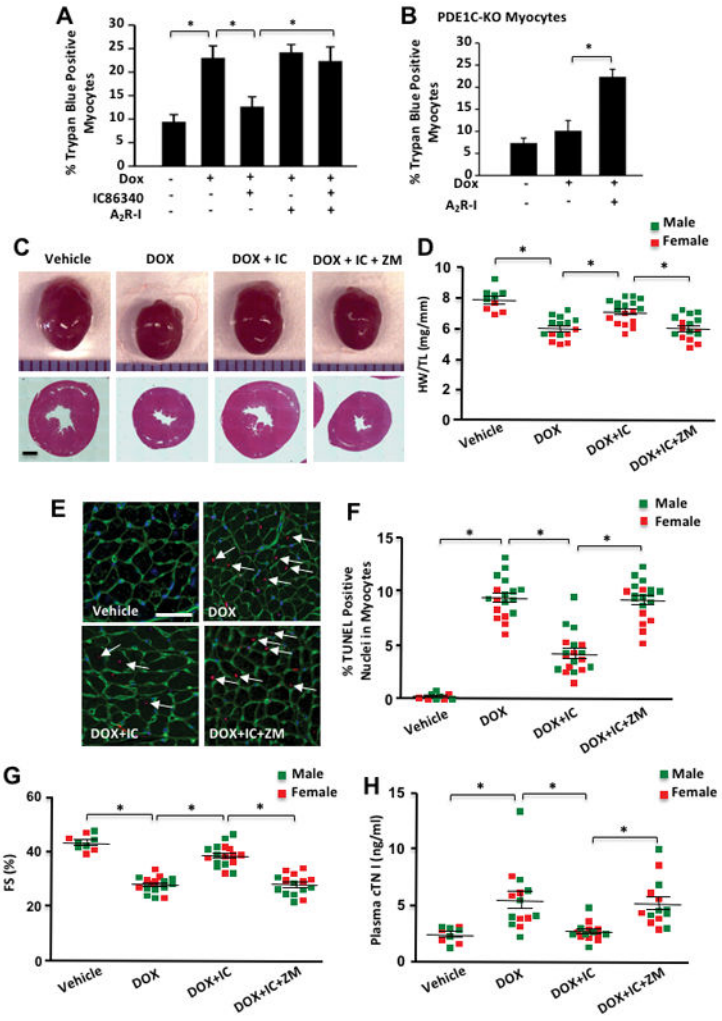

Figure 7. A_2R activation mediates the protective effect of PDE1 inhibition against doxorubicin-induced cardiomyocyte death and cardiac toxicity *in vitro* and *in vivo*

(A–B) Cell death was induced by doxorubicin (DOX, 1 μmol/L) in PDE1C-WT (A) or PDE1C-KO (B) cardiomyocytes in the presence of IC86340 (15 μmol/L), and/or A_2R-I (A_2R antagonist ZM241385 200 nmol/L). (C–H) Male or female C57BL/6J mice were treated with doxorubicin (DOX, 20 mg/kg, i.p.) in a single bolus via i.p. for 5 days and also treated with IC86340 (6 mg/kg/day), ZM241385 (10 mg/kg/day) and/or vehicle via i.p. 2 days before DOX and 5 days afterwards. (C) Representative images showing whole hearts and cross sections with H&E staining. Scale bars: 1 mm. (D) Quantification of heart weight/tibia length; (E) Representative image of TUNEL staining of heart sections. White arrows indicate TUNEL positive cardiomyocytes. Scale bars: 50 μm. (F) Quantification of TUNEL results. (G) Fractional shortening assessed by Echocardiogram at the time of termination. (H) Plasma troponin levels. Data are presented as mean ± SEM. *p<0.05 by one-way ANOVA with Bonferroni's correction. A and B: n=3 replicates from 3 mice. D,F, G, and H: n= 5–9 for male mice and n=4–9 for female mice.

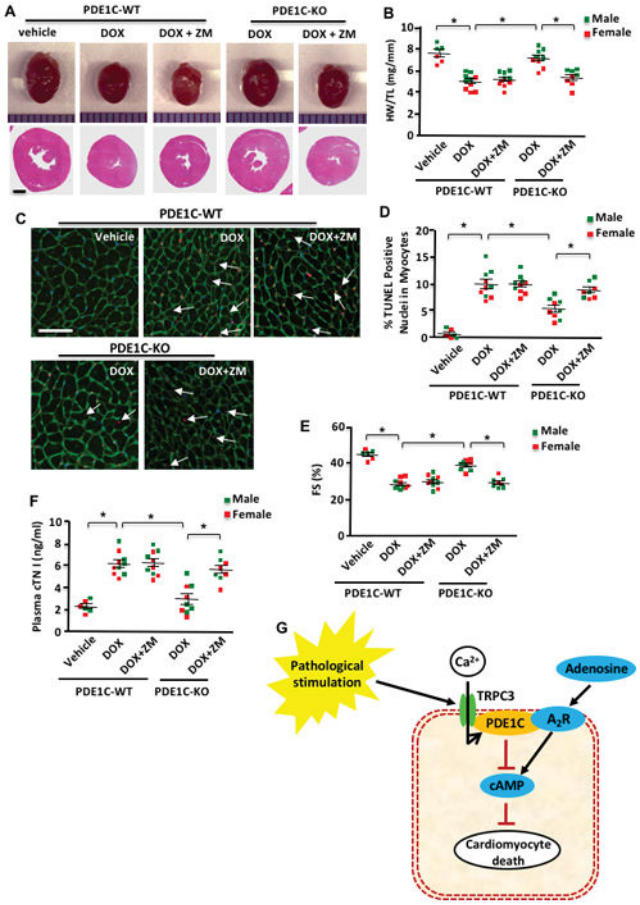

Figure 8. A_2R activation mediates the protective effect of PDE1C deficiency against doxorubicin-induced cardiac toxicity *in vivo*

Male or female PDE1C-WT or PDE1C-KO mice were treated with doxorubicin (DOX, 20 mg/kg, i.p.) in a single bolus via i.p., and also pretreated with ZM241385 (10 mg/kg/day) and/or vehicle via i.p. 2 days before DOX and continued for additional 5 days. (A) Representative images of whole hearts and cross sections with H&E staining. Scale bars: 1 mm. (B) Quantification of heart weight/tibia length. (C) Representative image of heart sections with TUNEL staining. White arrows point to TUNEL positive apoptotic cardiomyocytes. Scale bars: 50 μm. (D) Quantification of TUNEL staining results. (E) Fractional shortening assessed by echocardiography at the time of termination. (F) Plasma troponin I (cTnI) levels. Data are presented as mean ± SEM. *$p<0.05$ by one-way ANOVA with Bonferroni's correction. B, D, E, and F: n= 3–5 for male mice and n=3–5 for female mice. (G) Proposed model. Pathological stimuli, such as Ang II or DOX, activate TRPC3 and result in Ca^{2+} influx. This serves to activate PDE1C and subsequently increases the degradation of cAMP derived from A_2R activation, which promotes cell death.