

RESEARCH ARTICLE

# Inhibition of cAMP-Dependent PKA Activates $\beta_2$ -Adrenergic Receptor Stimulation of Cytosolic Phospholipase A<sub>2</sub> via Raf-1/MEK/ERK and IP<sub>3</sub>-Dependent Ca<sup>2+</sup> Signaling in Atrial Myocytes

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## Abstract

We previously reported in atrial myocytes that inhibition of cAMP-dependent protein kinase (PKA) by laminin (LMN)-integrin signaling activates  $\beta_2$ -adrenergic receptor ( $\beta_2$ -AR) stimulation of cytosolic phospholipase A<sub>2</sub> (cPLA<sub>2</sub>). The present study sought to determine the signaling mechanisms by which inhibition of PKA activates  $\beta_2$ -AR stimulation of cPLA<sub>2</sub>. We therefore determined the effects of zinterol (0.1  $\mu$ M; zint- $\beta_2$ -AR) to stimulate I<sub>Ca,L</sub> in atrial myocytes in the absence (+PKA) and presence (-PKA) of the PKA inhibitor (1  $\mu$ M) KT5720 and compared these results with atrial myocytes attached to laminin (+LMN). Inhibition of Raf-1 (10  $\mu$ M GW5074), phospholipase C (PLC; 0.5  $\mu$ M edelfosine), PKC (4  $\mu$ M chelerythrine) or IP<sub>3</sub> receptor (IP<sub>3</sub>R) signaling (2  $\mu$ M 2-APB) significantly inhibited zint- $\beta_2$ -AR stimulation of I<sub>Ca,L</sub> in -PKA but not +PKA myocytes. Western blots showed that zint- $\beta_2$ -AR stimulation increased ERK1/2 phosphorylation in -PKA compared to +PKA myocytes. Adenoviral (Adv) expression of dominant negative (dn) -PKC $\alpha$ , dn-Raf-1 or an IP<sub>3</sub> affinity trap, each inhibited zint- $\beta_2$ -AR stimulation of I<sub>Ca,L</sub> in +LMN myocytes compared to control +LMN myocytes infected with Adv- $\beta$ gal. In +LMN myocytes, zint- $\beta_2$ -AR stimulation of I<sub>Ca,L</sub> was enhanced by adenoviral overexpression of wild-type cPLA<sub>2</sub> and inhibited by double dn-cPLA<sub>2</sub><sup>S505A/S515A</sup> mutant compared to control +LMN myocytes infected with Adv- $\beta$ gal. In -PKA myocytes depletion of intracellular Ca<sup>2+</sup> stores by 5  $\mu$ M thapsigargin failed to inhibit zint- $\beta_2$ -AR stimulation of I<sub>Ca,L</sub> via cPLA<sub>2</sub>. However, disruption of caveolae formation by 10 mM methyl- $\beta$ -cyclodextrin inhibited zint- $\beta_2$ -AR stimulation of I<sub>Ca,L</sub> in -PKA myocytes significantly more than in +PKA myocytes. We conclude that inhibition of PKA removes inhibition of Raf-1 and thereby allows  $\beta_2$ -AR stimulation to act via PKC $\alpha$ /Raf-1/MEK/ERK1/2 and IP<sub>3</sub>-mediated Ca<sup>2+</sup> signaling to stimulate cPLA<sub>2</sub> signaling within caveolae. These findings may be relevant to the remodeling of  $\beta$ -AR signaling in failing and/or aging heart, both of which exhibit decreases in adenylate cyclase activity.

**Abbreviations:** LMN, laminin; cPLA<sub>2</sub>, cytosolic phospholipase A<sub>2</sub>; AA, arachidonic acid; ERK, extracellular-signal regulated kinase; MEK, mitogen-activated protein kinase kinase; Raf-1 (also known as c-Raf), MAP kinase kinase kinase; PLC, phospholipase C; FAK, focal adhesion kinase; PI-(3)K, phosphatidylinositol-3' kinase; Akt, protein kinase B; zint- $\beta_2$ -AR, zinterol  $\beta_2$ -adrenergic receptor;  $I_{Ca,L}$ , L-type Ca<sup>2+</sup> current; Adv, adenovirus; dn, dominant negative.

## Introduction

We previously reported that attachment of atrial myocytes to the extracellular matrix protein laminin (LMN) acts via  $\beta_1$  integrin receptors to decrease  $\beta_1$ -adrenergic receptor (AR) and increase  $\beta_2$ -AR stimulation of L-type Ca<sup>2+</sup> current ( $I_{Ca,L}$ ) [1]. Cell attachment to LMN decreases  $\beta_1$ -AR signaling by inhibiting adenylate cyclase activity and diminishing cAMP levels via integrin-dependent activation of focal adhesion kinase (FAK)/phosphatidylinositol-3' kinase (PI-3K)/protein kinase B (Akt) signaling [2]. We also reported that atrial cell attachment to LMN enhances  $\beta_2$ -AR signaling by activating Gi/ERK/cytosolic phospholipase A<sub>2</sub> (cPLA<sub>2</sub>)/arachidonic acid (AA) stimulation of  $I_{Ca,L}$  [3].  $\beta_2$ -AR activation of cPLA<sub>2</sub> signaling is dependent on concomitant LMN-mediated inhibition of adenylate cyclase/cAMP-dependent kinase (PKA) [3]. In other words, cell attachment to LMN acts via inhibition of adenylate cyclase/PKA to both inhibit  $\beta_1$ -AR signaling and enhance  $\beta_2$ -AR signaling through activation of cPLA<sub>2</sub>. In embryonic chick ventricular myocytes [4] and rat ventricular myocytes [5]  $\beta_2$ -AR stimulation also activates cPLA<sub>2</sub>/AA signaling. Moreover, these authors proposed that activation of  $\beta_2$ -AR/cPLA<sub>2</sub> signaling may compensate for depressed cAMP signaling [4]. Interestingly, in both of these studies by Pavoine et al. (1999) and Ait-Mamar et al., (2005) cardiomyocytes were cultured on LMN, supporting our findings that cell attachment to LMN may be responsible for inhibition of PKA and activation of  $\beta_2$ -AR/cPLA<sub>2</sub> signaling. However, the mechanism by which PKA inhibition activates  $\beta_2$ -AR/cPLA<sub>2</sub> signaling is not clear.

Our initial experiments indicated that in atrial myocytes  $\beta_2$ -AR activation of cPLA<sub>2</sub> is Ca<sup>2+</sup>-dependent and mediated via ERK1/2 signaling [3]. This is consistent with studies in embryonic chick ventricular myocytes (cultured on LMN) in which  $\beta_2$ -AR stimulation acts via ERK1/2 signaling to activate cPLA<sub>2</sub> [6]. Moreover, in a variety of cell systems Raf-1 activates downstream ERK1/2 and PKA inhibits Raf-1 [7, 8]. Therefore, inhibition of PKA should remove inhibition of Raf-1, thereby allowing  $\beta_2$ -AR stimulation to act via Raf-1/MEK/ERK1/2 signaling. Moreover, protein kinase C (PKC) activates Raf-1 [9, 10]. In other words, PKA inhibits and PKC activates Raf-1/MEK/ERK1/2 signaling. Based on these considerations we sought to determine whether inhibition of PKA facilitates  $\beta_2$ -AR stimulation to act via PKC/Raf-1/MEK/ERK1/2 to activate cPLA<sub>2</sub>. These findings may be relevant to the remodeling of  $\beta_2$ -AR signaling in the failing and/or aging heart, both of which exhibit decreases in adenylate cyclase activity.

## Materials and Methods

### Ethics Statement

The animal and experimental protocols used in this study were approved by the Institutional Animal Care and Use Committee (IACUC) of Loyola University Medical Center, Maywood, IL. IACUC prescribed the rules for the animal care and supervised their enforcement. Animals were obtained from a licensed vendor (R & R Research, Howard City, MI., USA), and housed and fed in our AAALAC approved Comparative Medicine Department. Adult cats of either sex (n = 32 cats) were anesthetized with sodium pentobarbital (50 mg/kg, IP).

### Isolation of atrial myocytes

Once fully anesthetized, a bilateral thoracotomy was performed, and the heart was rapidly excised and mounted on a Langendorff perfusion apparatus. After enzyme (collagenase; type II, Worthington Biochemical) digestion, atrial myocytes were isolated as previously reported [11].

## Perforated patch clamp experiments

Electrophysiological recordings from atrial myocytes were performed in the perforated (nystatin) patch whole-cell configuration at room temperature, as previously described [11]. L-type  $\text{Ca}^{2+}$  current ( $I_{\text{Ca,L}}$ ) was activated by depolarizing pulses from a holding potential of -40 mV to 0 mV for 200 ms every 5 s and measured in relation to steady-state current.  $\beta_2$ -AR stimulation was achieved by 0.1  $\mu\text{M}$  zinterol (zint- $\beta_2$ -AR), a specific  $\beta_2$ -AR agonist [12]. Agonist was applied for approximately 4 min and the effects on peak  $I_{\text{Ca,L}}$  amplitude were recorded at the steady-state response.

## Plating of atrial myocytes on LMN coated glass coverslips

Generally, we compared freshly isolated atrial myocytes obtained from the same hearts, as previously described [3]; atrial myocytes on uncoated glass coverslips in the absence of PKA inhibitor (+PKA) and atrial myocytes on uncoated glass coverslips exposed to the specific PKA inhibitor (1  $\mu\text{M}$ ) KT5720 (-PKA). Because pharmacological inhibition of PKA elicits signaling mechanisms that are similar to those elicited by LMN-integrin signaling, we performed some experiments on atrial myocytes attached to glass coverslips coated with laminin (+LMN; 40  $\mu\text{g/ml}$ ) for at least 2 hrs, as previously described [11]. Inhibition of PKA by KT5720 typically decreases basal  $I_{\text{Ca,L}}$  amplitude by 15–20% [3], consistent with the relatively high endogenous PKA activity in cat atrial myocytes [11]. In addition, a variety of experimental results indicate that atrial cell attachment to LMN is not restoring LMN-mediated signaling somehow lost during the cell isolation procedure. For example, control experiments have shown that atrial myocytes plated on poly-L-lysine, a non-specific substrate for cell attachment, fail to exhibit changes in  $\beta$ -AR signaling similar to cells attached to LMN [1]. Moreover, freshly isolated cardiomyocytes not plated on LMN exhibit responses to  $\beta$ -AR stimulation which are similar to multicellular cardiac preparations, i.e. exhibit predominantly  $\beta_1$ -AR over  $\beta_2$ -AR signaling [1]. However, cell attachment to LMN decreases the  $\beta_1$ -/ $\beta_2$ -AR signaling ratio resulting in predominantly  $\beta_2$ -AR over  $\beta_1$ -AR signaling [1]. Moreover, pharmacological inhibition of PKA in myocytes not attached to LMN mimics the effects of cell attachment to LMN [3].

## Adenoviral infection of atrial myocytes

In some experiments, atrial myocytes were attached to laminin (2h) and then infected (100 moi, 24h) with replication-defective adenovirus (Adv) prior to electrophysiological recording. PKC $\alpha$  was inhibited by infection with an Adv expressing kinase-inactive mouse PKC $\alpha$  [13], kindly provided by Dr. Trevor Biden, Garvan Institute of Medical Research, St. Vincent's Hospital, Sydney Australia. An Adv expressing a dominant-negative (dn) mutant of rabbit PKC $\epsilon$  [14] was kindly provided by Dr. Peipei Ping, University of California-Los Angeles. Adv expressing wild type, and a non-phosphorylatable mutant of human cPLA<sub>2</sub> (S515A/S505A double mutant) [15] were generously provided by Dr. K.U. Malik, University of Tennessee Health Science Center, Memphis, TN. An Adv expressing a dn mutant of human Raf-1 [9] was kindly provided by Dr. Dan Kuppaswamy, Medical University of South Carolina, Charleston, SC. A control Adv expressing nuclear-encoded  $\beta$ -galactosidase (Adv- $\beta\text{gal}$ ) was used to control for nonspecific effects of adenoviral infection [16]. Adenoviruses were amplified and purified using HEK293 cells, and the multiplicity of infection (moi) for each virus was determined by dilution assay in HEK293 cells grown in 96 well clusters, as previously described [16]. Preliminary experiments using 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal) staining of Adv- $\beta\text{gal}$  infected cells determined that a concentration of 100 moi infected  $93 \pm 3\%$  ( $n = 3$  expts, 400–700 cells/expt) of cultured myocytes. The  $\text{IP}_3$  affinity trap consists of the ligand binding domain of the rat type 1  $\text{IP}_3\text{R}$ . The construction of this vector and subsequent

production of adenovirus was previously described in detail [17]. Freshly isolated atrial myocytes were infected with Adv-IP<sub>3</sub> affinity trap or the Adv- $\beta$ gal (control) for 1 h, followed by 18 h short-term culture at 37°C.

## Western blot

Atrial myocytes were plated on poly-L-lysine, a biologically inactive substrate and therefore represent +PKA myocytes. Atrial myocytes were either untreated or treated with Zinterol, KT5720 alone (PKA inhibitor) and KT combined with Zinterol. A positive control showing ERK1/2 and p38MAPK phosphorylation in A7r5 cells is used after stimulation with 1  $\mu$ M angiotensin II (5 min). Briefly atrial homogenates were centrifuged at 10,000 g for 2 min and the supernatant was collected. The protein concentrations of the samples were determined using the Bradford protein assay (Bio-Rad). Aliquots of the samples (40  $\mu$ g) were dissolved in a Laemmli Sample buffer containing: 60 mM Tris-HCl, 2% SDS, 20% Glycerol, 5%  $\beta$ -mercaptoethanol, 0.01% bromophenol blue (pH 6.8) and the proteins were separated on a 4–15% Mini-PROTEAN TGX Gel (Bio-Rad). After transfer, the membrane was blocked with TBS-T Buffer containing 20 mM Tris pH 7.5, 150 mM NaCl, 0.05% Tween, and a 5% blocking powder (Bio Rad) at 4°C for 1 hr. The blot was probed with primary and secondary antibodies for phospho-ERK1/2 and phosphor-p38<sup>MAPK</sup> that were purchased from cell signaling technologies (Danvers, MA). The blot was subsequently probed with GAPDH primary antibody for 2 hrs, followed by HRP-conjugated goat anti-mouse secondary antibody (1:10,000, Santa Cruz Biotechnology) at 4°C for 1 hr. Specific binding was visualized by chemiluminescence (Immun-Star Western C Kit, Bio-Rad) using a ChemiDoc XRS imager (Bio-Rad Life Science Research, Hercules, CA). The intensities of the bands corresponding to each protein were quantified using ImageLab software (Bio-Rad). The relative intensity for each band was normalized to the intensity of the GAPDH staining.

## Chemicals

Zinterol, AACOCF<sub>3</sub>, GW5074, edelfosine (ET-18-OCH<sub>3</sub>), chelerythrine, 2-aminoethyl diphenyl borate (2-APB), KT5720, methyl- $\beta$ -cyclodextrin, ryanodine, thapsigargin (Sigma Chemical).

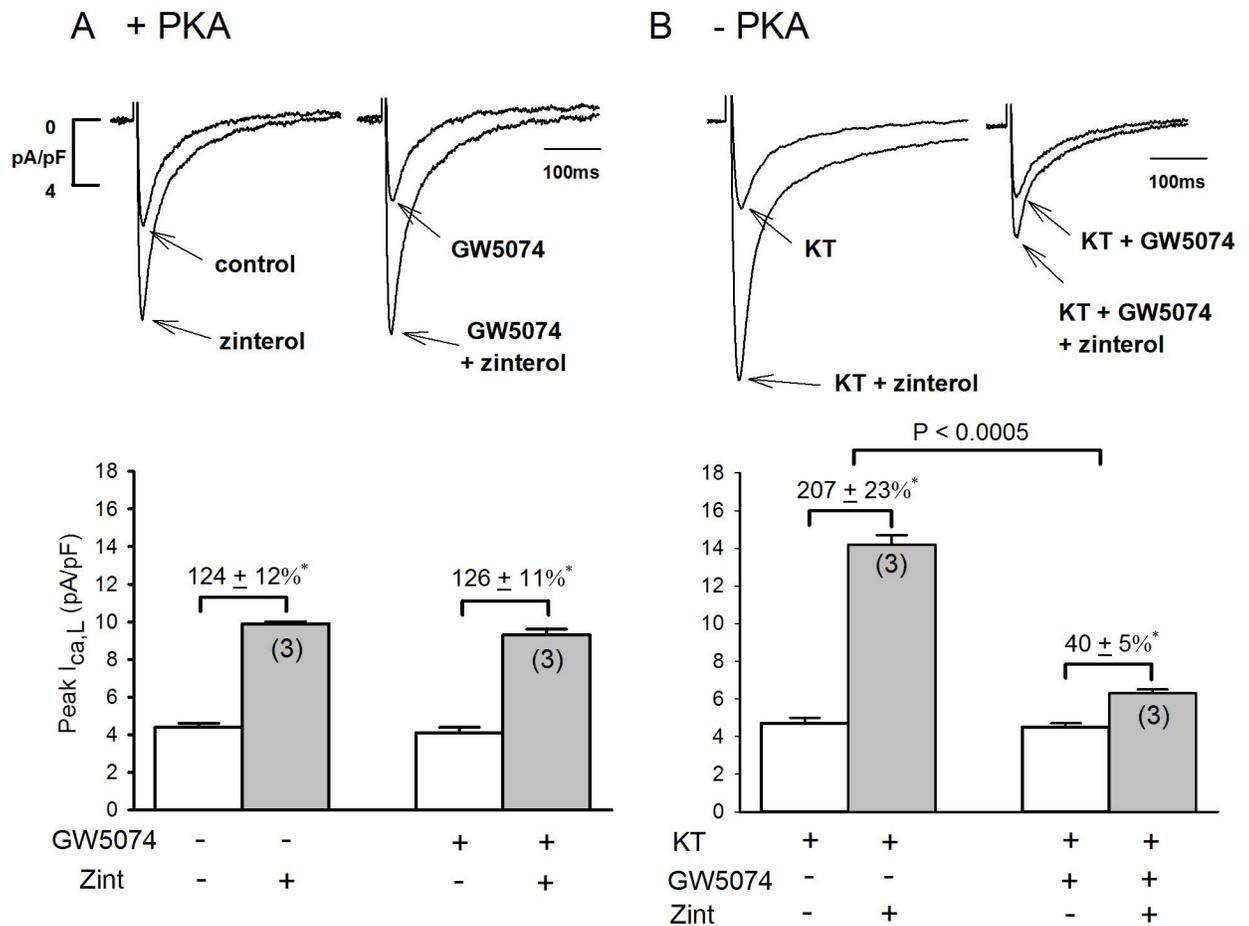
## Statistics

Data are mean  $\pm$  standard error (SE) of the mean. Measurements were analyzed using either paired or unpaired Student's *t* test for significance at  $P < 0.05$ . Multiple comparisons were performed by ANOVA followed by a Student–Newman–Keuls test with significance at  $P < 0.05$ .

## Results

### Role of Raf-1 in +PKA and –PKA atrial myocytes

As shown in Fig 1 we determined the effects of 10  $\mu$ M GW5074, a potent Raf-1 inhibitor [8] on zint- $\beta_2$ -AR stimulation of  $I_{Ca,L}$  in +PKA and –PKA atrial myocytes. In +PKA myocytes (A) 0.1  $\mu$ M zint- $\beta_2$ -AR stimulation elicited a typical increase in  $I_{Ca,L}$  ( $124 \pm 12\%$ ,  $N = 3$ ). In another group of +PKA myocytes from the same hearts (A), prior exposure to GW5074 for 30 min had no significant effects on basal  $I_{Ca,L}$  amplitude (open bar) or zint- $\beta_2$ -AR stimulation of  $I_{Ca,L}$  ( $126 \pm 11\%$ ,  $N = 3$ ). In contrast, as shown in panel B, control zint- $\beta_2$ -AR stimulation of  $I_{Ca,L}$  in –PKA myocytes (B;  $207 \pm 23\%$ ,  $N = 3$ ) was enhanced compared to +PKA myocytes (A;  $124 \pm 12\%$ ,  $N = 3$ ) due to activation of cPLA<sub>2</sub> signaling, as previously reported [4]. In –PKA myocytes, GW5074 had no effects on basal  $I_{Ca,L}$  amplitudes (open bars) but in contrast to +PKA myocytes, GW5074 significantly inhibited zint- $\beta_2$ -AR stimulation of  $I_{Ca,L}$  (B;  $40 \pm 5\%$ ,  $N = 3$ ).



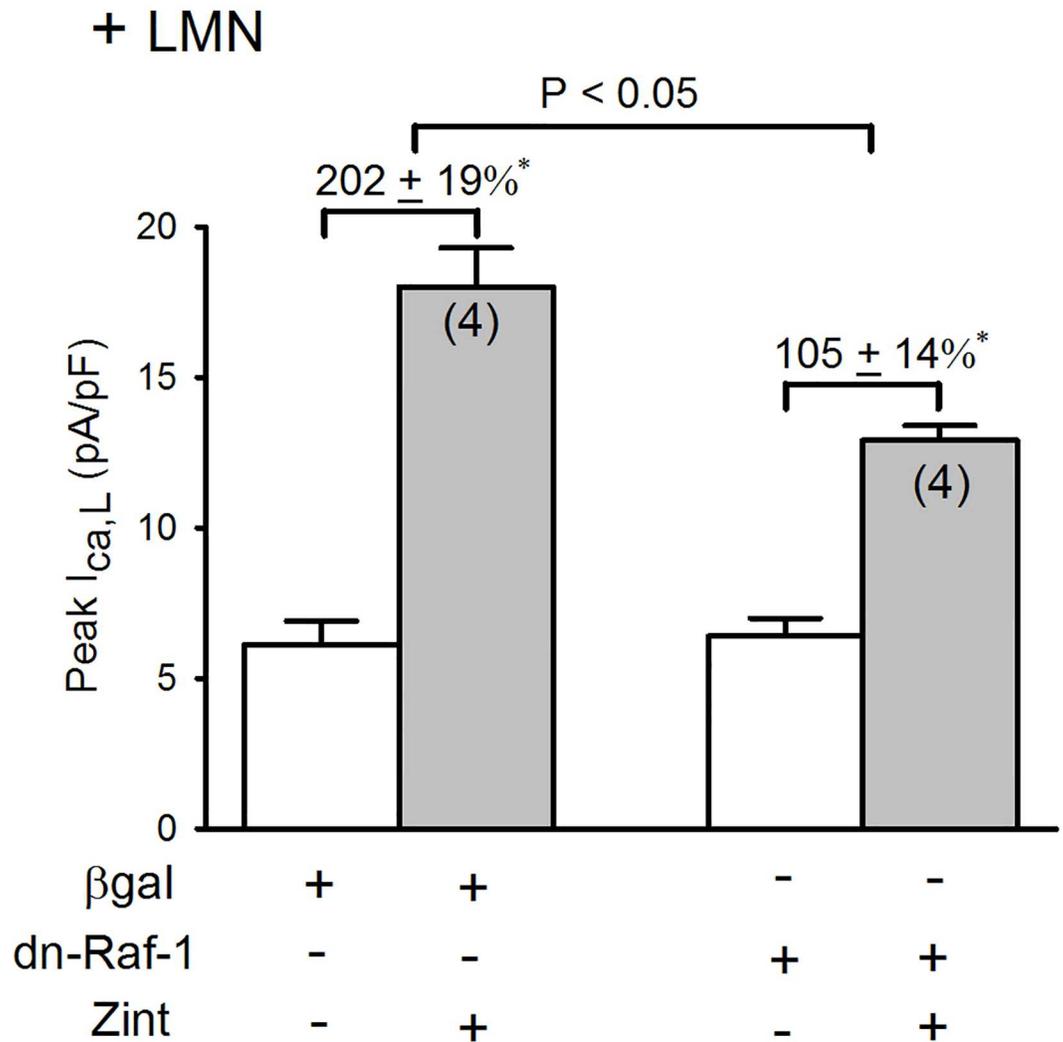
**Fig 1. Effects of Raf-1 inhibition (10  $\mu$ M GW5074) on zint- $\beta_2$ -AR stimulation of  $I_{Ca,L}$  in +PKA (A), -PKA (B) atrial myocytes.** A; In +PKA myocytes, GW5074 (30 min) had no significant effect on zint- $\beta_2$ -AR stimulation of  $I_{Ca,L}$ . B; In -PKA myocytes, zint- $\beta_2$ -AR stimulation of  $I_{Ca,L}$  was enhanced compared to control responses (A) and GW5074 significantly inhibited zint- $\beta_2$ -AR stimulation of  $I_{Ca,L}$ . Numbers in parentheses indicate the number of myocytes studied. \* =  $P < 0.05$ .

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Additional experiments showed that zint- $\beta_2$ -AR stimulation of  $I_{Ca,L}$  in +LMN myocytes produced results similar to those found in -PKA myocytes (control;  $192 \pm 13\%$ ,  $N = 3$ ) and (GW5074;  $33 \pm 9\%$ ,  $N = 3$ ) (see S1 Fig for data).

### Comparison of zint- $\beta_2$ -AR induced $I_{Ca,L}$ currents in the Adv- $\beta$ gal and dn-Raf-1 mutant infected atrial myocytes

To further establish the role of Raf-1, we infected atrial myocytes with an Adv that expresses a dn-Raf-1 mutant (generously provided by Dr. Kuppuswamy [9, 18]). Control cells were infected with Adv- $\beta$ gal. Infected cells were cultured on LMN overnight and therefore represent +LMN myocytes. As shown in Fig 2A, in control +LMN myocytes expressing  $\beta$ gal, zint- $\beta_2$ -AR stimulation elicited a typically enhanced increase in  $I_{Ca,L}$  ( $202 \pm 19\%$ ). In +LMN myocytes expressing the dn-Raf-1 mutant, zint- $\beta_2$ -AR stimulation of  $I_{Ca,L}$  was significantly inhibited ( $105 \pm 14\%$ ;  $P < 0.02$ ) compared to control. Together, these findings indicate that Raf-1 signaling plays no role in  $\beta_2$ -AR stimulation of  $I_{Ca,L}$  in freshly isolated atrial myocytes not attached to LMN (Fig 1A). However, when PKA is inhibited by either PKA inhibitor or cell attachment to LMN,  $\beta_2$ -AR stimulation acts via Raf-1 to activate cPLA<sub>2</sub> and stimulate  $I_{Ca,L}$ .

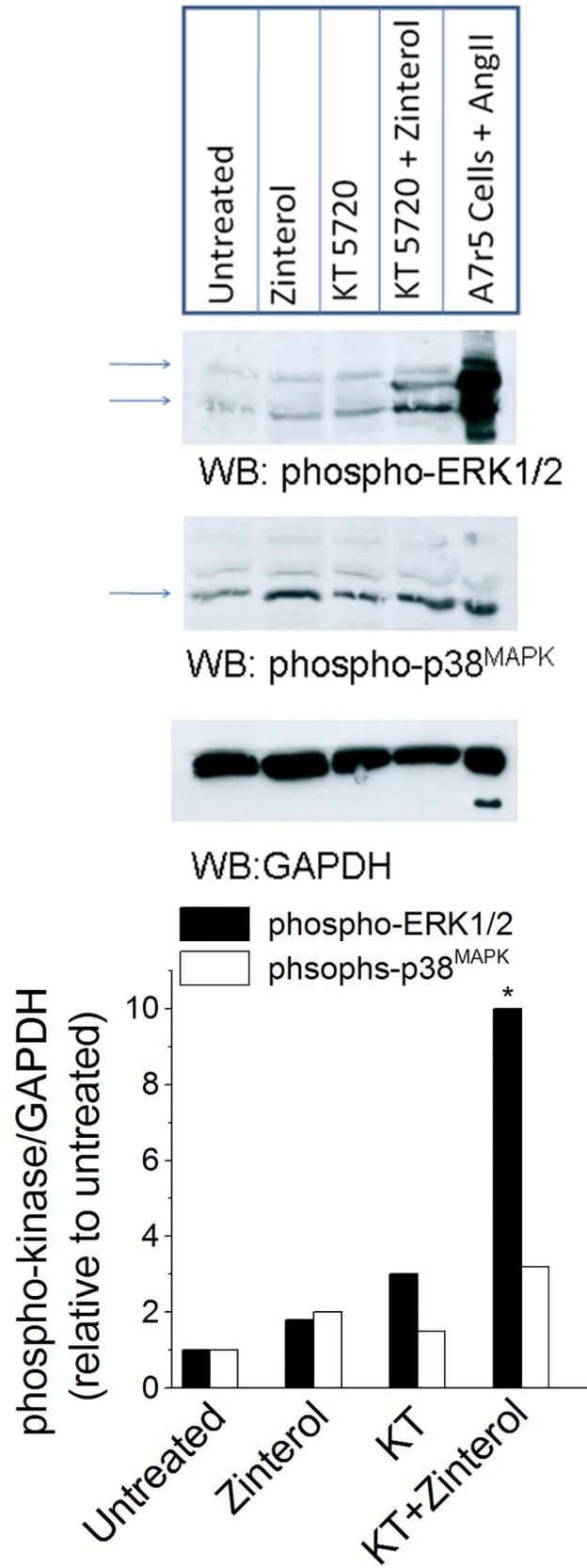


**Fig 2. Effects of dn-Raf-1-Adv on zint- $\beta_2$ -AR stimulation of  $I_{Ca,L}$  in +LMN myocytes.** In control +LMN myocytes ( $\beta$ gal) zint- $\beta_2$ -AR stimulation elicited a typically enhanced increase in  $I_{Ca,L}$ . In +LMN myocytes infected with dn-Raf-1-Adv zint- $\beta_2$ -AR stimulation of  $I_{Ca,L}$  was significantly inhibited compared with controls. Numbers in parentheses indicate that number of cells studied. \* =  $P < 0.05$ .

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### Inhibition of PKA, increases ERK1/2 phosphorylation in atrial myocytes

It is well established that Raf-1 activates downstream MEK/ERK1/2 signaling [7, 8]. Moreover, our previous results indicated that inhibition of MEK/ERK1/2 signaling by U0126 inhibited zint- $\beta_2$ -AR stimulation cPLA<sub>2</sub> signaling [3]. Therefore, inhibition of PKA should activate  $\beta_2$ -AR stimulation of ERK1/2 phosphorylation. As shown in Fig 3, we performed Western blots and probed for zint- $\beta_2$ -AR-mediated ERK1/2 and p38<sup>MAPK</sup> phosphorylation in the absence (+PKA) and presence of PKA inhibitor (-PKA). Atrial myocytes were plated on poly-L-lysine, a biologically inactive substrate and therefore represent +PKA myocytes. As shown in Fig 3 in control +PKA myocytes zint- $\beta_2$ -AR stimulation modestly increased ERK1/2 phosphorylation (solid bars). Exposure of +PKA myocytes to the PKA inhibitor (1  $\mu$ M) KT5720, (-PKA



**Fig 3. Effect of PKA inhibition on  $\beta_2$ -AR-mediated phosphorylation of ERK1/2.** Western blots of atrial cell homogenates show that in control -PKA myocytes zinterol modestly increased ERK1/2 phosphorylation (solid bars). Inhibition of PKA by 1  $\mu$ M KT5720 (KT) increased basal ERK1/2 phosphorylation. In the presence of KT5720, zinterol prominently stimulates ERK1/2 phosphorylation. Zinterol only modestly stimulated p38MAPK phosphorylation (open bars), and without significant effect by KT5720. The last lane on the Western blot is a positive control showing ERK1/2 and p38MAPK phosphorylation in A7r5 cells stimulated by 1  $\mu$ M angiotensin II (5 min). Similar results were obtained in a total of 3 experiments. \* =  $P < 0.05$ .

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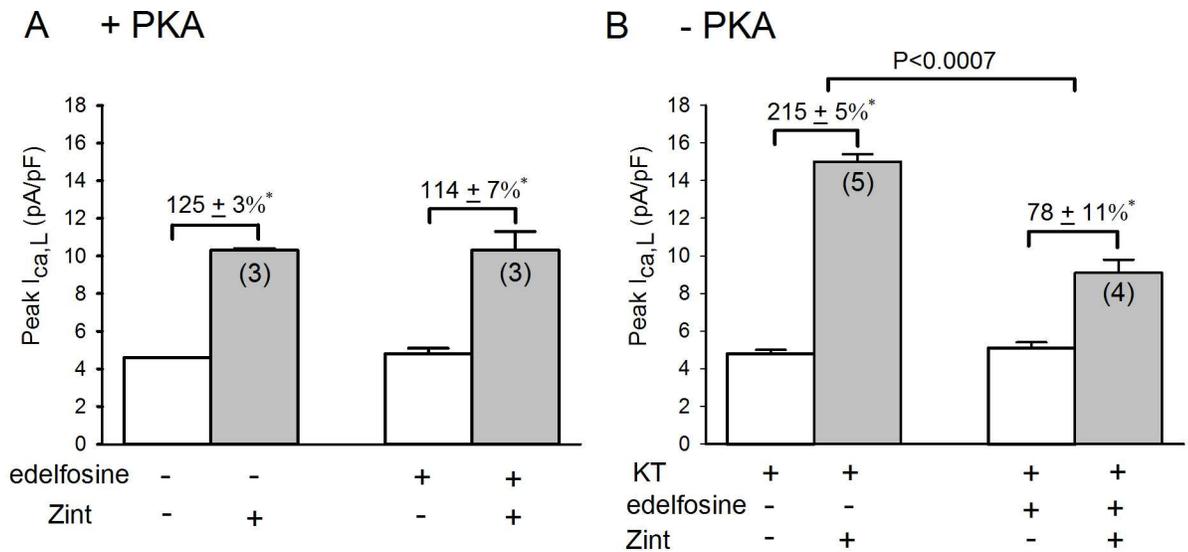
myocytes) further increased ERK1/2 phosphorylation, suggesting that inhibition of basal PKA activity removes PKA-induced inhibition of basal ERK1/2 phosphorylation. In -PKA myocytes, zint- $\beta_2$ -AR stimulation prominently stimulated ERK1/2 phosphorylation. Moreover, zint- $\beta_2$ -AR stimulation only modestly stimulated p38<sup>MAPK</sup> phosphorylation (open bars), and was not significantly affected by KT5720. The last lane on the Western blot is a positive control showing ERK1/2 and p38<sup>MAPK</sup> phosphorylation in A7r5 cells stimulated by 1  $\mu$ M angiotensin II (5 min). In conjunction with our previous findings [3], the present results indicate that inhibition of PKA preferentially activates  $\beta_2$ -AR stimulation of ERK1/2 signaling.

### Role of PLC in the enhanced zint- $\beta_2$ -AR induced $I_{Ca,L}$ currents in -PKA atrial myocytes

It is well established that Raf-1 activates downstream MEK/ERK1/2 signaling [7, 8]. Moreover, our previous results indicated that inhibition of MEK/ERK1/2 signaling by U0126 inhibited zint- $\beta_2$ -AR stimulation of cPLA<sub>2</sub> signaling [3]. This raises the question of how  $\beta_2$ -AR stimulation activates Raf-1. In a variety of cell systems [10], including cardiac muscle [9], protein kinase C (PKC) activates Raf-1/MEK/ERK1/2. Receptor-mediated activation of phospholipase C (PLC) and the hydrolysis of phospholipids results in the production of diacylglycerol and stimulation of PKC isoenzymes. Therefore, as shown in Fig 4 we determined the effects of 0.5  $\mu$ M edelfosine, a specific inhibitor of phospholipase C [19], on zint- $\beta_2$ -AR stimulation of  $I_{Ca,L}$  in +PKA and -PKA myocytes. In control cells (A), edelfosine had little effect on basal  $I_{Ca,L}$  amplitude (open bars) or zint- $\beta_2$ -AR stimulation of  $I_{Ca,L}$  (zint, 125 $\pm$ 3% vs edelfosine, 114 $\pm$ 7%). In -PKA myocytes (B) zint- $\beta_2$ -AR stimulation elicited a typically enhanced increase in  $I_{Ca,L}$  (215 $\pm$ 5%) compared to control responses (A) due to activation of cPLA<sub>2</sub> [3]. In contrast to +PKA myocytes, edelfosine now significantly inhibited zint- $\beta_2$ -AR stimulation of  $I_{Ca,L}$  (78 $\pm$ 11%;  $P < 0.0005$ ). Additional experiments in +LMN myocytes showed results similar to those found in -PKA myocytes, i.e. zint, 204 $\pm$ 2% vs edelfosine, 84 $\pm$ 8%;  $P < 0.03$  (see S2 Fig for data). In other words, inhibition of PKA allows  $\beta_2$ -AR stimulation to act via PLC to stimulate cPLA<sub>2</sub> signaling.

### Role of PKC in the enhanced zint- $\beta_2$ -AR induced $I_{Ca,L}$ currents in -PKA atrial myocytes

Because activation of PLC produces diacylglycerol and activation of PKC we next determined the effects of 4  $\mu$ M chelerythrine, an inhibitor of Ca<sup>2+</sup>-dependent and Ca<sup>2+</sup>-independent PKCs [20] on zint- $\beta_2$ -AR stimulation of  $I_{Ca,L}$  in +PKA and -PKA atrial myocytes. As shown in Fig 5, in +PKA myocytes (panel A), chelerythrine had no significant effects on basal  $I_{Ca,L}$  amplitude (open bars) or zint- $\beta_2$ -AR stimulation of  $I_{Ca,L}$  (control, 106 $\pm$ 8% vs cheler, 116 $\pm$ 3%). In -PKA myocytes (panel B) zint- $\beta_2$ -AR stimulation elicited a typically enhanced increase in  $I_{Ca,L}$  (278 $\pm$ 13%) compared to control (panel A) due to activation of cPLA<sub>2</sub> [3]. In contrast to +PKA myocytes, chelerythrine now significantly inhibited zint- $\beta_2$ -AR stimulation of  $I_{Ca,L}$  (71 $\pm$ 13%;  $P < 0.002$ ). These findings indicate PKC signaling plays no role in  $\beta_2$ -AR stimulation of  $I_{Ca,L}$  in freshly isolated atrial myocytes. However when PKA is inhibited  $\beta_2$ -AR stimulation acts via PKC to stimulate cPLA<sub>2</sub>, consistent with  $\beta_2$ -AR stimulation of PLC.



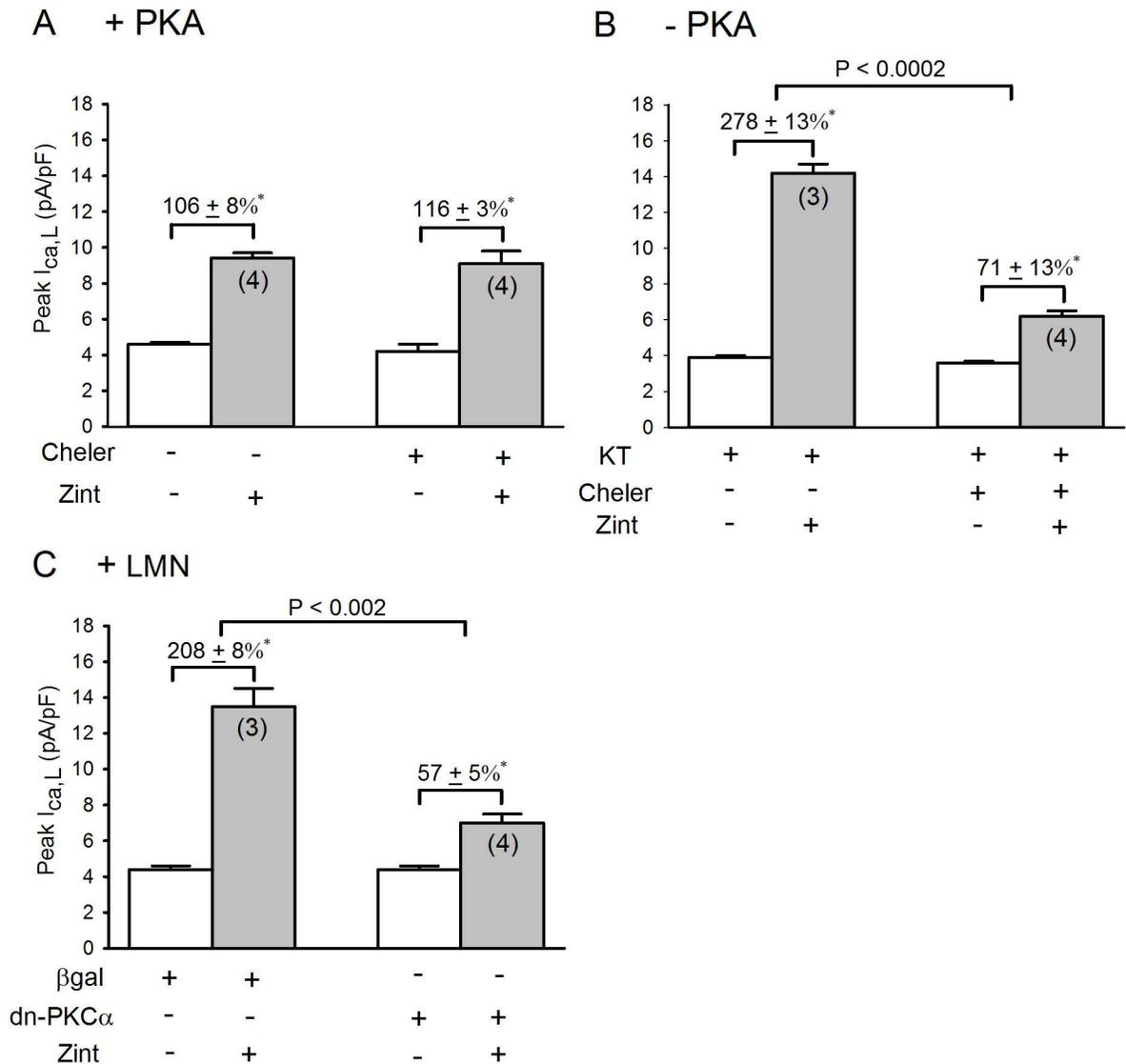
**Fig 4. Effects of 0.5  $\mu$ M edelfosine on zint- $\beta_2$ -AR stimulation of  $I_{Ca,L}$  in +PKA (A), -PKA(B) and atrial myocytes.** A; in control +PKA myocytes edelfosine had no significant effects on zint- $\beta_2$ -AR stimulation of  $I_{Ca,L}$ . B; in -PKA myocytes zint- $\beta_2$ -AR stimulation elicited a typically enhanced increase in  $I_{Ca,L}$  compared to controls (A) and edelfosine significantly inhibited zint- $\beta_2$ -AR stimulation of  $I_{Ca,L}$ . Numbers in parentheses indicate the number of myocytes studied. \* =  $P < 0.05$ .

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In another approach we infected atrial myocyte with an Adv expressing a dominant-negative mutant of  $Ca^{2+}$ -dependent PKC $\alpha$  (dn-PKC $\alpha$ ). Control cells were infected with Adv- $\beta$ gal. Infected cells were cultured overnight on LMN and therefore represent +LMN myocytes. As shown in Fig 5C, in control +LMN myocytes (Adv- $\beta$ gal) zint- $\beta_2$ -AR stimulation elicited a typically enhanced increase in  $I_{Ca,L}$  ( $208 \pm 8\%$ ) compared to +PKA myocytes (panel A). In +LMN myocytes expressing the dn-PKC $\alpha$  mutant, zint- $\beta_2$ -AR stimulation of  $I_{Ca,L}$  was significantly inhibited (control,  $208 \pm 8\%$  vs dn-PKC $\alpha$ ,  $57 \pm 5\%$ ;  $P < 0.002$ ). Similar experiments performed on +LMN myocytes expressing a dn-PKC $\epsilon$  mutant showed no differences with control +LMN ( $\beta$ -gal) myocytes (control,  $158 \pm 21\%$  vs dn-PKC $\epsilon$ ,  $165 \pm 31\%$ ,  $N = 4$ ). These findings provide additional support for the idea that  $\beta_2$ -AR stimulation acts via  $Ca^{2+}$ -dependent PKC $\alpha$  to activate cPLA<sub>2</sub>.

### Role of cPLA<sub>2</sub> in the enhanced zint- $\beta_2$ -AR induced $I_{Ca,L}$ currents in LMN plated atrial myocytes

Activation of cPLA<sub>2</sub> requires phosphorylation at serine sites S505 and S515 [21–23]. We therefore determined the effect of  $\beta_2$ -AR stimulation in +LMN myocytes infected with Adv that either expressed a non-phosphorylatable dominant-negative cPLA<sub>2</sub> (dn-cPLA<sub>2</sub><sup>S505A/S515A</sup>) mutant, wild-type cPLA<sub>2</sub> (wt-cPLA<sub>2</sub>) or  $\beta$ gal as control. Infected atrial myocytes were cultured on LMN overnight and therefore represent +LMN myocytes. Fig 6 shows that in control +LMN myocytes expressing  $\beta$ gal,  $\beta_2$ -AR stimulation elicited a typical increase in  $I_{Ca,L}$  ( $166 \pm 11\%$ ). In +LMN myocytes expressing the double dn-cPLA<sub>2</sub><sup>S505A/S515A</sup> mutant  $\beta_2$ -AR stimulation of  $I_{Ca,L}$  was significantly attenuated ( $84 \pm 8\%$ ) compared to control. In +LMN myocytes overexpressing wt-cPLA<sub>2</sub>,  $\beta_2$ -AR stimulation of  $I_{Ca,L}$  was significantly enhanced ( $307 \pm 8\%$ ) compared to either control or dn-cPLA<sub>2</sub><sup>S505A/S515A</sup>. These results confirm that cell attachment to LMN activates  $\beta_2$ -AR/cPLA<sub>2</sub> signaling and further indicates that  $\beta_2$ -AR stimulation of cPLA<sub>2</sub> requires serine phosphorylation at one or both sites.



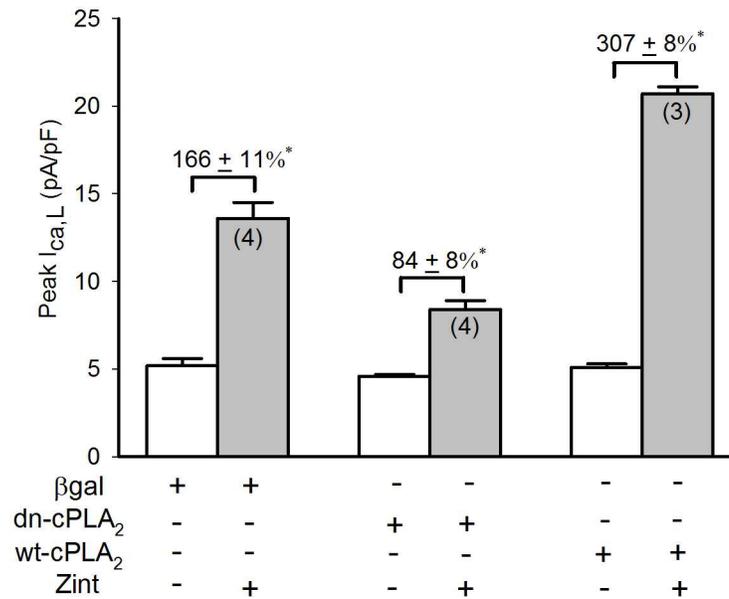
**Fig 5. Effects of 4  $\mu$ M chelerythrine (cheler) on zint- $\beta_2$ -AR stimulation of  $I_{Ca,L}$  in +PKA(A) and -PKA (B) myocytes and effects of dn-PKC $\alpha$ -Adv (C) on zint- $\beta_2$ -AR stimulation of  $I_{Ca,L}$  in +LMN myocytes.** A; in control +PKA myocytes, chelerythrine had no significant effects on zint- $\beta_2$ -AR stimulation of  $I_{Ca,L}$ . B; in -PKA myocytes, zint- $\beta_2$ -AR stimulation elicited a typically enhanced increase in  $I_{Ca,L}$  compared to controls (A) and chelerythrine significantly inhibited zint- $\beta_2$ -AR stimulation of  $I_{Ca,L}$ . C; in control +LMN myocytes ( $\beta$ gal) zint- $\beta_2$ -AR stimulation of  $I_{Ca,L}$  was typically enhanced compared to controls (A). In +LMN myocytes infected with dn-PKC $\alpha$ -Adv, zint- $\beta_2$ -AR stimulation of  $I_{Ca,L}$  was significantly inhibited. Numbers in parentheses indicate that number of cells studied. \* =  $P < 0.05$ .

doi:10.1371/journal.pone.0168505.g005

### Role of IP<sub>3</sub> receptor in the enhanced zint- $\beta_2$ -AR induced $I_{Ca,L}$ currents in -PKA atrial myocytes

Our previous work showed that  $\beta_2$ -AR stimulation of cPLA<sub>2</sub> signaling is dependent on intracellular  $Ca^{2+}$  [3]. The fact that  $\beta_2$ -AR stimulation acts via PLC to activate cPLA<sub>2</sub> (Fig 3) suggests the potential involvement of IP<sub>3</sub>-mediated  $Ca^{2+}$  signaling. We therefore determined the effects of 2  $\mu$ M 2-APB, a putative IP<sub>3</sub> receptor (IP<sub>3</sub>R) blocking agent [24], on  $\beta_2$ -AR stimulation of  $I_{Ca,L}$  in +PKA and -PKA myocytes. As shown in Fig 7A, in +PKA myocytes 2-APB had

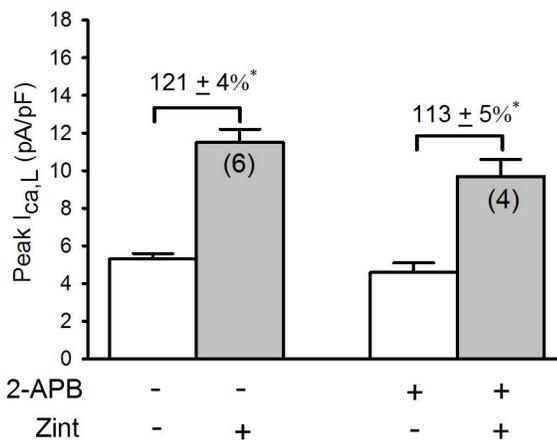
+ LMN



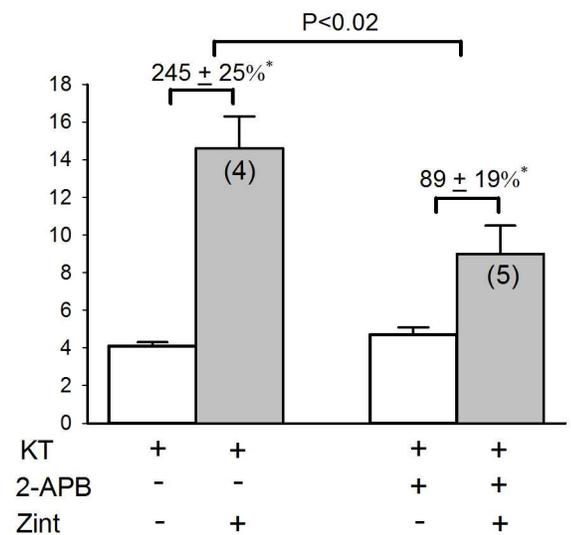
**Fig 6. Effects of dn-cPLA<sub>2</sub><sup>S515A/S505A</sup> and wt-cPLA<sub>2</sub> on zint-β<sub>2</sub>-AR stimulation of I<sub>Ca,L</sub> in +LMN myocytes.** Compared to control +LMN myocytes (βgal), zint-β<sub>2</sub>-AR stimulation of I<sub>Ca,L</sub> was significantly inhibited and enhanced in myocytes overexpressing dn-cPLA<sub>2</sub><sup>S515A/S505A</sup> and expressing wt-cPLA<sub>2</sub>, respectively. Numbers in parentheses indicate that number of cells studied. \* = P<0.05.

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A + PKA

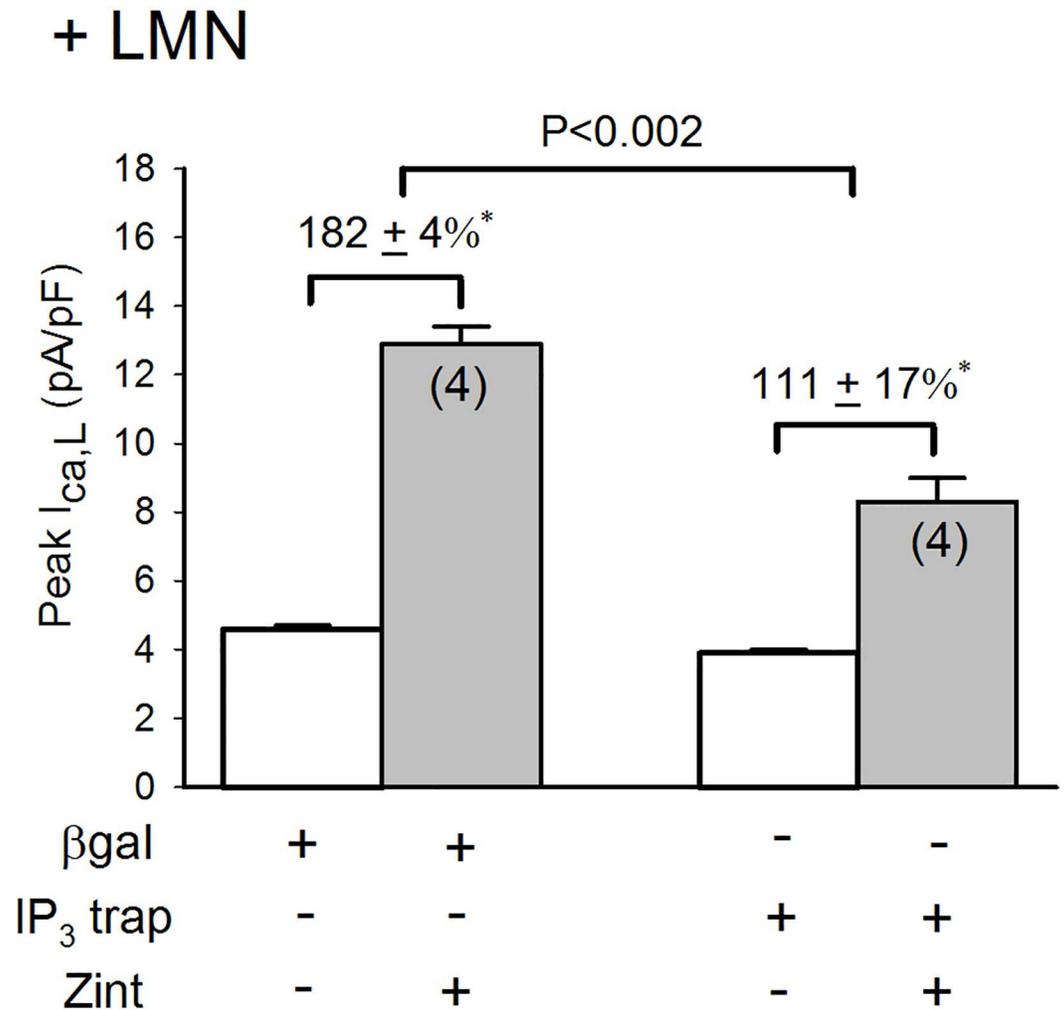


B - PKA



**Fig 7. Effects of 2 μM 2-APB on zint-β<sub>2</sub>-AR stimulation of I<sub>Ca,L</sub> in +PKA (A), -PKA atrial myocytes.** A; in control +PKA myocytes, 2-APB had no significant effects on zint-β<sub>2</sub>-AR stimulation of I<sub>Ca,L</sub>. B; in -PKA myocytes, zint-β<sub>2</sub>-AR stimulation elicited a typically enhanced increase in I<sub>Ca,L</sub> compared to controls (A) and 2-APB significantly inhibited zint-β<sub>2</sub>-AR stimulation of I<sub>Ca,L</sub>. Numbers in parentheses indicate the number of myocytes studied. \* = P<0.05.

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**Fig 8. Effect of adenovirus IP<sub>3</sub> affinity trap on zint- $\beta_2$ -AR stimulation of  $I_{Ca,L}$  in +LMN myocytes.** Compared with control +LMN myocytes ( $\beta$ gal), zint- $\beta_2$ -AR stimulation of  $I_{Ca,L}$  was significantly inhibited in +LMN myocytes infected with adenovirus expressing the IP<sub>3</sub> affinity trap. Numbers in parentheses indicate the number of myocytes studied. \* = P < 0.05.

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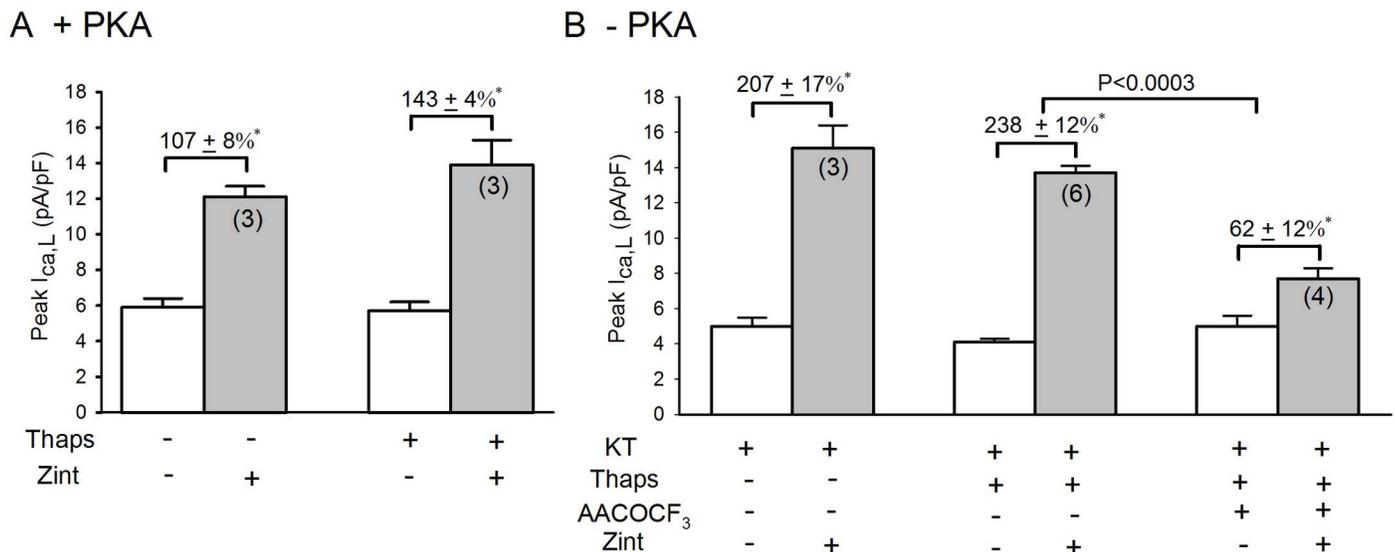
no significant effect on basal  $I_{Ca,L}$  (open bars) or zint- $\beta_2$ -AR stimulation of  $I_{Ca,L}$  (control, 121 ± 4% vs 2-APB, 113 ± 5%). In -PKA myocytes (B) zint- $\beta_2$ -AR stimulation elicited a typically enhanced increase in  $I_{Ca,L}$  compared to control responses (panel A). In contrast to +PKA myocytes, 2-APB significantly inhibited zint- $\beta_2$ -AR stimulation of  $I_{Ca,L}$  (control, 245 ± 25% vs 2-APB, 89 ± 19%; P < 0.02). Similar results were obtained in +LMN myocytes where zint- $\beta_2$ -AR stimulation of  $I_{Ca,L}$  also was significantly blocked by 2-APB (control, 186 ± 26%; N = 4 vs 87 ± 7%; N = 4, P < 0.01) (see S3 Fig online data).

In another approach, we infected atrial myocytes with an adenovirus that expresses an IP<sub>3</sub> affinity trap which binds to IP<sub>3</sub> in the cytosol and thereby inhibits IP<sub>3</sub>-mediated Ca<sup>2+</sup> signaling by preventing IP<sub>3</sub> from reaching and activating the IP<sub>3</sub> receptor (IP<sub>3</sub>R) [17]. Cells were cultured overnight on LMN and therefore represent +LMN myocytes. As shown in Fig 8, in control +LMN myocytes expressing  $\beta$ gal, zint- $\beta_2$ -AR stimulation elicited a typical increase in  $I_{Ca,L}$  (182 ± 4%). However, in +LMN myocytes expressing the IP<sub>3</sub> trap zint- $\beta_2$ -AR stimulation of  $I_{Ca,L}$  (111 ± 17%) was significantly inhibited compared to controls (P < 0.002).

Together, these findings suggest that IP<sub>3</sub>R signaling plays no role in zint- $\beta_2$ -AR stimulation of  $I_{Ca,L}$  in freshly isolated atrial myocytes. However, when PKA is inhibited by either PKA inhibitor (Fig 7) or cell attachment to LMN (Fig 8),  $\beta_2$ -AR stimulation of cPLA<sub>2</sub> is dependent on IP<sub>3</sub>-mediated Ca<sup>2+</sup> signaling.

### Role of IP<sub>3</sub> receptors located on SR or nuclear envelope in the enhanced zint- $\beta_2$ -AR induced $I_{Ca,L}$ currents in -PKA atrial myocytes

IP<sub>3</sub>Rs are thought to be located primarily on the sarcoplasmic reticulum (SR) and nuclear envelope membranes. Because the SR and nuclear envelope membranes are highly interconnected, inhibition of SR Ca<sup>2+</sup> uptake by thapsigargin depletes intracellular Ca<sup>2+</sup> stores from both sites [25]. Therefore, in Fig 9 we determined whether depletion of SR and nuclear Ca<sup>2+</sup> by 5  $\mu$ M thapsigargin (10 min; Thaps) inhibits  $\beta_2$ -AR stimulation of  $I_{Ca,L}$  in +PKA (A) and -PKA (B) myocytes. In +PKA myocytes (A) thapsigargin had no effect on basal  $I_{Ca,L}$  amplitude (open bars). Compared to control responses (107 $\pm$ 8%), thapsigargin slightly enhanced zint- $\beta_2$ -AR stimulation of  $I_{Ca,L}$  (143 $\pm$ 4%), although the change was not statistically significant. This modest increase in  $\beta_2$ -AR stimulation of  $I_{Ca,L}$  is consistent with the effects of thapsigargin to inhibit SR Ca<sup>2+</sup> release and thereby inhibit Ca<sup>2+</sup>-mediated inactivation of  $I_{Ca,L}$ . In fact, separate experiments showed that thapsigargin abolished SR Ca<sup>2+</sup> transients (data not shown). In -PKA myocytes (B), zint- $\beta_2$ -AR stimulation of  $I_{Ca,L}$  (207 $\pm$ 17%) was typically enhanced compared to control responses (107 $\pm$ 8%) obtained in +PKA myocytes (A). Interestingly, in -PKA myocytes treated with thapsigargin  $\beta_2$ -AR stimulation of  $I_{Ca,L}$  (238 $\pm$ 12%) was still enhanced. In other words, depletion of Ca<sup>2+</sup> from SR and nuclear membranes failed to prevent PKA inhibition from enhancing  $\beta_2$ -AR signaling. Moreover, the last column in Fig 9B shows that in -PKA myocytes treated with thapsigargin, AACOCF<sub>3</sub> (cPLA<sub>2</sub> inhibitor) significantly inhibited zint- $\beta_2$ -AR stimulation of  $I_{Ca,L}$  (62 $\pm$ 12%), indicating that the enhanced response to zint- $\beta_2$ -AR stimulation was in fact due to activation of cPLA<sub>2</sub> signaling and that depletion of SR and



**Fig 9. Effects of 5  $\mu$ M thapsigargin (10 min; Thaps) on zint- $\beta_2$ -AR stimulation of  $I_{Ca,L}$  in +PKA(A) and -PKA (B) myocytes.** A; in +PKA myocytes, compared to control responses, thapsigargin slightly enhanced zint- $\beta_2$ -AR stimulation of  $I_{Ca,L}$ . B; in -PKA myocytes, zint- $\beta_2$ -AR stimulation elicited a typically enhanced increase in  $I_{Ca,L}$  compared to control (A) that was not prevented by treatment with thapsigargin. The addition of 10  $\mu$ M AACOCF<sub>3</sub> (+ thaps) significantly inhibited zint- $\beta_2$ -AR stimulation of  $I_{Ca,L}$  indicating that thapsigargin did not prevent zint- $\beta_2$ -AR stimulation of cPLA<sub>2</sub>. Numbers in parentheses indicate the number of myocytes studied. \* = P<0.05.

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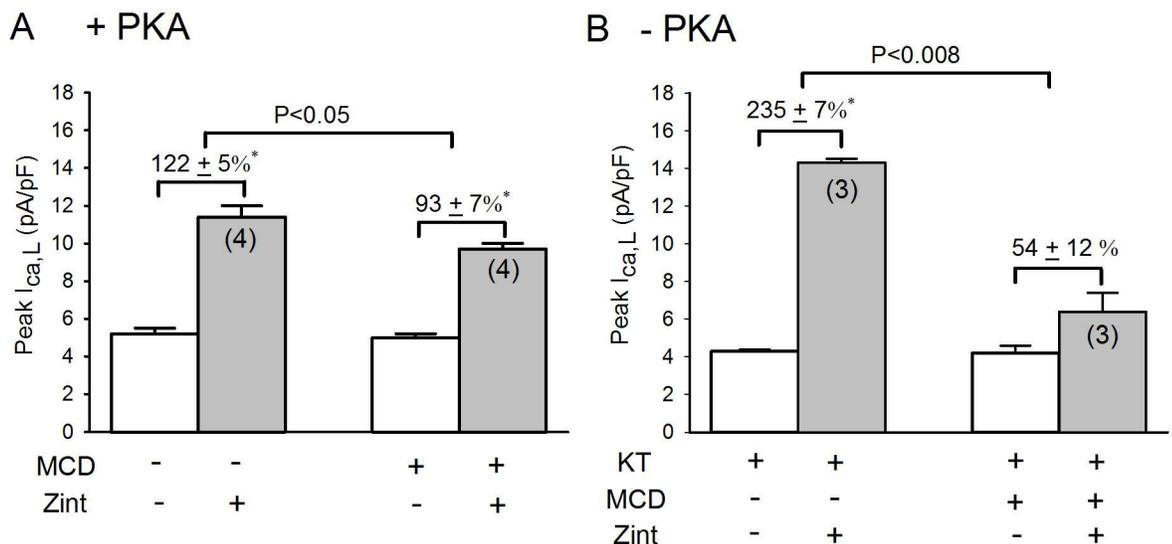
nuclear Ca<sup>2+</sup> stores failed to prevent  $\beta_2$ -AR/cPLA<sub>2</sub> signaling. Similar results were obtained when Ca<sup>2+</sup> stores were depleted by treatment (10 min) with 10  $\mu$ M ryanodine (data not shown). These results indicate that IP<sub>3</sub>-dependent Ca<sup>2+</sup> signaling is not mediated via IP<sub>3</sub>Rs located on SR or nuclear envelope membranes.

### Role of IP<sub>3</sub> receptors located in caveolae in the enhanced zint- $\beta_2$ -AR induced I<sub>Ca,L</sub> currents in -PKA atrial myocytes

Alternatively, IP<sub>3</sub>R protein is present in caveolae [26, 27], which are abundant in atrial myocytes [28]. We therefore determined the effects of 10 mM methyl- $\beta$ -cyclodextrin (MCD; 30 min), an agent that disrupts caveolae formation [29], on zint- $\beta_2$ -AR stimulation of I<sub>Ca,L</sub> in +PKA and -PKA myocytes. As shown in Fig 10A, in +PKA myocytes MCD caused a modest but significant inhibition of zint- $\beta_2$ -AR stimulation of I<sub>Ca,L</sub> (control, 122 $\pm$ 5% vs MCD, 93 $\pm$ 7%; P<0.05), representing a 24% decrease. This is consistent with the idea that  $\beta_2$ -ARs are normally localized to caveolae [30]. However, in -PKA myocytes (B) MCD elicited a significantly larger inhibition of zint- $\beta_2$ -AR stimulation of I<sub>Ca,L</sub> (control, 235 $\pm$ 7% vs MCD, 54 $\pm$ 12%; P<0.008), representing a 77% decrease. The fact that MCD elicited a significantly larger inhibition of  $\beta_2$ -AR signaling in -PKA compared to +PKA myocytes supports the idea that the IP<sub>3</sub>Rs that are essential for  $\beta_2$ -AR/cPLA<sub>2</sub> signaling are localized to the caveolae.

### Discussion

We previously reported that in atrial myocytes inhibition of adenylate cyclase/PKA by either cell attachment to LMN or inhibition of PKA in cells not attached to LMN activates  $\beta_2$ -AR/cPLA<sub>2</sub> signaling [2, 3]. The present study extends those findings by showing that when PKA is inhibited,  $\beta_2$ -AR stimulation acts via PKC $\alpha$ /Raf-1/MEK/ERK1/2 and IP<sub>3</sub>-dependent Ca<sup>2+</sup> signaling to activate cPLA<sub>2</sub>.



**Fig 10. Effect of 2 mM methyl- $\beta$ -cyclodextrin (MCD; 30 min) on zint- $\beta_2$ -AR stimulation of I<sub>Ca,L</sub> in +PKA (A) and -PKA (B) myocytes.** A; in +PKA myocytes, MCD significantly decreased zint- $\beta_2$ -AR stimulation of I<sub>Ca,L</sub> compared to control responses (-24%). B; in -PKA myocytes, zint- $\beta_2$ -AR stimulation elicited a typically enhanced increase in I<sub>Ca,L</sub> compared to +PKA myocytes (A) and the effect of MCD to decrease zint- $\beta_2$ -AR stimulation of I<sub>Ca,L</sub> was enhanced (-77%) compared to +PKA myocytes (A). Numbers in parentheses indicate the number of myocytes studied. \* = P<0.05.

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In the present experiments, we showed that inhibition of PKA prominently stimulates  $\beta_2$ -AR-mediated phosphorylation of ERK1/2, consistent with  $\beta_2$ -AR stimulation of  $I_{Ca,L}$  via MEK/ERK1/2 signaling [3]. Moreover, inhibition of Raf-1 by either GW5074 or adenoviral expression of a dn-Raf-1 mutant significantly inhibited  $\beta_2$ -AR stimulation of  $I_{Ca,L}$  in -PKA or +LMN myocytes but failed to affect  $\beta_2$ -AR signaling in +PKA myocytes. These findings can be explained by the fact that PKA inhibits Raf-1 and Raf-1 activates MEK/ERK1/2 signaling [7, 31, 32]. Because atrial myocytes normally exhibit relatively high endogenous levels of adenylylate cyclase/PKA activity [11], Raf-1 is normally inhibited and therefore  $\beta_2$ -AR stimulation is unable to activate Raf-1/MEK/ERK1/2 signaling in +PKA myocytes. However, when PKA is inhibited (-PKA or +LMN myocytes)  $\beta_2$ -AR stimulation acts via Raf-1/MEK/ERK1/2 to activate cPLA<sub>2</sub>. The present experiments also show that adenoviral overexpression of wt-cPLA<sub>2</sub> or expression of a dn-cPLA<sub>2</sub><sup>S505A/S515A</sup> mutant in +LMN myocytes significantly enhanced or inhibited, respectively,  $\beta_2$ -AR stimulation of  $I_{Ca,L}$  compared to control +LMN myocytes expressing  $\beta$ gal. In various cell systems including vascular smooth muscle and fibroblasts ERK1/2 phosphorylates cPLA<sub>2</sub> at S505 and S515 [15, 21–23]. In cardiac ventricular myocytes (cultured on LMN)  $\beta_2$ -AR stimulation acts via ERK1/2 and p38<sup>MAPK</sup> to phosphorylate cPLA<sub>2</sub> at S505 [6].

In atrial myocytes,  $\beta_2$ -ARs are coupled to both G<sub>s</sub>- and G<sub>i</sub>-mediated signaling pathways [12]. We [3] and others [4] have reported that  $\beta_2$ -ARs act via pertussis toxin-sensitive G<sub>i</sub> to activate cPLA<sub>2</sub>. In various cell systems, including rat ventricular myocytes [33] activation of PKA phosphorylates  $\beta_2$ -ARs and thereby switches  $\beta_2$ -AR coupling from G<sub>s</sub>-mediated adenylylate cyclase to G<sub>i</sub>-mediated ERK1/2 signaling [34, 35]. Moreover, inhibition of PKA by H-89 inhibited  $\beta_2$ -AR/G<sub>i</sub>-mediated ERK1/2 signaling. These findings are not consistent with the present results, which indicate that in adult atrial myocytes inhibition of PKA by H-89 or KT5720 activates (rather than inhibits)  $\beta_2$ -AR/G<sub>i</sub>-mediated ERK1/2 signaling [3]. However, our findings are consistent with reports that PKA inhibits Raf-1 and its downstream MEK/ERK1/2 signaling pathway [7, 31, 32]. Therefore, in atrial myocytes inhibition of PKA removes Raf-1 inhibition and thereby activates  $\beta_2$ -AR/G<sub>i</sub> stimulation of Raf-1/MEK/ERK1/2 signaling.

The present results also indicate that inhibition of PLC (edelfosine) or PKC (chelerythrine or dn-PKC $\alpha$ ) significantly inhibited  $\beta_2$ -AR stimulation of  $I_{Ca,L}$  in -PKA or +LMN myocytes but not in +PKA myocytes. Moreover, in +LMN myocytes expression of dn-PKC $\alpha$  significantly inhibited  $\beta_2$ -AR signaling while expression of dn-PKC $\epsilon$  had no effect, indicating that  $\beta_2$ -ARs act via Ca<sup>2+</sup>-dependent PKC $\alpha$  to activate cPLA<sub>2</sub>. These findings are consistent with reports that PKC activates Raf-1/MEK/ERK1/2 signaling in adult cardiac myocytes [9, 18] and that PKC activates cPLA<sub>2</sub> signaling [10].

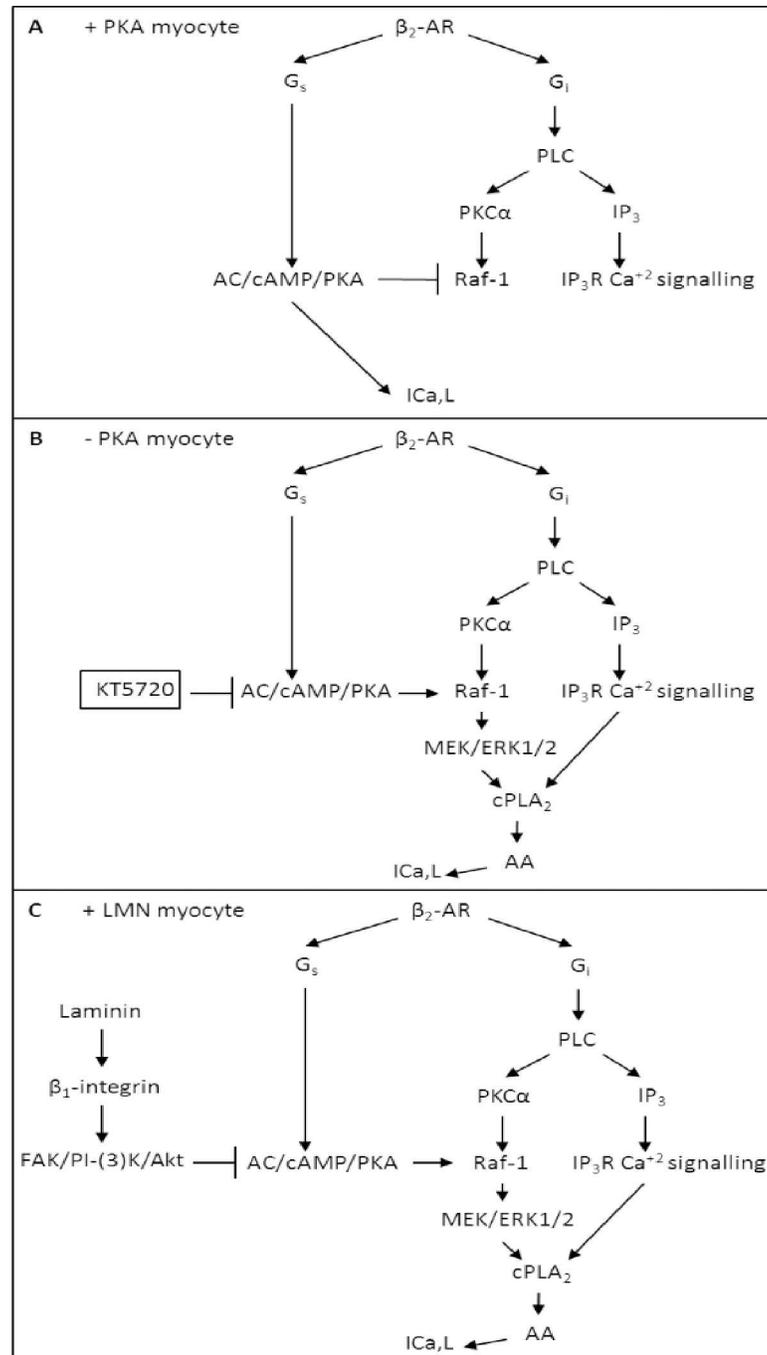
Our previous findings showed that strong chelation of intracellular Ca<sup>2+</sup> by BAPTA prevented  $\beta_2$ -AR stimulation via cPLA<sub>2</sub>. This is consistent with the fact that several of the signaling molecules involved in the proposed  $\beta_2$ -AR/cPLA<sub>2</sub> signaling cascade are Ca<sup>2+</sup>-dependent, including PLC, PKC $\alpha$  and cPLA<sub>2</sub>. In the present study, we investigated more specifically which source of intracellular Ca<sup>2+</sup> is required for  $\beta_2$ -AR/cPLA<sub>2</sub> signaling. We found that 2-APB, an agent that inhibits IP<sub>3</sub>-mediated Ca<sup>2+</sup> release in cat atrial myocytes [24] significantly inhibited  $\beta_2$ -AR stimulation of  $I_{Ca,L}$  in both -PKA and +LMN myocytes but not in +PKA myocytes. Moreover, adenoviral expression of an IP<sub>3</sub> affinity trap which inhibits IP<sub>3</sub>-mediated Ca<sup>2+</sup> signaling [17] resulted in a similar inhibition of  $\beta_2$ -AR signaling in +LMN myocytes. The fact that 2-APB had no effect on  $\beta_2$ -AR signaling in control +PKA myocytes and that it exerted effects similar to those of the Adv-IP<sub>3</sub> affinity trap suggests that 2-APB acted specifically to inhibit IP<sub>3</sub>-mediated Ca<sup>2+</sup> signaling. Together, these results indicate that  $\beta_2$ -AR stimulation of cPLA<sub>2</sub> is dependent on IP<sub>3</sub>-mediated Ca<sup>2+</sup> signaling. Although IP<sub>3</sub>Rs are typically located on SR and nuclear membranes, the present results showed that thapsigargin, an agent that depletes both

SR and nuclear envelope Ca<sup>2+</sup> stores [25] failed to prevent  $\beta_2$ -AR stimulation of I<sub>Ca,L</sub> via cPLA<sub>2</sub>. However, disruption of caveolae formation by methyl- $\beta$ -cyclodextrin elicited a significantly larger inhibition of  $\beta_2$ -AR stimulation in -PKA myocytes than control +PKA myocytes. We therefore conclude that IP<sub>3</sub>Rs on the SR and nuclear envelope membranes are not involved in the Ca<sup>2+</sup> signaling required for  $\beta_2$ -AR stimulation of cPLA<sub>2</sub>. Alternatively,  $\beta_2$ -AR stimulation of I<sub>Ca,L</sub> via cPLA<sub>2</sub> is dependent on IP<sub>3</sub>-mediated Ca<sup>2+</sup> signaling in caveolae. In endothelial and smooth muscle cells plasmalemmal caveolae contain IP<sub>3</sub>R protein that is speculated to mediate Ca<sup>2+</sup> influx through the caveolar membrane [26, 27]. Moreover, in rat ventricular myocytes (cultured on laminin)  $\beta_2$ -ARs stimulate translocation of cPLA<sub>2</sub> to the low density caveolin-3 enriched membrane fraction suggesting that cPLA<sub>2</sub> translocates to caveolae [5]. In fact, lipid rafts i.e. caveolae contain a wide variety of signaling components including  $\beta_2$ -ARs, G<sub>i</sub>, PKC, Raf-1, ERK1/2, IP<sub>3</sub>Rs [36], involved in  $\beta_2$ -AR/cPLA<sub>2</sub> signaling.

We therefore propose (see Fig 11) that under normal conditions (A; +PKA myocytes),  $\beta_2$ -AR/G<sub>s</sub> stimulation acts via adenylate cyclase (AC)/cAMP/PKA to stimulate I<sub>Ca,L</sub>. The relatively high basal levels of endogenous PKA activity and  $\beta_2$ -AR-stimulated PKA activity both inhibit Raf-1 signaling, thereby preventing  $\beta_2$ -AR stimulation from normally activating cPLA<sub>2</sub>. However, inhibition of PKA by either PKA inhibitor (B; -PKA myocytes) or by cell attachment to LMN (C; +LMN myocytes), removes inhibition of Raf-1. Under these conditions,  $\beta_2$ -AR/G<sub>i</sub> stimulation of PLC activates PKC $\alpha$  and IP<sub>3</sub>R-mediated Ca<sup>2+</sup> signaling in caveolae. With PKA inhibited, PKC $\alpha$  is now able to stimulate Raf-1/MEK/ERK1/2 signaling. Therefore,  $\beta_2$ -AR/G<sub>i</sub>-mediated activation of both ERK1/2 and IP<sub>3</sub>-dependent Ca<sup>2+</sup> signaling stimulates cPLA<sub>2</sub>/AA. We previously reported that arachidonic acid (AA) stimulates I<sub>Ca,L</sub> [3]. This scenario is consistent with the fact that cPLA<sub>2</sub> activation requires both elevation of submicromolar intracellular [Ca<sup>2+</sup>] and phosphorylation by various kinases (see also [21–23]).

## Conclusions

These findings may have important implications with respect to the aging and/or failing heart, both of which exhibit decreases in adenylate cyclase activity. In both animal models [37] and in the human right atrium [38], increasing age is associated with a decrease in  $\beta_1$ -AR function that results from a decrease in adenylate cyclase activity. Likewise, adenylate cyclase activity is depressed in the failing human heart [39] and canine pacing-induced heart failure [40, 41] and yet  $\beta_2$ -AR signaling is preserved. Previous studies by Nalli et al suggest that PKA and PKG phosphorylates PLC $\beta_3$  in gastric smooth muscle cells and decreases PI hydrolysis [42]. The decrease in PI hydrolysis has been suggested to diminish IP<sub>3</sub>-mediated Ca<sup>2+</sup> release decreasing muscle contraction. Along these lines, the present studies also suggest that feline atrial cardiomyocytes exhibit a similar regulation as that seen in gastric muscle. Our research also suggests that increases in extracellular matrix proteins, i.e. fibrosis, may contribute to decreases in adenylate cyclase/PKA activity in the aging and/or failing heart, which in turn switches  $\beta_2$ -AR signaling from cAMP/PKA to cPLA<sub>2</sub>/AA. This mechanism may be responsible for the preservation of  $\beta_2$ -AR signaling while  $\beta_1$ -AR signaling is depressed. Thus, the diminished PKA activity in cardiac fibrosis may decrease phosphorylation of PLC resulting in an increase in IP hydrolysis, Ca<sup>2+</sup> release and greater contraction. Indeed, our present results indicate that inhibition of PLC significantly inhibited  $\beta_2$ -AR stimulation of I<sub>Ca,L</sub> in -PKA or +LMN myocytes but not in +PKA myocytes. In addition, our results suggest that 2-APB, an agent that inhibits IP<sub>3</sub>-mediated Ca<sup>2+</sup> release in cat atrial myocytes [24] significantly inhibited  $\beta_2$ -AR stimulation of I<sub>Ca,L</sub> in both -PKA and +LMN myocytes but not in +PKA myocytes. Together, these results indicate that  $\beta_2$ -AR stimulation of cPLA<sub>2</sub> in both -PKA and +LMN myocytes is dependent on PLC/IP<sub>3</sub>-mediated Ca<sup>2+</sup> signaling. Given that cPLA<sub>2</sub>/AA signaling is a potentially pro-



**Fig 11. Schematic summary showing the proposed signaling mechanisms underlying zint- $\beta_2$ -AR stimulation of  $I_{Ca,L}$  in +PKA (A), -PKA (B) atrial myocytes.** A; in +PKA myocytes, zint- $\beta_2$ -AR stimulation acts via  $G_s$  to activate adenylate cyclase (AC)/cAMP-dependent kinase (PKA) which in turn stimulates  $I_{Ca,L}$ . Both basal and stimulated PKA activity inhibits Raf-1 signaling, thereby preventing zint- $\beta_2$ -AR stimulation of  $I_{Ca,L}$  via cPLA<sub>2</sub>. B; in cells not attached to LMN, inhibition of PKA by KT5720 removes inhibition of Raf-1. C; cell attachment to LMN acts via  $\beta_1$  integrins and FAK/PI-(3)K/Akt signaling to inhibit adenylate cyclase (AC)/cAMP-dependent kinase (PKA) activity, thereby removing inhibition of Raf-1.  $\beta_2$ -AR stimulation acts via  $G_i$  to stimulate PLC leading to activation of PKC $\alpha$  and IP<sub>3</sub>R-mediated Ca<sup>2+</sup> signaling within caveolae. With PKA inhibited, PKC $\alpha$  stimulates Raf-1/MEK/ERK1/2 signaling. Together, ERK1/2 and IP<sub>3</sub>-mediated Ca<sup>2+</sup> signaling activate cPLA<sub>2</sub>/AA, resulting in stimulation of  $I_{Ca,L}$ .

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inflammatory mediator, the present findings suggest that fibrosis in the aging and/or failing heart may predispose inflammation and atrial dysfunction. In fact, AA is reported to slow atrial conduction and has been implicated in the development of postoperative atrial fibrillation [43]. On the other hand, activation of the Raf/MEK/ERK pathway may be cardioprotective [44]. In fact, inhibition of adenylate cyclase/PKA and subsequent activation of Raf-1/MEK/ERK1/2 signaling enhances cardiac resistance to oxidative stress, increases cell survival, and extends lifespan [45]. Therefore, inhibition of adenylate cyclase/PKA activity and the remodeling of  $\beta_2$ -AR signaling to activate Raf/MEK/ERK1/2 may help ameliorate the deterioration of function that occurs in the aging and/or failing atrium. Previous studies have indicated that, beside inhibition of COX, aspirin has COX-independent mechanisms that plays a role in tumor suppression [46, 47]. Aspirin reportedly inhibits PI3K/Akt kinase activity in epithelial ovarian cancer cells [48]. Aspirin down regulates the expression of PI3K, Akt and ERK in rodent model of acute pulmonary embolism [49] and in turn inhibit the release of inflammatory cytokines [50]. On the other hand, our studies indicate that laminin acts via FAK/PI(3)K/Akt signaling to inhibit adenylate cyclase-mediated stimulation of  $I_{Ca,L}$ . Although, there is no direct evidence suggesting that aspirin or NSAIDs play a role in cardiomyocyte aging, we hypothesize that aspirin or NSAIDs may influence cPLA<sub>2</sub>/AA pathway and this requires further investigation.

## Supporting Information

**S1 Fig. Effects of Raf-1 inhibition (10  $\mu$ M GW5074) on zint- $\beta_2$ -AR stimulation of  $I_{Ca,L}$  in +LMN atrial myocytes.** A; In +LMN myocytes, zint- $\beta_2$ -AR stimulation of  $I_{Ca,L}$  was enhanced and GW5074 significantly inhibited zint- $\beta_2$ -AR stimulation of  $I_{Ca,L}$ . Numbers in parentheses indicate the number of myocytes studied. \* =  $P < 0.004$ . (TIF)

**S2 Fig. Effects of 0.5  $\mu$ M edelfosine on zint- $\beta_2$ -AR stimulation of  $I_{Ca,L}$  in +LMN atrial myocytes.** A; in +LMN myocytes zint- $\beta_2$ -AR stimulation elicited a typically enhanced increase in  $I_{Ca,L}$  and edelfosine significantly inhibited zint- $\beta_2$ -AR stimulation of  $I_{Ca,L}$ . Numbers in parentheses indicate the number of myocytes studied. \* =  $P < 0.03$ . (TIF)

**S3 Fig. Effects of 2  $\mu$ M 2-APB on zint- $\beta_2$ -AR stimulation of  $I_{Ca,L}$  in +LMN atrial myocytes.** A; +LMN myocytes, zint- $\beta_2$ -AR stimulation elicited a typically enhanced increase in  $I_{Ca,L}$  and 2-APB significantly inhibited zint- $\beta_2$ -AR stimulation of  $I_{Ca,L}$ . Numbers in parentheses indicate the number of myocytes studied. \* =  $P < 0.01$ . (TIF)

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