We have recently demonstrated that in human heart, β2-adrenergic receptors (β2-ARs) are biochemically coupled not only to the classical adenyl cyclase (AC) pathway but also to the cytosolic phospholipase A2 (cPLA2) pathway (Pavoine, C., Behforouz, N., Gauthier, C., Le Govueillo, S., Roudot-Thoraval, F., Martin, C. R., Pawlak, A., Feral, C., Defer, N., Houel, R., Magne, S., Amadou, A., Loïsance, D., Duvaldestin, P., and Pecker, F. (2003) Mol. Pharmacol. 64, 1117–1125). In this study, using Fura-2-loaded cardiomyocytes isolated from adult rats, we showed that stimulation of β2-ARs triggered an increase in the amplitude of electrically stimulated [Ca2+]i transients and contractions. This effect was abolished with the PKA inhibitor, H89, but greatly enhanced upon addition of the selective cPLA2 inhibitor, AACOCF3. The β2-AR/cPLA2 inhibitory pathway involved Gβγ and MSK1. Potentiation of β2-AR/AC/PI(4,5)P2-induced [Ca2+]i responses by AACOCF3 did not rely on the enhancement of AC activity but was associated with eNOS phosphorylation (Ser177) and l-NAME-sensitive NO production. This was correlated with PKA-dependent phosphorylation of PLB (Ser16). The constraint exerted by the β2-AR/cPLA2 pathway on the β2-AR/AC/PI(4,5)P2-induced Ca2+ responses required integrity of caveolar structures and was impaired by Filipin III treatment. Immunoblot analyses demonstrated zinterol-induced translocation of cPLA2 and its cosedimentation with MSK1, eNOS, PLB, and sarcoplasmic reticulum Ca2+ pump (SERCA) 2a in a low density caveolin-3-enriched membrane fraction. This inferred the gathering of β2-AR signaling effectors around caveolae/sarcoplasmic reticulum (SR) functional platforms. Taken together, these data highlight cPLA2 as a cardiac β2-AR signaling pathway that limits β2-AR/AC/PI(4,5)P2-induced Ca2+ responses in adult rat cardiomyocytes through the impairment of eNOS activation and PLB phosphorylation.

Cardiovascular diseases are becoming the leading cause of death worldwide, with heart failure as a major symptom. Most patients with heart failure show dysregulation of the sympathetic system, and the resulting chronic elevation of plasma catecholamine levels correlates with the prognosis (1). Among the identified cardiac catecholamine receptors, β1-adrenergic receptors (β1-AR)1 and β2-AR received the most interest as mainstay regulators of cardiac performance. Noteworthy, β1-AR and β2-AR functionally diverge because of clear discrepancies in signaling transduction pathways (2). Chronic over-stimulation of cardiac β1-AR, associated with exclusive activation of the Gs/adenylyl cyclase (AC) pathway, is detrimental and leads to hypertrophy, fibrosis, and heart failure (3). In sharp contrast, sustained β2-AR stimulation protects cardiomyocytes against apoptosis. In addition, transgenic mice with cardiac moderate β2-AR overexpression display not only long term improved cardiac function but also normal life expectancy (4–6). The beneficial effect of β2-AR stimulation appears to rely on the ability of β2-AR to couple with pertussis toxin (PTX)-sensitive G proteins (Gi12 and Go5), leading to a β2-AR/Gi/AC signaling spatially confined to the caveolar structures and functionally moderated, as compared with the β1-AR/Gs/AC pathway (7–9). Identification of β2-AR-Gi-downstream effectors is of major interest to elucidate, and to further exploit the cardiac protective β2-AR potential.

Recently, on human cardiac ventricular and auricular biopsies, we identified a new β2-AR-stimulated pathway, namely the Gi/cytosolic phospholipase A2 (cPLA2) pathway, in addition to the classical β2-AR/Gs/AC pathway (10). cPLA2 is a high molecular mass enzyme (85 kDa), activated by submicromolar concentrations of Ca2+, that displays a unique selectivity for arachidonyl in the sn-2 position of phospholipids (11). cPLA2 is fully activated by both phosphorylation by mitogen-activated protein kinase and increases in [Ca2+]i. cPLA2 activation is associated with its translocation from the cytosol to intracellular membranes, such as endoplasmic/sarcoplasmic reticulum, Golgi apparatus, and nuclear envelope. The essential role of cPLA2 in inflammation, asthma, neurodegenerative diseases, and bleomycin-induced pulmonary fibrosis is now well documented, and cPLA2 has been identified as an attractive therapeutic target in the development of new inflammatory drugs (12–15). In contrast, a dearth of information exists on the possible functional impact of the cPLA2 and its role in the development of cardiac diseases. Only one recent study has established the antihypertrophic potential of the cPLA2 pathway in cardiac and skeletal muscles, using the model of the

1 The abbreviations used are: β1-AR, β1-adrenergic receptor; AC, adenyl cyclase; FITC, fluorescein isothiocyanate; MES, 4-morpholineethanesulfonic acid; SERCA, sarcoplasmic reticulum Ca2+ pump; SR, sarcoplasmic reticulum; eNOS, endothelial nitric-oxide synthase; PLB, phospholamban; PKA, cAMP-dependent kinase; PLA2, phospholipase A2; PTX, pertussis toxin; Pi3K, phosphatidylinositol 3-kinase; DAPI, 4’,6-diamidino-2-phenylindole; DAF-FM (4-aminom-5-methyl-amino-2’,7’-difluorofluorescein).

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cPLA₂ knockout mice (16). In the present study, we examined the impact of β₂-AR-G/cPLA₂ and β₂-AR-G/Ac pathways on Ca²⁺ signaling and contraction in isolated adult rat cardiomyocytes. We show that β₂-AR agonists trigger an increase in the amplitude of electrically stimulated [Ca²⁺], transients that rely on the β₂-AR-G/Ac/PKA pathway and is under the negative control of the β₂-AR-G/MSK1/cPLA₂ pathway. We provide evidence that upon β₂-AR stimulation cPLA₂ translocates to caveolae/sarcoplasmic reticulum (SR)-functional platforms and bridges phosphorylation of endothelial nitric-oxide synthase (eNOS) and phospho-lamban (PLB). Using fluorescence imaging, we showed that the β₂-AR/cPLA₂ pathway neutralizes NO production. Our study highlights the essential physiological role of the β₂-AR-G/cPLA₂ pathway in limiting β₂-AR/Ac/PKA-induced Ca²⁺ responses.

**EXPERIMENTAL PROCEDURES**

**Materials**

Zolerox was kindly supplied by Bristol-Myers Squibb Co (Stanford, CA). 9,10-Fluorene-2,7-dicarboxylic acid (FDCA) was synthesized in the laboratory. Aldrich, Sigma, Prolabo, and Upstate Biotechnology (Lake Placid, NY) were purchased by Molecular Probes (Montluçon, France). Rabbit polyclonal antibodies against human phospho-eNOS (Ser1177) and rabbit polyclonal antibodies against human phospho-MSK1 (Thr581) were from Cell Signaling Technology (Beverly, MA). Sheep immunofluorescence-purified IgG antibodies against human MSK1 were from Upstate Biotechnology (Lake Placid, NY); mouse monoclonal IgG1 antibodies against rat caveolin-3 were from BD Biosciences Pharmingen (Le Pont De Claix, France); mouse monoclonal IgG antibodies against mouse caveolin-1 (clone A11) and rabbit polyclonal antibodies against phosphorylated phospholamban (Ser16), and phosphorylated phospho-lamban (Thr17) were from Cyclacel (Dundee, UK); mouse monoclonal antibodies against the N-terminal part of the human cPLA₂ were from Santa Cruz Biotechnology. Peroxidase-conjugated goat anti-rabbit IgG and peroxidase-conjugated donkey anti-sheep IgG were purchased from Jackson Immunoresearch Laboratories (West Grove, PA), and peroxidase-conjugated rabbit anti-mouse IgG were from Biosys (Compiegne, France). Goat anti-mouse IgG (H+L) highly cross-absorbed coupled to Alexa Fluor® 594 and goat anti-rabbit IgG (H+L) highly cross-absorbed coupled to Alexa Fluor® 647 were from Molecular Probes. Fluorescein isothiocyanate (FITC)-conjugated phalloidin was from Sigma and Vectashield mounting medium containing DAPI was from Vector Laboratories (Burlingame, CA). Polyethylene glycol (PEG) 6000 and caveolin-3 isoform were prepared from isolated adult rat cardiomyocytes according to a detergent-free purification scheme as described previously (24, 25). Freshly isolated cardiomyocytes (1.5 × 10⁶ cells-condition) were submitted to a 10 min of pretreatment with or without 10 μM A20693 followed by a 10-min incubation with or without 1 μM zinterol in BSS buffer at 37 °C. All subsequent experiments were performed at room temperature, and cells were resuspended in 1 ml of solution A (0.5 mM Na₂CO₃, pH 11, containing phosphahtase inhibitors (10 mM NaF, 100 μM Na₂VO₄, and 5 mM Na₃P₂O₇) were sequentially disrupted by homogenization with a loose fitting Dounce homogenizer (10 strokes), a Polytron tissue grinder (three 10-s bursts), and a bath sonicator (one 5-min burst). 1 ml of homogenate was then adjusted to 45% sucrose (% Brix measured with a refractometer) by adding 3 ml of 80% sucrose prepared in 2 volumes of MES-buffered saline (MBS: 25 mM MES, pH 6.5, and 0.15 M NaCl) and 1 volume of solution A. The 45% sucrose fraction was placed on the bottom of an ultracentrifuge tube, overlaid with a 5–30% continuous sucrose gradient, and subjected to ultracentrifugation (750,000 × g, 16 h, 4 °C). The resulting fractions were collected from the top of the gradient. 1 ml of the 30–40% fraction was then dialyzed against triphasic buffer (25 mM MES, pH 6.5, and 0.15 M NaCl) and centrifuged at 39,000 rpm for 18 h at 4 °C in a SW41Ti rotor (Beckman Coulter). After centrifugation, eleven 1-ml fractions were collected from the top of the gradient, concentrated by precipitation with trichloroacetic acid (addition of 75 μl of a 100% solution). After centrifugation at 10,000 × g for 15 min at 4 °C, pellets were resuspended in 50 mM Tris, pH 8.8 added with phosphatase inhibitors (10 mM NaF, 100 μM Na₂VO₄, and 5 mM Na₃P₂O₇) and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 2 μg/mL leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 2 μg/mL aprotinin). Following determination of the protein content, fractions were dissolved in Laemmli loading buffer.

**Immunoblot Analysis of the Phosphorylation State of MSK1, eNOS, and PKA and Detection of Cavelcin-3, PLB, MSK1, eNOS, SERCA 2A, and cPLA₂**—Samples, 50 μg of each cellular extract (Fig. 5) or 5 μg of each gradient fraction (Fig. 7A), were subjected to SDS-PAGE (15% acrylamide gels) and proteins electro transferred to polyvinylidine di-
fluoride membranes (0.22 μm, Millipore). Membranes were first incubated with antibodies against caveolin-3 (1:5,000) (Fig. 7A), or P-(Ser18)-PLB (1:1,000) (Fig. 5), or P-(Thr21)-PLB (1:1,000) (not shown). Blots were treated with secondary peroxidase-conjugated rabbit anti-mouse (1:20,000) or goat-anti rabbit (1:20,000) antibodies. The peroxidase activity was visualized with an enhanced chemiluminescent detection kit (Supersignal West Dura). 65 μg of each caveolin-3-enriched fraction (fraction 7) were loaded on preparative SDS-PAGE (8–16% acrylamide) and transferred to Hybond C Super (Amersham Biosciences) nitrocellulose. Immunoblots were cut in small pieces and analyzed with antibodies for cPLA₂ (1:500), MSK1 (1:1,000), P-(Thr57)-MSK1 (1:500), PLB (1:5,000), P-(Ser18)-PLB (1:1,000), SERCA 2a (1:1,000), eNOS (1:2,500), and P-(Ser177)-eNOS (1:1,000). Blots were then treated with secondary peroxidase-conjugated rabbit anti-mouse (1:20,000 dilution), goat-anti rabbit (1:20,000 dilution), or donkey anti-sheep (1:20,000 dilution) antibodies.

**Immunofluorescence and Confocal Laser Scanning Microscopy**

Immunofluorescence Staining—Indirect immunofluorescence was performed on freshly isolated myocytes fixed with 4% formaldehyde at room temperature as described previously (26). Briefly, myocytes were incubated in phosphate-buffered saline containing 5% bovine serum albumin for 30 min to block nonspecific binding sites, followed by overnight incubation with a solution of mouse monoclonal antibodies against caveolin-3 (clone 26, 1:100) and rabbit polyclonal antibodies against SERCA 2a (1:100, see Ref. 27). After washes, cells were first incubated for 1 h with an excess of the secondary antibody goat anti-mouse IgG (H+L) highly cross-absorbed coupled to Alexa Fluor® 594 (1:100). Myocytes were then washed and treated with the secondary antibody goat anti-rabbit IgG (H+L) highly cross-absorbed coupled to Alexa Fluor® 647 (1:100) for 1 h. This was followed after washes by incubation with FITC-conjugated-phallolidin (1/30) for 90 min. After a final wash, coverslips were mounted in Vectashield mounting medium containing DAPI. Labeling of cells with secondary antibodies alone was carried out as a negative control.

Confocal Microscopy and Image Processing—Images were collected with a Zeiss LSM-510 multitracking laser scanning confocal microscope (Carl Zeiss SAS, Frankfurt, Germany). Fluorochromes were detected sequentially using laser lines 488 nm (FITC), 543 nm (Alexa Fluor® 594) and 633 nm (Alexa Fluor® 647). DAPI was visualized by UV excitation. Offset and gain for each channel was set in order to avoid any cross-talk between the three fluorochrome emission spectra. The images were coded white (FITC), red (Alexa Fluor® 594), and green (Alexa Fluor® 647) giving yellow co-localization in merge images (Alexa Fluor® 594 and Alexa Fluor® 647). The oil objective used was ×63 (NA 1.4), giving a resolution of 100 nm in the x,y plane and 300 nm in the z axis (pinhole, 72 μm).

Quantification of Overlapping—We studied 10 individual myocytes and analyzed 55 ± 5 successive single sections acquired in each image stack. Overlapping was quantified with the LSM 510 3 software (Carl Zeiss). The overlapping of SERCA 2a and caveolin-3 stainings in the x,y plane was determined owing to analysis of fluorescence intensity profiles as previously described by Bolte et al. (28). Positive structures were evaluated using threshold values of 152 ± 17 (caveolin-3) and 144 ± 5 (SERCA 2a) pixel units on a grayscale of 0–255. A scatterplot of the individual pixels from each paired images was generated, and overlapped pixels were characterized by an overlap coefficient value of 1. The number of overlapped pixels was compared with the number of total positive pixels for each labeling (caveolin-3 or SERCA 2a).

Statistical Analysis—Results were analyzed by the unpaired two-tailed Mann-Whitney test. Differences were considered statistically significant at a value of P < 0.05.

**RESULTS**

The β₂-AR/cPLA₂ Pathway Restrains the β₂-AR/AC/PKA-induced Ca²⁺- and Contraction Responses in Isolated Cardiomyocytes—The amplitude of [Ca²⁺], transients was measured in electrically stimulated adult rat cardiomyocytes and loaded with Fura 2-AM, in response to the β₂-AR selective agonist, zinterol. Zinterol induced a dose-dependent stimulatory effect on the amplitude of [Ca²⁺], transients (maximal response of 182 ± 11% with zinterol (1 μM)) (Fig. 1A and B). The cPLA₂ inhibitor, ACOCCF₃ (10 μM), had no effect on basal [Ca²⁺], transient amplitude (Fig. 1B), but enhanced zinterol-induced Ca²⁺ responses (maximal response of 307 ± 28% with 1 μM zinterol) (Fig. 1A and B). Typical traces of [Ca²⁺], transients and fractional shortening showed the correlation between the increase in the amplitude of [Ca²⁺], transients and that of contraction in cardiomyocytes in response to zinterol added alone, or after treatment with ACOCCF₃ (Fig. 1A). Inhibition of PKA with H89 (3 μM) blunted the zinterol effect on the amplitude of [Ca²⁺], transients and contraction, in the absence (not shown) as well as in the presence of ACOCCF₃ (Fig. 1A). These results identified PKA activation as an essential feature of zinterol-induced Ca²⁺ and contraction responses, and demonstrated that the cPLA₂ pathway exerted a constraint on PKA-dependent events.

Among the possible targets of the cPLA₂ pathway, we examined AC activity. In membranes purified from isolated cardiomyocytes, zinterol produced a limited but significant dose-dependent activation of AC activity (maximal 125 ± 5% with zinterol (1 μM)), compared with the 216 ± 21, 356 ± 5, and 1435 ± 10% stimulations elicited by isoproterenol (10 μM) (mainly β₂-AR), NA (1 mM), and forskolin (10 μM), respectively (Fig. 1C). The restricted zinterol-induced AC activation was unmodified by preincubation with ACOCCF₃ (maximal 123 ± 4% activation; Fig. 1C), rejecting AC as a possible target of the β₂-AR/cPLA₂ pathway.

The cPLA₂ Constraint Is Specific to β₂-AR and Does Not Affect β₁-AR-induced Ca²⁺ Response—The maximal increases in the amplitude of [Ca²⁺], transients elicited by two non-selective β-AR stimuli, namely epinephrine (1 μM) added with the α-AR antagonist, prazosin (1 μM) and isoproterenol (0.3 μM), were significantly enhanced in the presence of ACOCCF₃ (Fig. 2). Maximal Ca²⁺ responses reached 302 ± 37 and 275 ± 18% in the presence of ACOCCF₃, compared with 182 ± 12 and 204 ± 17%, in its absence, respectively. The β₂-AR stimulus, isoproterenol (0.3 μM) associated with the β₂-AR antagonist, CGP 20712A (0.3 μM), elicited a 119 ± 8% increase in the amplitude of [Ca²⁺], transients, that was markedly amplified (167 ± 19%) in the presence of the cPLA₂ inhibitor (Fig. 2). In contrast, selective β₁-AR stimulation triggered by isoproterenol (0.3 μM) associated with the β₂-AR antagonist, ICI 118551 (0.1 μM), induced similar increases in the amplitude of [Ca²⁺], transients in the absence or in the presence of ACOCCF₃ (262 ± 21 versus 271 ± 47%, respectively) (Fig. 2). These results indicated that the constraint exerted by the cPLA₂ pathway selectively targeted the β₂-AR-induced Ca²⁺ response, without affecting the β₁-AR-induced Ca²⁺ response.

Involvement of a PTX-sensitive G Protein and MSK1 in the β₂-AR/cPLA₂ Inhibitory Pathway—In heart, PTX treatment ADP-ribosylates the α-subunits of G, and G, proteins, leading to their blockade in the trimeric form (αβγ) and their inhibition. As shown in Fig. 3, and in accordance with published data (29), PTX treatment potentiated the effect of zinterol (1 μM) on the amplitude of [Ca²⁺], transients (330 ± 32 versus 179 ± 9% in PTX-ununtreated cells), mimicking ACOCCF₃ effect. Furthermore, PTX treatment rendered the β₂-AR-induced Ca²⁺ response insensitive to ACOCCF₃ (Fig. 3). This suggested, in line with our previous study in human cardiac tissue (10), that a PTX-sensitive G protein-mediated stimulation of the cPLA₂ activity by β₂-AR agonists in rat.

Previous studies using purine agonists in adult rat cardiomyocytes identified cPLA₂ as a downstream target of the mitogen-activated protein kinase, MSK1, (30, 31). To examine the role of MSK1 in β₂-AR induced Ca²⁺ responses, we treated cardiomyocytes with a MSK1 inhibitor, RO 318220. RO 318220 did not affect basal amplitude of [Ca²⁺], transients (not shown) but potentiated the zinterol-induced Ca²⁺ response, thus mimicking ACOCCF₃ action. The effects of RO 318220 and ACOCCF₃ were not additive (Fig. 3). These results suggested that


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\text{cPLA}_2 \text{ and } \beta_2\text{-AR-induced Ca}^{2+} \text{ Responses in Rat Cardiomyocytes}
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\text{The } \beta_2\text{-AR/cPLA}_2 \text{ Pathway Restrains NOS Activity and PLB Phosphorylation—In cardiomyocytes isolated from adult rats, } \beta_2\text{-AR stimulation has been reported to induce an increase in the L-type Ca}^{2+} \text{ current (Ica,L) because of NO production (32, 33). In the cat atrial cardiomyocytes, NO acts via cGMP-mediated inhibition of the type III phosphodiesterase (PDE) activity, enhancing cAMP-dependent stimulation of Ica,L. Among NO-generating enzymes expressed in the cardiomyocytes, eNOS can be activated upon PKA- or PI3K-dependent phosphorylation of the Ser1177 residue (34). Because in adult rat cardiomyocytes, both PKA and PI3K activities are activated upon } \beta_2\text{-AR stimulation (35), we investigated whether zinterol did trigger NO production that could be amplified by AACOCF}_3\text{ treatment. Cells were loaded with the NO-sensitive fluorescent indicator, DAF-FM diacetate, and Fura 2-AM, to correlate NO and Ca}^{2+} \text{ responses. In rat cardiomyocytes that were electrically stimulated, no change in DAF fluorescence was detected within the first 20 min of incubation time following addition of zinterol alone (not shown). In contrast, exposure to zinterol combined with AACOCF}_3\text{ produced a marked increase in DAF fluorescence (Fig. 4A), that reached a mean } 2.1 \pm 0.2\text{-fold maximal increase in } F/F_0 \text{ after } 8 \pm 1 \text{ min. The increase in DAF fluorescence was blocked in the presence of the NO synthase inhibitor, L-NAME (not shown), and could thus be attributed to NO production. An increase in the amplitude of } [\text{Ca}^{2+}]_i \text{ transients occurred together with NO release in cells stimulated with zinterol plus AACOCF}_3\text{. Interestingly, L-NAME treatment did not modify the increase in the amplitude of } [\text{Ca}^{2+}]_i \text{ transients in response to zinterol alone (Fig. 4B) but partially reversed the potentiation of zinterol-induced } [\text{Ca}^{2+}]_i \text{ responses elicited by AACOCF}_3\text{. These results suggested that, in adult rat cardiomyocytes, basal zinterol-induced Ca}^{2+} \text{ responses were unrelated to NO production. In contrast, inhibition of the } \beta_2\text{-AR/cPLA}_2 \text{ pathway by AACOCF}_3\text{ reversed NO release in response to zinterol. The latter took part in the mechanism of amplification of PKA-dependent Ca}^{2+} \text{ responses.}
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\text{The activity of SERCA is essentially controlled by its inhibitor, PLB. Phosphorylation of PLB reverses this inhibition, thereby accelerating Ca}^{2+} \text{ uptake into the SR (for a review see Ref. 36). In adult rat cardiomyocytes, enhancement of } \beta_2\text{-AR-induced activation of the cPLA}_2 \text{ pathway relied on MSK1 stimulation.}
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\text{FIG. 1. The } \beta_2\text{-AR-induced increase in amplitude of } [\text{Ca}^{2+}]_i \text{, transients and fractional shortening in electrically stimulated adult rat cardiomyocytes is enhanced by AACOCF}_3\text{ and abrogated by H89. AC is not the target of AACOCF}_3\text{ action. Cardiomyocytes, isolated from adult rats were loaded with Fura 2-AM, were electrically stimulated at 0.5 Hz, and exposed for 3 min to the } \beta_2\text{-AR agonist, zinterol, in the absence (control) or in the presence of AACOCF}_3\text{ (10 } \mu\text{M) added with or without H89 (3 } \mu\text{M), as described under "Experimental Procedures." A, typical traces of recorded } [\text{Ca}^{2+}]_i \text{ responses are representative of at least six cells obtained from three different isolations. B, amplitude of } [\text{Ca}^{2+}]_i \text{ transients and fractional shortening are normalized to control values determined at time 0. Values are mean } \pm \text{ S.E. of effects observed on at least six cells obtained from three different isolations. C, adenylyl cyclase activity was measured in membranes prepared from isolated cardiomyocytes, as described under "Experimental Procedures." Results were obtained from triplicate determinations. *, } p < 0.05 \text{ versus control.}
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control experiments performed with a \( \beta_2 \)-AR selective stimulus, namely 100 nM isoproterenol added with 100 nM ICI 118,551, showed a marked PLB phosphorylation on Ser16, that was insensitive to AACOCF3 treatment (not shown). Thus, the cPLA2 constraint was specific to \( \beta_2 \)-AR- and did not affect \( \beta_2 \)-AR-induced Ser16-PLB phosphorylation. It should be noted that, in quiescent rat ventricular myocytes, nonspecific \( \beta_2 \)-AR stimulation (in response to norepinephrine) has been shown to increase PKA-dependent phosphorylation of PLB at Ser16 with little impact on the CaMKII-dependent phosphorylation of Thr17. In fact, in contrast with our previous observations in isolated spontaneously beating heart (18), we did not detect herein any phosphorylation of PLB on Thr17 in response to either \( \beta_2 \)- or \( \beta_2 \)-AR stimulation, in the absence as well as in the presence of AACOCF3 (not shown). Such an observation agrees with the reported pacing-dependence of the phenomenon (36, 38).
Integrity of Caveolae Is Required to Observe the cPLA₂-dependent Constraint of β₂-AR-induced Ca²⁺ Responses—In adult rat cardiomyocytes, β₂-ARs are preferentially concentrated in caveolae, together with G proteins (i.e. Gₐ, Gᵢ), effectors (i.e. AC), kinases (i.e. PKA, protein kinase C, mitogen-activated protein kinase) and adaptor proteins (i.e. AKAP, NHERF). Importantly, such a localization has been shown to dictate β₂-ARs downstream signaling pathways (25, 39, 40). Caveolae are characteristic flask-shaped invaginations of the plasma membrane that are distinctively enriched in cholesterol (41). Next, experiments were designed to evaluate the impact of caveolar structures on the constraint exerted by the cPLA₂ pathway on β₂-AR/AC/PKA-induced Ca²⁺ responses. We used Filipin III, a detergent that disrupts caveolar structures by binding to cholesterol. Cardiomyocytes loaded with Fura 2-AM were pretreated with Filipin III (0.2 μg/ml) for 30 min, including a pretreatment with or without AACOCF₃ (10 μM) during the last 10 min, and then exposed to zinterol (1 μM). Filipin III by itself produced a 149 ± 7% increase in the amplitude of basal [Ca²⁺], transients, that might reflect the accessibility of new targets to messengers (i.e. cAMP) or effectors (i.e. PKA) previously sequestered inside caveolar structures (Fig. 6). Subsequent addition of zinterol (1 μM) led to a final 198 ± 10% increase. However, zinterol action was no more sensitive to AACOCF₃ treatment (10 μM) during the last 10 min of incubation. Cardiomyocytes were then exposed for 3 min to the β₂-AR agonist zinterol (1 μM). The amplitude of [Ca²⁺] transients was normalized to control values determined at time 0, before the addition of Filipin III. Values are mean ± S.E. of effects observed on at least six cells obtained from two different isolations. *, p < 0.05 versus control

Next, experiments were performed using the most enriched caveolin-3 fraction, namely fraction 7, which was separated because of a 32 ± 1.5% sucrose density and corresponded to a mean 14 ± 3% of the total cellular proteins (Fig. 7A). Identical quantities of proteins from fraction 7, isolated from cells submitted to the four different treatments (described above), were separated on preparative gels. Following electrophoretic separation, proteins were cut in small pieces and analyzed, in parallel, for caveolin-3-enriched fractions recovered in light sucrose gradient fractions that contained only a minor portion of total cellular protein (Fig. 7A).

FIG. 6. Treatment with Filipin III abrogates the enhancing effect of AACOCF₃ on zinterol-induced Ca²⁺ response. Cardiomyocytes, isolated from adult rats, were loaded with Fura 2-AM and electrically stimulated at 0.5 Hz. They were treated for 30 min with Filipin III (0.2 μg/ml), and with or without AACOCF₃ (10 μM) during the last 10 min of incubation. Cardiomyocytes were then exposed for 3 min to the β₂-AR agonist zinterol (1 μM). The amplitude of [Ca²⁺] transients was normalized to control values determined at time 0, before the addition of Filipin III. Values are mean ± S.E. of effects observed on at least six cells obtained from two different isolations. *, p < 0.05 versus control

FIG. 5. AACOCF₃ treatment uncovers phosphorylation of (Ser¹⁶⁶)-PLB residue in response to zinterol. Cardiomyocytes, isolated from adult rats, were (or not) pretreated for 10 min, maintained with AACOCF₃ (10 μM), and treated for 10 min with or without zinterol (1 μM). Cardiomyocytes obtained from each of four distinct conditions of incubation (control, zinterol, AACOCF₃, AACOCF₃ + zinterol) were homogenized, and protein samples (50 μg) were subjected to SDS-PAGE. Membranes were immunostained with antibodies against P-(Ser¹⁶)-PLB, as described under “Experimental Procedures.” A representative immunoblot is shown in the upper panel. Average data (densitometric evaluation with arbitrary units) are presented in the bottom panel (mean ± S.E. of four experiments. *, p < 0.05 versus AACOCF₃ treatment.}

**cPLA₂ Recruit to Caveolae/SR Platforms Hinders Zinterol-induced Phosphorylation of eNOS and PLB—**We sought to further investigate the role of caveolae in β₂-AR signaling pathways. We incubated isolated adult cardiomyocytes with or without zinterol (1 μM), in the presence or in the absence of AACOCF₃. We prepared a low density caveolin-3-enriched membrane fraction using extraction in detergent-free alkaline sodium carbonate buffer followed by centrifugation in a continuous sucrose gradient, as described recently (25). As expected caveolin-3-enriched fractions were recovered in light sucrose gradient fractions that contained only a minor portion of total cellular protein (Fig. 7A).
eNOS is a well known marker of caveolae, and the phosphorylation of eNOS at Ser<sup>1177</sup> is usually correlated with eNOS activation. Similar levels of eNOS were detected in the four fraction 7 samples. Interestingly, a large increase in eNOS phosphorylation level was observed, in response to zinterol added together with AACOCF<sub>3</sub>. Thus, zinterol-induced AACOCF<sub>3</sub>-dependent production of NO (Fig. 4) could be related with phosphorylation and activation of eNOS, sedimented in the caveolin-3-enriched membrane fraction.

Interestingly, all caveolin-3-enriched fraction 7 obtained from the different treatments contained comparative amounts of PLB, with phosphorylation of PLB on Ser<sup>16</sup> detected only in response to zinterol added with AACOCF<sub>3</sub>, in accordance with results described in Fig. 5. Similarly, SERCA 2a was also detected in equal amounts in the different caveolin-3-enriched fraction 7. These results argued for an association between caveolae and a portion of SR membranes in adult rat cardiomyocytes, close enough to lead to their pull-down together during cellular fractionation.

We performed confocal microscopy in isolated adult rat cardiomyocytes immunostained with SERCA 2a and caveolin-3 antibodies in order to compare intracellular distribution of the two proteins (Fig. 8). Immunostained cardiomyocytes displayed a striated and regular actin pattern as illustrated by phalloidin staining (Fig. 8A, panel b). As previously reported in adult mouse cardiomyocytes (27, 42), SERCA 2a exhibited a longitudinal and transverse distribution and was also concentrated around the nucleus (Fig. 8A, panel c). Caveolin-3 was present all along the peripheral cellular membrane, the intercalated disks and exhibited a punctuated distribution within the T-tubules (Fig. 8A, panel d). The merge image (Fig. 8A, panel a) showed partial overlapping of the two fluorescent stainings (yellow). Analysis of the overlapping of SERCA 2a and caveolin-3 stainings, using distribution of fluorescence intensities, as shown in the typical histogram in Fig. 8B, demonstrated a mean overlapping distance of 640 ± 36 nm largely above the limits of resolution of the confocal microscope (100 nm). Overlapping pixels represented 25 ± 5% of green pixels (SERCA 2a).
and 43 ± 7% of red pixels (caveolin-3) (Fig. 8C, area 3). In addition to the well known major distribution of the SERCA 2a along the longitudinal SR and around the nucleus, these results strongly supported the close vicinity of a significant portion of SERCA 2a-enriched membranes (SR) with a portion of caveolin-3-enriched membranes (caveolae).

Taken together, these results show that cPLA2 limits phosphorylation of eNOS and PLB via its translocation toward caveolae/SR functional platforms, gathering 2-AR signaling effectors.

DISCUSSION

The new findings of this study are 3-fold. First, we show that β2-AR, through stimulation of a PTX-sensitive G protein and MSK1, evokes cPLA2 activation. Second, we give evidence that β2-AR-induced cPLA2 activation is linked to the translocation of the enzyme to low density caveolin-3-enriched membrane fractions that are closely associated with SR membranes and constitute functional signaling platforms. Inside those platforms we identified two components of the β2-AR/cPLA2 pathway, namely eNOS and PLB, showing that the blockade of cPLA2 unmasks zinterol-induced phosphorylation of eNOS and PLB. Finally, we demonstrate that the β2-AR/cPLA2 pathway, through inhibition of NOS phosphorylation and NO production, associated with the inhibition of PLB phosphorylation, selectively constrains the β2-AR/AC/PKA-induced Ca2+ response.

In rat cardiomyocytes, cPLA2 has been identified as a target of MSK1 in response to purinergic agonists stimulation (43, 44). We report for the first time that β2-AR agonists trigger phosphorylation of MSK1 that we find associated within caveolin-3/SR platforms.

Caveolae constitute a unique endocytic and exocytic compartment of the cell membrane, capable of importing molecules, delivering them to specific locations within the cell, and compartmentalizing a variety of signaling activities (41). Caveolae are a site of Ca2+ storage and entry into the cell. Our study provides evidence for β2-AR-induced translocation of the cPLA2 to platforms constituted of caveolae tightly connected to SR membranes. PLA2 activity has been implicated in constitutive membrane trafficking (45); however, only one recent study reported the presence of the cPLA2 in caveolin 1-enriched membrane fractions isolated from hippocampal preparations (46). Most caveolin-interacting proteins identified so far contain a caveolin-binding motif located within their enzymatically active catalytic site (41). As a matter of fact, such a caveolin-binding motif is present within the catalytic domain of the cPLA2. Nevertheless, up to date, activation of cPLA2 has been essentially associated with its Ca2+-dependent translocation from the cytosol to intracellular membranes, including perinuclear, endoplasmic, or SR membranes, but not to plasma membranes (47). We observe that treatment with AACOCF3 abrogates the association of the cPLA2 to caveolin-3/SR-platforms. One possible explanation relies on recent data showing that the cPLA2 catalytic domain modulates membrane association and membrane residence time of the enzyme (48). Because AACOCF3 is expected to target the catalytic site of the enzyme, competing with endogenous substrates, it might also affect interaction between caveolin-3 or SR membranes and cPLA2.

This study describes the cosedimentation of caveolae markers (caveolin-3 and eNOS) with SR markers (PLB and SERCA

FIG. 8. Confocal microscopic analysis of SERCA 2a and caveolin-3 distribution in adult rat cardiomyocytes. A, double immunostaining (visualized by confocal microscopy) of isolated adult rat cardiomyocytes with antibodies directed against SERCA 2a (green) and caveolin-3 (red) and DAPI coloration of nuclei (panel a). Details of immunolabeling are shown in panel c (SERCA 2a) and panel d (caveolin-3). Phalloidin and DAPI labeling (panel b) were used to reveal actin distribution and nuclei, respectively. Bar, 20 μm. B, overlapping of SERCA 2a and caveolin-3 staining in the area previously indicated in A, panel c and A, panel d. The upper panel presents the region of interest, and the lower panel details distribution of green and red fluorescence intensities along the white line (about 3.5 μm). C, typical scatterplot of the individual pixels from paired images. The threshold levels of green (139) and red (169) signals determined the overlapping region (area 3); areas 2 and 1 corresponding to green and red pixels, respectively, with no color mixing.
2a), suggesting the possible close interactions between the two types of membranes in cardiomycocytes. It is noteworthy that SR Ca$^{2+}$ uptake is reported to take place mainly in the longitudinal SR (away from caveolae), which contains the highest SERCA 2a density (49). In contrast, most of the SR Ca$^{2+}$ release would occur in proximity of the terminal cisternae (junctional and corbular SR), close to the T-tubules (49), where ryadonide receptors are concentrated (50). We provide confocal microscopic support for the major distribution of the SERCA 2a along the longitudinal SR and around the nucleus in adult rat cardiomycocytes. However, our experiments also demonstrate the close vicinity of a pool of SERCA 2a with caveolin-3-enriched membranes. These results are in agreement with the confocal microscopic study of Vangheluwe et al. (27), in adult mice cardiomycocytes, describing a close vicinity between SERCA and ryanodine receptor molecules, in an area close to the T-tubules. A similar localization of SERCA has been previously reported by Greene et al. (42). Interestingly, recent ultrastructural studies also describe the vicinity of the SR membranes with caveolae in airway smooth muscle cells (51), and caveolae are proposed to provide a platform of interaction between SR and plasmalemmal ion channels (52).

This study suggests that all components of the β$_2$-AR-cAMP pathway are located in caveolin-3/SR-interacting platforms where they can also be regulated by other signaling pathways, such as cPLA$_2$. The potentiating effect of AACTOF$_3$, which neutralizes cPLA$_2$ action, seems to rely on phosphorylating of target proteins already present inside such platforms rather than on a recruitment of effectors toward platforms. It is noteworthy that caveolae have been reported to retain eNOS in the inactive state, because eNOS activity is inhibited by binding to the caveolin scaffold domain. However, our results identify the phosphorylated form of eNOS in the caveolin-3-enriched membrane fraction, which argues for its activation inside caveolae. In fact, parallel imaging studies show that adult rat cardiomycocytes treated with AACTOF$_3$ plus zinterol produce NO.

Our findings that β$_2$-AR-induced cPLA$_2$ activation impairs β$_2$-AR-induced NO release agree with the previously reported inhibition of NO release by AA in PC12 cells (53). NO release, evoked by β$_2$-AR agonists in the presence of the cPLA$_2$ inhibitor, AACTOF$_3$, is associated with an augmented Ca$^{2+}$ response. Data from the literature point out the complexity of the modulatory effects of NO on the contractile function. NO influences the positive β-adrenergic inotropic effect in a bimodal fashion, depending not only on its concentration but also on that of catecholamines (54). And the group of Lipsius, in particular, reported an activatory role of NO on β$_2$-AR-mediated contractile effects (32, 33). Several mechanisms can underlie the potentiation of the Ca$^{2+}$ response by NO, including inhibition of the cG$_1$-PDE that would favor cAMP pathways (55), and nitrosylation of the L-type Ca$^{2+}$ channel (56) and the ryanodine receptor that would stimulate channel activities (57). Furthermore, through nitrosylation of enzymes of the respiratory complexes, NO enhances electromechanical coupling (58). It should be noted that potentiation by NO of the β$_2$-AR/PKA-induced Ca$^{2+}$ response, through the blockade of the cPLA$_2$ pathway, described herein in isolated adult rat cardiomycocytes, is not observed in cat atrial myocytes. In the latter, detectable NO production in response to zinterol does not require the blockade of cPLA$_2$ (32, 33).

Xiao and co-workers (35) previously identified PI3K as a downstream target of β$_2$-AR signaling, in adult rat cardiomycocyte. Our study highlights clear homology between β$_2$-AR/PI3K and β$_2$-AR/cPLA$_2$ pathways since they both confine and negate concurrent β$_2$-AR/G$_i$-mediated PKA signaling, without affecting β$_1$-AR-induced responses. PI3K inhibition, as well as cPLA$_2$ inhibition, enables the β$_2$-AR/PKA signaling to reach and to phosphorylate intracellular substrates such as phospholamban, and markedly enhance the β$_2$-AR-positive contractile response in cardiac myocytes. Likewise, both potentiating effects are mediated through a PTX-sensitive G protein and are not accompanied by an increase in cAMP formation. Because of their similar impact on β$_2$-AR/PKA signaling, a link between the two mechanisms cannot be ruled out. One hypothesis would be that both pathways share a common effector. One of the potential targets of arachidonic acid might be the protein phosphatase 5, a SerThr phosphatase expressed in the heart, that is activated by lipids and calyculin A (59–61). Note that Xiao and co-workers have suggested that β$_2$-AR-induced PI3K activation resulted in the stimulation of a calyculin-sensitive protein phosphatase activity. However, a number of studies argue in favor of the divergence of cPLA$_2$ and PI3K signaling pathways. Thus, studies on transgenic mice have shown that β$_2$-AR signaling bifurcates at the level of two different PTX-sensitive G$_i$ proteins, G$_{ai_2}$ and G$_{ai_3}$, leading to different effects depending of the G$_i$ isoform. G$_{ai_2}$ takes an essential protective part in the chronic signaling of overexpressed β$_2$-AR, leading to prolonged survival and delayed cardiac pathology. In contrast, G$_{ai_3}$ is supposed to mediate β$_2$-AR induced reduction of Ca$^{2+}$ channel activity (8), potentially through the activation of the PI3K isoform, a downstream target of G$_{ai_3}$-β$_3$ signaling (32, 56). Knockout of the PI3Ky gene results in a net increase in cardiac contractility without impact on cardiomyocyte hypertrophy (35, 62). In contrast, the major role attributed to the cPLA$_2$ is a critical regulation of muscular cell size (16), and the knockout of the cPLA$_2$ gene produces cardiac hypertrophy without change in basal cardiac contractility. Studies performed in cardiomycocytes indicate that activation of PI3K stimulates rather than inhibits NO production (63, 64). In this report, we demonstrate that eNOS inhibition is an essential component of the cPLA$_2$ limiting action.

Our present study in adult rat ventricular cardiomycocytes further substantiate the major role in the heart of the β$_2$-AR/cPLA$_2$ pathway (65), beyond that of the β$_2$-AR/AC/PKA pathway, a role that we previously suspected from studies in human tissues (10) and embryonic chick cardiomycocytes (23, 66). It appears that the respective roles of each, β$_2$-AR/cPLA$_2$ and β$_2$-AR/AC/PKA pathways, depend on the species or the pathological state. Thus, in adult rat cardiomycocytes, the β$_2$-AR/AC/PKA pathway mediates the Ca$^{2+}$ response triggered by β$_2$-AR agonists, and is constitutively depressed because of the concomitant activation of the β$_2$-AR/cPLA$_2$ pathway. In contrast, in embryonic chick heart cells, cPLA$_2$ activation exclusively mediates β$_2$-AR stimulation of [Ca$^{2+}$]$^{+}$ cycling and cell contraction, (23, 66). In human tissue, β$_2$-AR activates both AC and cPLA$_2$ activities, the β$_2$-AR/cPLA$_2$ pathway being favored under conditions of altered β$_2$-AR/AC/PKA signaling (10). Taken together, those data pose the question as to the contractile impact of the cPLA$_2$ in human heart, and its possible evolution in the course of heart failure or aging that both represent physiopathological situations associated with a degradation of the β$_2$-AR/AC coupling (1). Another remaining important question is whether activation of the cPLA$_2$ takes part in the cardiac protective effect of β$_2$-AR stimulation, with eNOS inhibition as an essential component of its limiting action on the AC/PKA pathway.

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