We have recently reported that arachidonic acid mediates beta-adrenergic receptor (AR) stimulation of [Ca^{2+}]_i cycling and cell contraction in embryonic chick ventricular cardiomyocytes (Pavoine, C., Magne, S., Sauvadet, A., and Pecker, F. (1999) J. Biol. Chem. 274, 628–637). In the present work, we demonstrate that beta-AR agonists trigger arachidonic acid release via translocation and activation of cytosolic phospholipase A_2 (cPLA_2) and increase caffeine-releasable Ca^{2+} pools from Fura-2-loaded cells. We also show that beta-AR agonists trigger a rapid and dose-dependent phosphorylation of both p38 and p42/44 MAPKs. Translocation and activation of cPLA_2, as well as Ca^{2+} accumulation in sarcoplasmic reticulum stores sensitive to caffeine and amplification of [Ca^{2+}]_i cycling in response to beta-AR agonists, were blocked by inhibitors of the p38 or p42/44 MAPK pathways (SB203580 and PD98059, respectively), suggesting a role of both MAPK subtypes in beta-AR signaling. In contrast, beta-AR stimulation of [Ca^{2+}]_i cycling was rather limited by the MAPKs, clearly proving the divergence between beta-AR and beta_1-AR signaling systems. This study presents the first evidence for the coupling of beta-AR to cardiac cPLA_2 and points out the key role of the MAPK pathway in the intracellular signaling elicited by positive inotropic beta-AR agonists in heart.

**Beta-2-Adrenergic Receptor Agonists Increase Intracellular Free Ca^{2+} Concentration Cycling in Ventricular Cardiomyocytes through p38 and p42/44 MAPK-mediated Cytosolic Phospholipase A_2 Activation**

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‡ The abbreviations used are: AR, adrenergic receptor; AA, arachidonic acid (cis-5,8,11,14-eicosatetraenoic acid); SR, sarcoplasmic reticulum; A2COCF_3, arachidonyl trifluoromethyl ketone; PL�_2, phospholipase A_2; cPLA_2, cytosolic phospholipase A_2; sPLA_2, secreted phospholipase A_2; PL2A, Cyto PL_2A, Ca^{2+}-independent phospholipase A_2; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; PMA, phorbol 12-myristate 13-acetate; [Ca^{2+}]_i, intracellular free Ca^{2+} concentration; HLESS, Halse-nol lactate suicide substrate; PBS, phosphate-buffered saline; PC, 1-stearoyl-2-arachidonylphosphatidylethanolamine; DTT, dithiothreitol; PC2, polyvinylidene difluoride; MEK, mitogen-activated protein ki-nase/extracellular signal-regulated kinase kinase; MSK, Mitogen- and stero-activated protein kinase; MNK, MAPK-interacting kinase; PTX, pertussis toxin.

MAPKs are a family of Ser/Thr kinases that comprises the extracellular signal-regulated kinases (ERKs or p42/44 MAPKs), the c-Jun N-terminal kinases (JNKs), and p38 cyclase via coupling to G_s (1–4). However, the role of cAMP in the functional response to beta-AR stimulation is controversial, and there is accumulating evidence that beta_2-AR agonists could modulate cardiac excitation-contraction coupling via a cAMP-independent mechanism (1). We have recently identified an alternative pathway, relying the positive inotropic effect of beta_2-AR agonists in embryonic chick heart cells (5). In these cells, stimulation of beta_2-ARs triggers the production of arachidonic acid (AA) via a pertussis toxin-sensitive G protein pathway. We also demonstrated that AA induces 45Ca^{2+} accumulation in sarcoplasmic reticulum (SR) stores sensitive to caffeine, a mechanism likely to support positive inotropy (6). The beta_2-AR elicited contractile effect is independent of adenylyl cyclase activation and cAMP production and totally relies on AA release. Although inhibition of the beta_2-AR-induced responses by A2COCF_3 suggested the role of a Ca^{2+}-dependent cytosolic phospholipase A_2 (cPLA_2) as the effector of beta_2-AR, direct evidence for the coupling of beta_2-AR to cPLA_2 activation remained to be established.

Phospholipase A_2 comprises a large family of enzymes that hydrolyze the sn-2-fatty acyl ester bonds of membranous phospholipids, leading to the liberation of free fatty acids including AA. PLPL_2 enzymes are classified according to localization (extracellular versus intracellular), sequence homology, and biochemical characteristics (7). Extracellular PLPL_2 enzymes, also referred to as secreted PLPL_2 (sPLPL_2) enzymes, represent a growing family of enzymes with five distinct mammalian sPLPL_2 enzymes already identified (8). To date, four intracellu-lar PLPL_2 sequences have been reported: two Ca^{2+}-dependent PLPL_2 enzymes (cPLA_2α and the recently identified cPLA_2β) (9, 10) and two Ca^{2+}-independent PLPL_2 enzymes (iPLA_2 and cPLA_2γ) (11, 12). Of particular interest is the Ca^{2+}-dependent cPLA_2α (referred to as cPLA_2 below), which plays an essential role in hormone-induced AA release (13) with major implications in reproductive function and inflammation, as confirmed recently by studies using cPLA_2-deficient mice (14, 15). cPLA_2 is characterized by a molecular mass of 85 kDa, activation by low (micromolar) concentrations of calcium, selectivity for arachidonyl in the sn-2-position of phospholipids, and sensitivity to inhibitors such as A2COCF_3 and methyl arachidonyl fluorophosphate (MAFP) (16, 17). cPLA_2 is fully activated by both phosphorylation and increases in intracellular calcium, which drive its translocation from the cytosol to membranes in a process utilizing a Ca^{2+}-dependent phospholipid-binding domain (C2 domain) in the N-terminal region of the enzyme. In a variety of cell types, phosphorylation of cPLA_2 is achieved by p42/44 mitogen-activated protein kinases (MAPKs) and/or the MAPK homolog p38 (16, 18).


MAPks (for a review, see Ref. 19). In cardiac myocytes, the ERK cascade is activated principally by G protein-coupled receptor agonists and peptide growth factors. JNKs and p38 MAPks are stimulated by hyperosmotic shock, hypoxia/reoxygenation, reactive oxygen species, and mechanical stress, but also by G protein-coupled receptors such as endothelin-1, phenylephrine (α-AR agonist), and angiotensin II (for a review, see Ref. 20). There is considerable evidence that all MAPks participate in the long-term myocyte hypertrophic response triggered by endothelin-1, phenylephrine, and phorbol 12-myristate 13-acetate (PMA). Thus, the literature clearly demonstrates the immense potential significance of the regulation of MAPk pathways in the myocardium with respect to its reactions to pathological stresses (e.g. hypoxia, ischemia, reperfusion injury, hypertension, and inflammatory diseases). In contrast, the participation of MAPks in cardiac physiological intracellular signaling is poorly documented.

In this study, we show that, in embryonic chick heart cells, β2-AR stimulation triggers cPLA2 translocation to the membranes, AA release, Ca2+ accumulation in SR stores sensitive to caffeine, and amplification of [Ca2+]cytosol wave activity. Thus, the literature clearly demonstrates the immense potential significance of the regulation of MAPk pathways in the myocardium with respect to its reactions to pathological stresses (e.g. hypoxia, ischemia, reperfusion injury, hypertension, and inflammatory diseases). In contrast, the participation of MAPks in cardiac physiological intracellular signaling is poorly documented.

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buffer A supplemented with 5% fetal calf serum, were plated in 60-mm plates and incubated in humidified 5% CO₂ and 95% air at 37 °C. After 24 h, the culture medium was changed to serum-free buffer A. A 24 h later, cells were exposed to various agonists and/or enzymatic inhibitors diluted in buffer A and incubated for various periods of time at 37 °C. Incubation was terminated by the removal of the medium and the inclusion of 2 mM EGTA (no added EGTA) or its absence (2 mM EGTA added) in the ice-cold lysis buffer. Cells were washed and resuspended in ice-cold lysis buffer (50 mM HEPES (pH 7.4), 10% (v/v) glycerol, 135 mM NaCl, 1 mM EGTA, 10 mM NaF, 10 mM NaOAc, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 1 μg/ml pepstatin, 40 μg/ml β-glycero-phosphate, and 100 μg/ml DTT). Cells were wrapped and allowed to lyse by vortexing X-5000 for 15 min at 4 °C. In the first series of experiments (cPLA₂ study; see Fig. 5), whole homogenates were used. In the second series of experiments (MAPK study; see Fig. 4), cell homogenates were transferred to microcentrifuge tubes and centrifuged at 20,000 × g for 15 min at 4 °C. Samples of the 20,000 × g supernatant containing 50 μg of proteins (MAPK study; see Fig. 4) or whole cell homogenates containing 100 μg of proteins (cPLA₂ study; see Fig. 5) were taken, lyophilized, resuspended in Laemmli loading buffer, frozen in liquid nitrogen, and stored at −80 °C. Samples were boiled for 5 min prior to electrophoresis.

**Immunoblot Analysis of the Distribution of PLA2 Activities in the 100,000 × g Supernatant**

Electrophoresis was performed on 4%–15% SDS-polyacrylamide gel (25 mA/gel). Proteins were transferred to Protran nitrocellulose membrane (Schleicher & Schuell) by electroblotting using Tris/glycine/SDS buffer containing 20% methanol (120 mA for 1 h at 4 °C). Protein transfer was evaluated by staining the gel with Coomassie Blue. Equal loading of proteins in each lane was checked by Ponceau red staining of the membrane. The nitrocellulose blots were agitated for 30 min at room temperature in TBST (10 mM Tris-HCl (pH 8), 150 mM NaCl, and 0.05% Tween 20) and then for 1 h in TBST supplemented with 5% nonfat dry milk prior to overnight incubation with primary antibodies against phosopho-MAPKs (1:2000 dilution) in TBST supplemented with 5% nonfat dry milk at 4 °C. Membranes were washed three times with TBST and incubated with peroxidase-conjugated swine anti-rabbit IgG (1:1000 dilution) for 1 h at room temperature in TBST containing 5% nonfat dry milk. Membranes were washed three times with TBST, and the peroxidase activity was determined using the enhanced chemiluminescence Western blot detection system (ECI, Amersham Pharmacia Biotech).

**Immunofluorescence Microscopy**

Embryonic chick heart cells (3 × 10⁵ cells/ml), suspended in buffer A supplemented with 5% fetal calf serum, were plated on plastic chamber slides (Lab-Tek, Nunc) coated with laminin (1 μg/ml) and incubated in humidified 5% CO₂ and 95% air at 37 °C. After 24 h, the culture medium was changed to serum-free buffer A. After a 1-h pretreatment with or without MAPK inhibitors and/or a 10-min preincubation with or without β₂-AR antagonists, cells were incubated for the indicated periods of time with or without selective β₂-AR agonists. Cells were washed twice briefly with PBS and allowed to dry. Cells were fixed for 10 min at room temperature in aceton, washed with PBS, and then permeabilized in 0.2% Triton X-100 and PBS for 15 min at 4 °C. After three brief washes, blocking was performed in PBS containing 10% sheep serum for 30 min at 37 °C. Cells were incubated with anti-cPLA₂ antibody (1:250 dilution) overnight at 4 °C and washed three times for 5 min with PBS at room temperature. Incubation with Cy3-conjugated anti-rabbit antibody (1:500 dilution) was then performed for 1 h at room temperature in the dark. The cells were washed 3 × 10 min with PBS and rinsed for 5 min with water. 10 μl of Vectashield mounting medium was applied to the cell surface, and coverslips were mounted. Controls were carried out by replacing the primary antibody with PBS. Slides were viewed by fluorescence microscopy on a Zeiss Axioscan microscope (magnification ×630).

Statistics—Results are expressed as means ± S.E. of n experiments and analyzed by unpaired Student’s t test, one-way analysis of variance, or non-parametric analysis of variance, as appropriate, with p < 0.05 considered to be significant.

**RESULTS**

**Distribution of PLA2 Activities in the 100,000 × g Fractions of Embryonic Chick Ventricular Cells—**We measured PLA2 activities in the 100,000 × g fractions prepared from embryonic chick ventricular cells. We used the preferential substrate of cPLA₂, 1-stearyl-2-[1-¹⁴C]arachidonylphosphatidylcholine, in the absence or presence of specific inhibitors of the different PLA₂ isoforms, viz. DTT, HELOSS, and AAOCCF₃, to block the sPLA₂, iPLA₂, and cPLA₂ activities, respectively. In the absence of inhibitors, total PLA₂ activity in the 100,000 × g supernatant fraction was 4–7 times higher than in the 100,000 × g pellet fraction. Thus, we used the 100,000 × g supernatant fraction to determine optimal conditions for the measurement of the AAOCCF₃-sensitive PLA₂ activity (cPLA₂). As shown in Fig. 1A, in the absence of inhibitors, the AAOCCF₃-sensitive PLA₂ activity (cPLA₂) represented 73% of the total PLA₂ activity. The addition of the sPLA₂ inhibitor DTT strongly and selectively improved the AAOCCF₃-sensitive cPLA₂ activity by 200%, presumably through stabilization of this oxidation-sensitive enzyme (25). In contrast, the cPLA₂ activity was not significantly affected upon addition of HELOSS. In the presence of DTT and HELOSS, cPLA₂ assay was linear with respect to time and protein (data not shown) and displayed Ca²⁺-dependent characteristics of cPLA₂, with maximal activation obtained at 1 mM Ca²⁺ and half-maximal stimulation occurring at 0.3 mM Ca²⁺ (Fig. 1B). Thus, the cPLA₂ activity for cPLA₂ was validated in both the 100,000 × g supernatant and pellet fractions of embryonic chick heart cells by the total blockade of PLA₂ activity upon addition of 10 μM AAOCCF₃ (Fig. 1C).

β₂-AR Agonists Activate cPLA₂ in Embryonic Chick Heart Cells—Embryonic chick heart cells were exposed to β₂-AR stim-


**β₂-AR/MAPK/cPLA₂ Pathway in Cardiomyocytes**

**FIG. 1.** PLA₂ activities in the 100,000 × g subcellular fractions of embryonic chick ventricular cells. A, effect of DTT and HELSS on the AACOCF₃-sensitive PLA₂ activity in the 100,000 × g supernatant fraction; B, Ca²⁺-dependent stimulation of PLA₂ activity measured in the presence of DTT and HELSS in the 100,000 × g supernatant fraction; C, AACOCF₃-sensitive PLA₂ activity measured in the presence of DTT and HELSS. The 100,000 × g supernatant and pellet fractions of embryonic chick ventricular cells were prepared, and PLA₂ activity was measured using 1-stearoyl-2-[1-³⁴C]arachidonylphosphatidylcholine as substrate as described under “Experimental Procedures.” The reaction was carried out with 30–50 μg of proteins from the 100,000 × g supernatant or pellet fraction. Assays were performed in the absence or presence of 10 μM AACOCF₃ and/or 2 mM DTT and/or 10 μM HELSS as indicated. In B, free Ca²⁺ concentrations were prepared using CaCl₂/EGTA buffers and determined by measurement of Fura-2 fluorescence. PLA₂ activity is expressed in total disintegrations/min (A and C) or as a percentage of maximal stimulation (B, with 100% maximal stimulation corresponding to 10,240 ± 275 total dpm). Total disintegrations/min corresponded to 450, 870, and 995 μg of total proteins in supernatants (A–C, respectively) and 660 μg of total proteins in the pellet (C). Total [³⁴C]PC radioactivity added to assays was 150,000 dpm (A) and 200,000 dpm (B and C). Values are the means ± S.E. of triplicate determinations from a typical experiment that was repeated twice. *, p < 0.01.

**FIG. 2.** β₂-AR stimulation elicits redistribution of cPLA₂ activity from the 100,000 × g supernatant fraction to the 100,000 × g pellet fraction (A) and induces [³⁴H]AA release (B). A, cPLA₂ activity was measured in 100,000 × g fractions (30–50 μg of proteins/assay) in the presence of 2 mM DTT and 10 μM HELSS after hormonal stimulation had been performed for 20 min as described under “Experimental Procedures.” PLA₂ activity is expressed as a percentage of the control (100% values were 2200 ± 400 and 12,200 ± 2000 total dpm in the pellet and supernatant fractions, respectively, and corresponded to 528 ± 40 and 970 ± 96 μg of total proteins, respectively). Values are the means ± S.E. of nine experiments, performed in triplicate. *, p < 0.01. zint, zinterol; iso, isoproterenol; CGP, 20712A. B, embryonic chick heart cells were labeled for 24 h with 1.5 μCi/ml [³⁴H]AA, washed twice with saline buffer containing 0.2% fatty acid-free bovine serum albumin, and incubated for the indicated periods of time with 30 nM zinterol as described under “Experimental Procedures.” [³⁴H]AA release in control cells corresponded to 1337 ± 187 dpm. Results expressed in disintegrations/min were corrected for the release in control cells and are the means ± S.E. of three different experiments.

induced a dose-dependent decrease in cPLA₂ activity in the 100,000 × g supernatant fraction, with a maximal effect observed at 30 nm zinterol (Fig. 2A). In parallel, 30 nm zinterol elicited an increase in cPLA₂ activity in the 100,000 × g pellet fraction (Fig. 2A). Zinterol effects on cPLA₂ activity were reproduced with a distinct β₂-AR stimulus, achieved with isoproterenol, a non-selective but complete β-agonist, added together with CGP 20712A, a selective β₂-AR antagonist (Fig. 2A). AACOCF₃ abrogated the effects of β₂-AR agonists on cPLA₂ activity (data not shown). Experiments performed in embryonic chick heart cells prelabeled with [³⁴H]AA indicated that zinterol stimulation resulted in a rapid (detected within 30 s) and sustained AA release over the 15 min examined (Fig. 2B).

Zinterol-induced translocation of cPLA₂ was examined by immunofluorescent staining of myocytes using anti-cPLA₂ antibody (Fig. 3). Unstimulated cells showed diffuse fluorescence throughout the cell (Fig. 3B). This staining specifically represented cPLA₂ as demonstrated by the negative staining pattern obtained when the anti-cPLA₂ primary antibody was omitted (Fig. 3A). In contrast, after stimulation with 30 nm zinterol, the

[Image 69x281 to 277x729]

[Image 316x419 to 547x729]
staining appeared to be concentrated at a perinuclear region of the cells (Fig. 3, C–F). These results are in agreement with a zinterol-induced redistribution of cPLA2 from the cytoplasm to the nuclear and/or SR membranes, both networks being tightly associated in our cells. cPLA2 translocation was almost maximal as soon as 30 s after stimulation with zinterol (Fig. 3C) and persisted during the first 30-min period examined (Fig. 3F). In keeping with these results, the linear kinetics of zinterol-induced AA release shown in Fig. 2 also argued for maximal activation of cPLA2 as soon as 30 s after zinterol application. It should be noted that immunofluorescent staining of cPLA2 in zinterol-treated cells appeared to be frequently and consistently more intense than in untreated cells. This was most likely due to a greater loss of soluble cPLA2 than of membrane-bound cPLA2 throughout the staining procedure. A similar interpretation has already been proposed in at least two other studies (see Ref. 26). Alternatively, as proposed by Schievelbein et al. (26), binding of cPLA2 to membrane structures might result in a better exposure of the epitope for the primary antibody, allowing a more efficient antibody binding.

p38 and p42/44 MAPKs Are Phosphorylated upon β2-AR Stimulation—Since phosphorylation of p38 and p42/44 MAPKs is a prerequisite for MAPK activation, we evaluated the phosphorylation state of either p38 or p42/44 MAPK in response to β2-AR stimulation using specific anti-phospho-MAPK antibodies. Zinterol increased the phosphorylation of both p38 and p42/44 MAPKs in a dose-dependent manner, with maximal phosphorylation of p42/44 and p38 MAPKs occurring at 3 and 30 nM, respectively (Fig. 4A). The phosphorylation of both MAPK subtypes in response to 30 nM zinterol was totally prevented when cells were preincubated for 10 min with the selective β2-AR antagonist ICI 118551 at 100 nM prior to zinterol application, suggesting that zinterol induction of MAPK phosphorylation results from a strict β2-AR agonist effect (Fig. 4B).

β2-AR Stimulation Induces cPLA2 Activation through a p38 and p42/44 MAPK Pathway—We next used SB203580, the p38 MAPK inhibitor acting at the catalytic site of the p38 MAPK enzyme (27), and PD98059, the MEK kinase inhibitor reported to impair p42/44 MAPK phosphorylation and activation (28), to investigate the impact of the p38 and p42/44 MAPK pathways on zinterol-induced AA release and cPLA2 translocation. As shown in Fig. 6, the presence of either 1 mM PD98059 or 1 mM SB203580 totally blocked zinterol-induced AA release. Similarly, treatment of cardiomyocytes with either 1 mM PD98059 or 1 mM SB203580 did not affect the pattern of cPLA2 in unstimulated cells (Fig. 7, C and D compared with A), but prevented its translocation in response to zinterol (D and F compared with B). Taken together, these results demonstrate that, upon β2-AR challenging, both p38 and p42/44 MAPKs play a critical role upstream of cPLA2 translocation and activation.

To examine the contribution of [Ca2+]i in zinterol-induced cPLA2 translocation, cells were pretreated for 10 min with 1 mM EGTA. Incubation of the cells with EGTA did not affect the
pattern of cPLA$_2$ in unstimulated cells (Fig. 7, G compared with A). In contrast, it impaired zinterol-induced translocation of cPLA$_2$ (Fig. 7, H compared with B), suggesting that preservation of basal [Ca$^{2+}$]i is a crucial factor for redistribution of the enzyme in response to zinterol.

**β$_2$-AR Stimulation of [Ca$^{2+}$]i Cycling Requires p38 and p42/44 MAPK Activation and Relies on Zinterol-induced Calcium Loading of Caffeine-sensitive Stores—**As previously demonstrated (5), zinterol stimulation triggered a rapid increase in the amplitude of [Ca$^{2+}$]i transients of electrically stimulated embryonic chick ventricular cells (Fig. 8D), which was totally blunted upon addition of the cPLA$_2$ inhibitor AOCOCF$_3$ (Fig. 8C). We show here that the zinterol effect on [Ca$^{2+}$]i cycling was unaffected in the presence of HELSS, a selective iPLA$_2$ blocker (Fig. 8F). Thus, our present data rule out the possible participation of iPLA$_2$ and confirm the selective role of cPLA$_2$ in β$_2$-AR-induced responses. The aim of the next experiments was to evaluate the role of p38 and p42/44 MAPKs in the effect of β$_2$-AR agonists on [Ca$^{2+}$]i cycling. Preincubation for 1 h with 1 μM SB203580 (Fig. 8E), an inhibitor of p38 MAPK, or 1 μM PD98059 (Fig. 8D), an inhibitor of the p42/44 MAPK pathway, totally blunted the zinterol effect. These results demonstrate that, in electrically stimulated cells, zinterol-induced stimulation of [Ca$^{2+}$]i cycling requires p38 and p42/44 MAPKs and selective cPLA$_2$ activation.

In the absence of electrical stimulation, it is noteworthy that zinterol was without effect on [Ca$^{2+}$]i (Fig. 8A). To evaluate the role of SR calcium stores as targets for zinterol action, we used caffeine, previously reported to increase the opening probability of the calcium release channels of the SR compartments (29). We examined the effect of zinterol on [Ca$^{2+}$]i transients triggered by caffeine in Fura-2-loaded cells in the absence of electrical stimulation. The application of 10 mM caffeine produced a unique [Ca$^{2+}$]i transient (Fig. 9A). In contrast, the application of caffeine together with zinterol resulted in a train of [Ca$^{2+}$]i transients (Fig. 9B). These experiments demonstrate that, in the absence of electrical stimulation, zinterol action leads to Ca$^{2+}$ loading of caffeine-sensitive SR stores. Zinterol potentiation of caffeine-induced Ca$^{2+}$ mobilization from the SR was blunted in the presence of AOCOCF$_3$, PD98059, or SB203580 (Fig. 9, C–E, respectively), whereas it was insensitive to HELSS (Fig. 9F). Taken together, these results demonstrate that zinterol induces Ca$^{2+}$ loading of caffeine-sensitive SR stores through the activation of p38 and p42/44 MAPKs and selective cPLA$_2$ activation.
cPLA₂. It is noteworthy that Ca²⁺ loading of SR stores induced by zinterol did not impact on basal cytosolic [Ca²⁺] in the absence of electrical stimulation (Fig. 8A). In contrast, the release of Ca²⁺ accumulated in SR stores is likely to support zinterol-induced amplification of electrically stimulated [Ca²⁺] cycling.

β₂-AR/MAPK/cPLA₂ Pathway in Cardiomyocytes

We previously demonstrated that, in embryonic chick ventricular cells, the β₁-AR-mediated increase in [Ca²⁺] cycling and contraction relies on a rise in intracellular cAMP via activation of adenyl cyclase and is independent of AA production (5). In particular, β₁-AR stimulation does not trigger [³H]AA release (5). However, as previously reported by others in adult rat cardiac myocytes, we observed that β₂-AR stimulation induced a rapid phosphorylation of p38 and p42/44 MAPKs in embryonic chick heart cells (data not shown). Next, imaging
and perfused in the presence of 100 nM isoproterenol with 300 nM CGP 20712A. This dose dependence correlates with our previous data on zinterol-induced AA release (5). At such doses, zinterol is unequivocally known to act through β2-AR. Accordingly, zinterol-induced MAPK phosphorylation (this study) and [Ca^{2+}]_{i} cycling stimulation (5) are totally blocked in the presence of 100 nM ICI 118551, a selective β2-AR antagonist. Furthermore, zinterol effects are reproduced by a distinct β2-AR stimulus, viz. the combination of isoproterenol and CGP 20712A, a selective β1-AR antagonist.

Our results argue for the requirement of cPLA2 activation in the β2-AR stimulation of [Ca^{2+}]_{i} cycling. We show that β2-AR stimulation triggers a rapid translocation of cPLA2 that results in an increase in cPLA2 activity associated with the 100,000 × g pellet subcellular fraction and subsequent release of AA, leading to SR Ca^{2+} loading. The β2-AR-induced responses (cPLA2 translocation, AA release, and SR loading) are detected as soon as 30 s following the initiation of β2-AR stimulation, in keeping with amplification of [Ca^{2+}]_{i}, cycling detected within 1 min.

Our results highlight the essential role of the p38 and p42/44 MAPK pathways in the regulation of cPLA2 activity. Inhibitors of either the p38 or p42/44 MAPK pathway (SB203580 and PD98059, respectively) neutralize all zinterol-induced responses, viz. cPLA2 translocation, induction of AA release, increase in SR Ca^{2+} loading, and amplification of [Ca^{2+}]_{i} cycling. Indeed, our results are the first to suggest that p38 and p42/44 MAPKs are key steps in the transduction of β2-AR signaling and β2-AR-mediated inotropic responses in embryonic chick heart cells. This study indicates that p38 and p42/44 MAPKs act independently and simultaneously to activate cPLA2 and AA release. Similar dual MAPK activation has been reported in rat cardiomyocytes for cPLA2 stimulation by ATP (30) and in human neutrophils for cPLA2 activation by opsonized zymosan (31).

Our data indicate that the MAPK prerequisite concerns only β2-AR-triggered pathways. β1-AR-mediated effects on [Ca^{2+}]_{i} cycling are rather amplified upon inhibition of the MAPK pathways. We have previously demonstrated that, in embryonic chick heart cells, as in myocytes of other species, β1-AR-induced amplification of [Ca^{2+}]_{i} cycling is mediated by cAMP and is neither associated with AA release nor sensitive to cPLA2 blockers. However, we observed that β1-AR stimulation elicits phosphorylation of p38 and p42/44 MAPKs in embryonic chick heart cells (data not shown). This suggests that MAPK activation by itself is not sufficient to induce cPLA2 activation and that additional events are involved in β1-AR-induced responses. An additional possibility is that the β1-AR agonist second messenger, viz. cAMP, exerts a dominant-negative effect and mutes the activating effect of MAPK pathways on cPLA2. In fact, we have previously demonstrated that cAMP exerts a negative effect on the cPLA2/AA pathway in embryonic chick heart cells (5). Furthermore, inhibition of cPLA2 activity upon protein kinase A-mediated phosphorylation has been reported in smooth muscle cells (32).

Several phosphorylation sites on cPLA2 have been identified (33). Most studies have focused on MAPK-dependent phosphorylation of Ser505 since it results in a characteristic decrease in electrophoretic mobility of the enzyme. The importance of Ser505 phosphorylation in cPLA2 activation appears to be cell type- and agonist-specific (for a review, see Ref. 34); although it is essential for Ca^{2+} ionophore 4-bromo-A23187-induced AA release in Chinese hamster ovary cells overexpressing human cPLA2 (26), it is not required for AA release from thrombin-stimulated platelets (35). cPLA2 activation by MAPKs, independent of Ser505 phosphorylation, has been reported in mouse peritoneal macrophages and in human neutrophils (34). In fact, it has been shown that kinases downstream of MAPKs, MSK1...
and MNK1 (30, 36), can activate cPLA2. These kinases are likely to phosphorylate cPLA2 at sites other than Ser505 (in particular, Ser727) without impact on cPLA2 gel mobility (18). Our results suggest that, in embryonic chick heart cells, cPLA2 can be phosphorylated at Ser505 in response to the MAPK activator PMA. In contrast, activation of cPLA2 by zinterol, which also relies on MAPK activation, seems unrelated to the phosphorylation of Ser505 since it occurs in the absence of detectable impact on cPLA2 gel mobility. However, a limited Ser505 phosphorylation of cPLA2 in response to zinterol cannot be ruled out due to technical problems associated with detection of the chicken cPLA2 sequence (see “Methods”).

It is well documented that calcium induces binding of cPLA2 to membranes through the C2 domain. Basal cPLA2 activity in embryonic chick heart cells assayed in vitro exhibited a typical Ca²⁺ requirement (Fig. 1). In addition, a supraphysiological increase in [Ca²⁺], promoted by the addition of the Ca²⁺ ionophore 4-bromo-A23187 (2 μM for 10 min) led to the translocation of the enzyme to membranes (data not shown). In many cell models, agonist-induced cPLA2 activity and AA release require Ca²⁺ elevation (34). However, in S9 cells, okadaic acid-induced AA release requires basal Ca²⁺ levels, but is observed without an increase in [Ca²⁺]. (37). Similarly, in embryonic chick heart cells that were not submitted to electrical stimulation, i.e. in the absence of detectable [Ca²⁺], elevation, zinterol induced cPLA2 translocation and AA release. Disruption of cellular Ca²⁺ homeostasis by incubating cells with EGTA prevented zinterol-induced cPLA2 translocation. This suggests that, in response to zinterol stimulation, basal [Ca²⁺], is sufficient to support cPLA2 translocation. Thus, our results indicate that, at normal basal [Ca²⁺], MAPK activation plays an essential role in regulating zinterol-induced translocation of cPLA2. Whether additional mechanisms are involved remains to be elucidated.

Zinterol-induced Ca²⁺ accumulation in SR stores could result from the blockade of SR Ca²⁺ efflux through ryanodine receptors or the potentiation of SR Ca²⁺ influx via the SR Ca²⁺ pump. However, another attractive hypothesis concerns the possible involvement of sarcoplasmic reticulum AA-activated Ca²⁺ channels. In fact, a non-capacitative Ca²⁺ entry pathway, gated by AA and independent of store depletion, has been identified in non-excitable cells and in A7r5 smooth muscle cells (38, 39). Whether AA-activated Ca²⁺ channels exist in cardiac cells and are responsible for zinterol-induced SR loading remains an open question.

The coupling of β₂-AR to MAPKs has already been documented. However, studies focused on the long-term role of MAPKs, in particular in the hypertrophic and/or mitogenic responses or in receptor desensitization mechanisms. In heart, p42/44 MAPK activation is involved in the induction of cardiomyocyte hypertrophy in response to isoproterenol, a non-specific β-AR agonist (40). Studies performed in β₂-AR-transfected HEK293 cells have demonstrated an activation of p42/44 MAPKs by those receptors. In HEK293 cells, p42/44 MAPK activation follows β₂-AR desensitization and relies on a cAMP-mediated event; protein kinase A-mediated phosphorylation of β₂-AR uncouples the phosphorylated receptor from the adenyl cyclase stimulatory G protein (G₁₅), a process termed heterologous desensitization, and switches the β₂-AR coupling to G₁₅ with subsequent stimulation of p42/44 MAPK (41). Daaka and co-workers (42–44) have proposed that this provides one mechanism by which β₂-ARs not only regulate their own internalization, but also initiate an additional signal transduction pathway, in which the desensitized receptor might function as a structural component of a mitogenic signaling complex. Thus, mechanistically, β₂-AR-mediated MAPK activation in fibroblasts requires sequential coupling to G₁₅ and G₁₆. In the absence of any coupling of β₂-AR to the G₁₃/adenylyl cyclase pathway, the situation in embryonic chick heart cells is clearly different. Our results argue for a primary coupling of β₂-AR to a PTX-sensitive G protein (hypothetically G₁₅) and MAPKs. In addition, our data suggest that β₂-AR coupling to MAPK will represent an alternative to adenyl cyclase in supporting cardiac contractility rather than a termination signal.

Alternatively, other studies performed in rat, dog, and mouse hearts have suggested a dual coupling of β₂-AR to concurrent G₁₅ and PTX-sensitive G₁₆ signaling pathways (45–47). In those species, the β₂-AR/G₁₆ pathway seems to exert a negative control on the β₂-AR/G₁₅ pathway. Indeed, stimulant effects of β₂-AR agonists on murine myocyte contractions can be detected only after PTX treatment (46). In rat and dog, PTX enhances the positive inotropic response of β₂-AR agonists. Xiao and Lakatta (45) proposed that G₁₆ signaling limits the G₁₅ pathway, inducing the compartmentalization of β₂-AR-induced cAMP signaling.

Our data clearly demonstrate that, in chicken, an exclusive coupling to a PTX-sensitive pathway mediates the positive inotropic response of β₂-AR agonists. The relative coupling efficiency of β₂-AR to either G₁₅/cyclase or G₁₆/cPLA2 would determine the nature of the messenger, cAMP or AA. This could vary depending on the cardiac tissue, the animal species, and/or the pathophysiological state of the heart. In healthy human hearts, β₂-AR is essentially coupled to G₁₆, and the positive inotropic response is mediated by cAMP, with a limited negative influence of β₂-AR coupled to G₁₅. An attractive hypothesis is that β₂-AR/cPLA2 coupling would be effective in mediating inotropic responses in the case of defective coupling to adenyl cyclase. Thus, congestive human heart failure is associated with alterations in the activation of the β₂-AR/cAMP pathway. Part of the remaining contractile effect of β₂-AR agonists could rely on the alternative production of AA. In keeping with this hypothesis, preliminary results from our laboratory clearly indicate the relevance of β₂-AR coupling to cPLA2 in human under certain pathophysiological circumstances.

In conclusion, our results confirm that the β₂-AR pathway diverges from the β₂-AR pathway in embryonic chick ventricular cells. They establish the selective role of the cPLA2/AA pathway and p38 and p42/44 MAPKs in β₂-AR-mediated effects on [Ca²⁺], cycling. This study emphasizes that, apart from their involvement in long-term β-AR-induced hypertrophy, MAPKs may play a major role as physiological regulators of the β₂-AR-mediated contractile responses in heart. A complete understanding of the cellular mechanisms involved in the coupling of β₂-AR to cPLA2 should identify novel targets for therapeutic intervention in failing hearts, known to present an uncoupling of both β₂-AR and β₂-AR from the adenyl cyclase system.

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β₂-Adrenergic Receptor Agonists Increase Intracellular Free Ca\textsuperscript{2+} Concentration Cycling in Ventricular Cardiomyocytes through p38 and p42/44 MAPK-mediated Cytosolic Phospholipase A\textsubscript{2} Activation

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