Evidence for a β₂-Adrenergic/Arachidonic Acid Pathway in Ventricular Cardiomyocytes

REGULATION BY THE β₁-ADRENERGIC/CAMP PATHWAY*

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The signaling pathway mediating the contractile effect of β₂-adrenergic receptors (β₂-AR) in the heart is still matter of debate. By using embryonic chick ventricular cardiomyocytes that express both functional β₁- and β₂-ARs, we show here that the specific β₂-AR agonist, ziniterol, increases the amplitude of Ca²⁺ transients and cell contraction of electrically stimulated cells. Ziniterol, up to 10 μM, did not stimulate adenylyl cyclase activity, and its effect on Ca²⁺ transients was unmodified by the specific cAMP antagonist, (Rp)-cAMPS. In contrast, ziniterol (10–100 nM) triggered arachidonic acid (AA) release from [³H]AA-loaded cells via the activation of the cytosolic phospholipase A₂ (cPLA₂). Stimulation of the Ca²⁺ transients by ziniterol was abolished by the cPLA₂ inhibitor, AACOCF₃, and was mimicked by AA (0.3–3 μM). Both stimulations of [³H]AA release and of [Ca²⁺], by ziniterol were abolished after treatment of the cardiomyocytes with pertussis toxin. Although cell responses to β₂-AR stimulation were mediated by AA, they were under cAMP control as follows: (i) the β₁-AR stimulation exerted a cAMP-mediated negative constraint on the β₂-AR/cPLA₂ pathway; (ii) cAMP potentiated AA action downstream β₂-AR stimulation. We conclude that, in cardiomyocytes, β₂-AR is coupled to cPLA₂ activation via a pertussis toxin-sensitive G protein. These results demonstrate the involvement of the cPLA₂/AA pathway in mediating positive inotropic effects, which could potentially compensate for a defective cAMP pathway.

β₁- and β₂-Adrenergic receptors (β₁- and β₂-ARs) coexist in the hearts of various animal species, including humans. However, their relative amount and their respective participation in the positive chronotropic and inotropic effects of adrenaline and noradrenaline vary depending on the cardiac tissue, the animal species, and/or the pathophysiological state (1, 2). In the non-failing human left ventricle, β₁-ARs represent 80% of the total β-ARs but mediate about 60% only of β-adrenergic-induced ventricular contractility (3). In the human failing heart, the β₁/β₂-AR ratio decreases, and the contribution of β₂-AR to the contractile responses becomes predominant over that of β₁-AR, in particular at low adrenaline concentrations (3, 4). For these reasons, the potential role of β₂-AR for improving cardiac performance has received considerable attention. In fact, the myocardial-targeted overexpression of β₂-ARs in transgenic mice significantly enhanced myocardial left ventricular contractility (5).

It is well documented that β₁-AR and β₂-AR subtypes are coupled to adenylyl cyclase activation and that stimulation of both receptors generally leads to an increase in cellular cAMP (4, 6, 7). In human healthy heart, β₂-ARs are more efficiently coupled to adenylyl cyclase than β₁-ARs (6–10). However, during cardiac failure, β₂-AR subtypes are partially uncoupled from adenylyl cyclase (6, 7), whereas their contribution to the positive inotropic effects of adrenaline and noradrenaline is increased to 63% (7). In addition, studies in the rat heart (11, 12) and in the non-failing and failing canine heart (13) have demonstrated a dissociation between the inotropic effect of β₂-AR and cellular cAMP increase. Based on those observations, Xiao et al. (12) proposed that unidentified signal transduction pathway(s), other than adenylyl cyclase and cAMP, could be involved in the cardiac inotropic response to β₂-AR stimulation.

Angiotensin II (14, 15), bradykinin (16, 17), and endothelin (15, 18), which exert positive inotropic responses, evoke AA release in heart. Furthermore, in a recent study, we have demonstrated that glucagon action relies not only on cAMP but also on the synergistic support of AA, by activation of the cPLA₂ which hydrolyzes the sn-2 fatty acyl ester bonds of membranous phospholipids (15).

The aim of the present study was to investigate the respective role of cAMP and AA in the cardiac response to β₂-adrenergic agonists. We used the model of embryonic chick ventricular cardiomyocytes that has been widely exploited for studies on metabolism, contractile physiology, electrophysiology, and examination of pathophysiologic states such as ischemia (19). We show that those cells, in addition to expressing β₁-AR (19, 20), also respond to β₂-AR stimulation. We compared the β₁- and β₂-AR-mediated effects on adenylyl cyclase, [Ca²⁺], transients, cell contraction, and AA release. Our results demonstrate that cAMP is the messenger of β₁-AR responses. In contrast, cell responses to β₂-AR stimulation were mediated by AA but under cAMP control.

EXPERIMENTAL PROCEDURES

Materials

Ziniterol and CGP 20712A were kindly supplied by Squibb and CIBA-Geigy (Basel, Switzerland), respectively. Mini-glucagon was obtained from ICN (Orsay, France). (Rp)-cAMPS, H89, HELSS, and AACOCF₃ were purchased from Biomol (Plymouth Meeting, PA). Penicillin/streptomycin, antibiotic solution, trypsin, nucleotides, (5)-isoproterenol, bovine serum albumin, arachidonic acid, ICI 118551, and pertussis toxin were obtained from Sigma (Saint Quentin Fallavier, France). Fura-2/AM was from Molecular Probes (Interchim, Montluçon, France). Fetal
calf serum and phosphate-buffered saline 2040 medium were purchased from Life Technologies, Inc. (Cergy Pontoise, France). M199 medium was obtained from Eurobio (Les Ulis, France). [α-32P]ATP (30 Ci/mmol) and [5-3H]cAMP (38–50 Ci/mmol) were purchased from Amersham Corp. (Les Ulis, France). [5,6,8,9,11,12,14,15-3H]Arachidonic acid (180–240 Ci/mmol) was obtained from NEN Life Science Products (Les Ulis, France).

Methods

Primary Culture of Embryonic Chick Ventricular Cardiomyocytes—Fecundated eggs were obtained from the Haas farm (Kaltenhouse, France). Primary monolayer cultured heart cells were prepared from 13-day-old chick embryo ventricles as described previously (15, 21, 22). Briefly, cells were dissociated by repeated cycles of trypsinization. The resulting cell suspension (5–7 × 10^6 cells/ml) was bubbled with 5% CO₂, 95% air, at 4 °C, and kept in buffer A (M199 medium containing 0.1% (w/v) NaHCO₃, 0.01% (w/v) L-glutamine, 0.1% penicillin/streptomycin antibiotic solution) until used, up to 5 days.

Fura-2 Loading and [Ca²⁺] Imaging—Cells were plated on plastic dishes, the bottom of which was replaced by a glass coverslip coated with laminin (1 μg/ml), and were incubated at 37 °C in humidified 5% CO₂, 95% air for 17–24 h.

Cells, attached to laminin, were bathed in 2 ml of saline buffer B (10 mM glucose, 130 mM NaCl, 5 mM KCl, 10 mM Hepes buffered at pH 7.4 with NaOH) containing 1 mM CaCl₂, 0.1 mM EGTA, and resuspended in saline buffer B containing 2 mM CaCl₂ and stimulated until a steady-state level of the Ca²⁺ transients was achieved, before addition of drugs and peptides to the perfusion medium.

Contractility Measurements—Experiments were performed in conditions similar to Ca²⁺ imaging, but cells were illuminated with visible light and images transmitted through a solid-state camera (CCD, black and white) at 640-cm high sensitivity) connected to the sideport of the microscope, as described previously (15). Contractions of single stimulated (0.5 Hz) myocytes were displayed on a video monitor, and the corresponding images (pixel × pixel) were recorded at a frequency of 9/s. Contractility measurements were determined by assessing changes in cell length using the Morphostar II software, developed by the IMSTAR (Paris, France).

Adenylyl Cyclase Assay—A particulate fraction of embryonic chick ventricular cardiomyocytes was obtained from cells washed twice in saline buffer B, disrupted by sonication, and centrifuged for 30 min at 30,000 × g. The pellet was resuspended in 50 mM Hepes, pH 7.4, and stored in liquid nitrogen. Adenylyl cyclase activity was measured as described previously (24). The assay medium, contained in a final volume of 60 μl, 50 mM Tris-HCl, pH 7.4, 5 mM MgCl₂, 1 mM EDTA, 1 mM [α-32P]ATP (10⁶ cpm), 1 mM [5-3H]cAMP (20,000 cpm), 50 μM GTP, 0.2 mM methylisobutyxanthine, 25 mM creatine phosphate, 1 mg/ml creatine kinase. The incubation was initiated by the addition of 20–70 μg of proteins and run at 37 °C. The reaction was terminated by adding 0.2 ml of 0.5 N HCl. Samples were boiled for 6 min and thereafter buffered with 0.2 ml of 1.5 M imidazole. [5-3H]cAMP formed was separated from [32P]ATP by chromatography on alumina columns according to the procedure of White (25). Results were obtained from triplicate determinations.

[3H]Arachidonic Acid Labeling—Embryonic chick ventricular cardiomyocytes (5 × 10⁶ cells/ml) suspended in buffer A, were plated in 24-well plates, left for 24 h in humidified 5% CO₂, 95% air, at 37 °C, and then incubated with 1.5 μCi/ml [3H]AA (6.75 nm). After 24 h incubation with [3H]AA, the cells were washed twice in saline buffer B containing 0.2% fatty acid-free bovine serum albumin and resuspended in saline buffer B.

Measurements of [3H]Arachidonic Acid Release in Intact Cells—At time 0 of the experiment, [3H]AA labeled-cells were exposed to various peptides and/or enzymatic inhibitors and incubated for various periods at 37 °C. Incubation was terminated by the addition of ice-cold EDTA (2 mM final), and the media were immediately transferred to microcentrifuge tubes. Centrifugation at 17,600 × g for 20 min in a Sigma centrifuge (model 2K15) at 4 °C was performed to pellet any cells or debris inadvertently collected with the extracellular medium. The amount of radioactivity in the supernatant was quantitated by liquid scintillation counting.

Analysis of the lipids released in the incubation medium was performed as described (26). At the end of the incubation period, the reaction mixture was acidified to pH 3.0 with HCl, and the products were extracted twice with ethyl acetate. The dried extracts were dissolved in ethanol/chloroform (1:2, v/v) and chromatographed on silica gel thin layer plate (Whatman LK5) in ethyl acetate/isooctane/water/acetic acid (11:5:10:2, v/v) as the solvent system. Standard concentrations of AA, prostaglandins E, and hydroxyeicosatetraenoic acids were co-chromatographed and visualized by exposing the plates to ultraviolet light. The area corresponding to each visualized spot was carefully extracted, and the radioactivity was determined by liquid scintillation counting.

RESULTS

β₁- and β₂-AR Stimulations Increase [Ca²⁺], Cycling and Contractility in Electrically Stimulated Embryonic Chick Ventricular Myocytes—The effect of increasing concentrations of isoproterenol was examined on [Ca²⁺], cycling of electrically stimulated embryonic chick ventricular myocytes. A dose-dependent increase in the amplitude of [Ca²⁺], transients was observed, reaching a maximal (210 ± 9%) stimulation at 1–10 μM isoproterenol (Fig. 1). Preincubation for 10 min with 100 nM of the selective β₂-AR antagonist, ICI 118551 (7), significantly reduced the stimulation evoked by 10 μM isoproterenol (28% inhibition) but was poorly effective in inhibiting the effect of lower concentrations. In contrast, 300 nM of the selective β₁-AR antagonist, CGP 20712A (7), markedly blocked the effect of low isoproterenol concentration, leading to a rightward-shifted dose-response curve of isoproterenol effect. CGP 20712A also reduced by 54% the maximal effect of 10 μM isoproterenol (Fig. 1).

β₂- and β₁-AR Agonist at Concentrations below 100 nM, and as a mixed β₁/β₂-AR agonist in the micromolar range of concentrations.

Zinterol, a specific, partial β₂-AR agonist (8, 27), elicited a
dose-dependent increase in \([\text{Ca}^{2+}]_i\), transient amplitude of electrically stimulated embryonic chick cardiomyocytes (Fig. 2A). A maximal, 144 ± 3%, increase was observed at 30 nM zinterol, with a half-maximal response occurring at 10 nM zinterol. As illustrated by the typical traces of \([\text{Ca}^{2+}]_i\) transients (Fig. 2B), the effect of zinterol was reversed by 100 nM of the selective \(\beta_2\)-AR antagonist, ICI 118551, but was not affected by 300 nM of the selective \(\beta_1\)-AR antagonist, CGP 20712A, and perfused without or with 30 nM zinterol. The traces are representative of at least 20 cells obtained from two different isolations.

Both \(\beta_1\)- and \(\beta_2\)-AR stimulatory effects on \([\text{Ca}^{2+}]_i\), cycling were correlated with increases in the amplitude of cell contraction; 30 nM zinterol (\(\beta_2\)-AR agonist) and 100 nM isoproterenol (at a concentration at which the agonist functioned as a \(\beta_1\)-AR agonist) increased the amplitude of cell contraction by 80 and 150% over basal, respectively (Fig. 3, A and B). Furthermore, as shown in the normalized and superimposed tracings of contraction (Fig. 3C), zinterol, like isoproterenol, markedly accelerated the kinetics of relaxation.

\(\beta_2\)-AR Stimulation of \([\text{Ca}^{2+}]_i\), Cycling Does Not Rely on Adenylyl Cyclase Activation—The effects of zinterol and isoproterenol on adenylyl cyclase activity were examined in a particular fraction of embryonic chick ventricular myocytes. Isoproterenol, at 10 \(\mu M\), elicited a maximal 1.8-fold stimulation of adenylyl cyclase activity, the half-maximal effect being obtained at 0.15 \(\mu M\) isoproterenol (Fig. 4A). This effect was totally blocked by 300 nM of the \(\beta_1\)-AR antagonist, CGP 20712A. Under the same assay conditions, zinterol had no effect on adenylyl cyclase activity (Fig. 4A). These results suggest that, in embryonic chick ventricular cardiomyocytes, adenylyl cyclase is specifically coupled to \(\beta_1\)-ARs but not to \(\beta_2\)-ARs.

To analyze further cAMP dependence of \([\text{Ca}^{2+}]_i\), cycling modulations in response to \(\beta_2\)- and \(\beta_1\)-AR stimulations, we used the cell-permeable selective cAMP antagonist, (R)p-cAMPS, (28). Following preincubation for 1 h with 10 \(\mu M\) (R)p-cAMPS, the increase in amplitude of \([\text{Ca}^{2+}]_i\), transients in response to 300 nM prenalterol, a specific \(\beta_1\)-AR agonist, was reduced by 83% (from 142 ± 7 to 107 ± 1% of control amplitude, Fig. 4B). The cAMP antagonist produced a similar reduction in the \(\beta_1\)-AR-mediated effect of 100 nM isoproterenol (from 178 ± 9 to 118 ± 3%, Fig. 4B). In contrast, (R)p-cAMPS failed to inhibit the \(\beta_2\)-AR response to either zinterol (30 nM) or fenoterol (100 nM) (Fig. 4B). These findings dem-

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**Fig. 2.** Zinterol, the specific \(\beta_2\)-AR agonist, increases the amplitude of \([\text{Ca}^{2+}]_i\) transients in electrically stimulated cells. Embryonic chick ventricular cardiomyocytes, loaded with Fura-2, were electrically stimulated at 0.5 Hz as described under “Experimental Procedures.” A, cells were perfused with increasing concentrations of zinterol, each concentration being applied for 3 min. Values are means ± S.E. of the effects observed on 20–30 cells, obtained from three different isolations. B, cells were preincubated for 10 min in the absence or in the presence of either 100 nM of the specific \(\beta_2\)-AR antagonist, ICI 118551 or 300 nM of the specific \(\beta_1\)-AR antagonist, CGP 20712A, and perfused without or with 30 nM zinterol. The traces are representative of at least 20 cells obtained from two different isolations.
demonstrate that adenylyl cyclase and cAMP govern β1-AR agonist-induced stimulation of [Ca^{2+}], cycling but are not involved in β2-AR-mediated effects.

Zinterol Stimulates [3H]AA Release from Embryonic Chick Ventricular Cardiomyocytes—The next series of experiments was performed to assess the possible involvement of AA in mediating β2-AR effects. Embryonic chick ventricular myocytes were labeled for 24 h with [3H]AA before the addition of agonist. As shown in Fig. 5A, zinterol evoked a dose-dependent release of [3H]AA, which reached a maximal (147 ± 4%) increase with 30 nM zinterol, the half-maximal effects occurring at 5 nM zinterol (Fig. 5A). The β1-AR agonist prenalterol, as well as isoproterenol ≤100 nM, when functioning in the β1-AR mode, had no effect on [3H]AA release (Fig. 5A). Only at concentrations above 1 μM did isoproterenol, functioning in the mixed β1/β2-AR mode, evoke a limited, 10% increase over basal in AA release.

Resolution on thin layer chromatography (TLC) of the [3H]AA produced by the [3H]AA labled cellular material, identified [3H]AA as the major product, both in control and zinterol-treated cells (75 and 79%, respectively) (Table I). Non-enzymatic degradation or contaminants of standard [3H]AA represented 4–19% of the total radioactivity recovered in supernatants; lipoxygenase and cycloxygenase products represented 4–24 and 1–8%, respectively (Table I).

Since AA formation in the heart is essentially attributed to PLA2 activity, we examined the effect of AACOCF3, a specific inhibitor of the cPLA2. The addition of 10 μM AACOCF3 to the perfusion medium dramatically reduced [3H]AA release evoked by zinterol (from 206 to 136% of control [3H]AA release, Table I). This inhibitory effect correlated with a blockade of the stimulatory effects on [Ca^{2+}], transients of both specific β2-AR agonists zinterol and fenoterol (Fig. 5B). In contrast, AA-AACOCF3 did not affect the β1-AR-mediated increase in [Ca^{2+}], cycling triggered by either prenalterol or isoproterenol at 100 nM (Fig. 5B). Taken together, these findings further supported the notion that β2-AR stimulation elicited AA release by stimulating the cPLA2, sensitive to ACOOF3. It may be noted that ACOOF3 completely inhibited β2-AR-mediated effects on [Ca^{2+}], cycling, whereas it had only a partial effect on β2-AR-stimulated AA release (Table I). This may suggest that the onset of the Ca^{2+} response requires the cellular AA level to reach a threshold.

Importantly, exogenous application of micromolar concentrations of AA reproduced the effect of β2-AR agonists on [Ca^{2+}], transients; at 3 μM, AA evoked a 140% increase in amplitude of [Ca^{2+}], transients (Fig. 6). The activating effect of AA on [Ca^{2+}], cycling was potentiated by 8-Br-cAMP (Fig. 6).

The β1-AR/cAMP Pathway Occludes Cell Responses to β2-AR Stimulation—Next, we looked for a possible cross-talk between β1- and β2-AR responses. In a first series of experiments, cells were electrically stimulated and exposed to 300 nM of the β1-agonist, prenalterol. The time course of the amplitude of [Ca^{2+}], transients is illustrated in Fig. 7. [Ca^{2+}], transient amplitude increased for the first minutes of exposure to prenalterol, reaching a maximal 50% increase over basal at 10 min. After 15 min, a decline in stimulation of [Ca^{2+}], cycling occurred, and after 30 min, the β1-AR-mediated effect was no more detectable (Fig. 7). Such a waning of a stimulated response in the face of continuous agonist exposure is typical of a desensitization phenomenon (29).

In a second series of experiments, we examined the response to β2-AR stimulation of cells under two extreme conditions: (i) after 3 min exposition to prenalterol, when β1-ARs are fully activated; (ii) after 45 min exposition to prenalterol, when β1-ARs are desensitized. As shown in the insets of Fig. 7, after
3 min incubation with prenalterol, the addition of zinterol did not produce further increase in [Ca$^{2+}$]$_{i}$ transient amplitude. In contrast, zinterol added to $\beta_{2}$-AR-desensitized cells evoked a 2-fold increase in the amplitude of [Ca$^{2+}$]$_{i}$ transients. Those results suggested a negative constraint exerted by $\beta_{1}$-AR activation on the $\beta_{2}$-AR-stimulated [Ca$^{2+}$]$_{i}$ cycling.

8-Br-cAMP reproduced prenalterol effect and inhibited the $\beta_{2}$-AR-mediated effects on [Ca$^{2+}$]$_{i}$ cycling (Fig. 8A). In addition, the cAMP antagonist, (Rp)-cAMPS, as well as the PKA inhibitor, H89, blocked the inhibitory effect of the $\beta_{1}$-AR agonist, prenalterol, on the cell response to $\beta_{2}$-AR stimulation (Fig. 8B and C). Taken together, those data suggest that cAMP, via PKA activation, exerts an inhibitory constraint on $\beta_{2}$-AR stimulation.

$\beta_{2}$-Adrenergic/cPLA$_{2}$ Pathway in Cardiomyocyte

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Embryonic chick heart cells were labeled with 1.5 μCi/ml [3H]AA as described under “Experimental Procedures.” After two washings in saline buffer containing 0.2% fatty acid-free bovine serum albumin, [3H]AA-labeled cells were incubated for 30 min in the presence or in the absence of 30 nM zinterol, and with or without 10 μM AACOCF₃. Analysis of the [3H]-lignids released in the incubation medium was performed following extraction and chromatography on silica gel thin layer plate (TLC plate) as described under “Experimental Procedures.” Results, corrected for yield of extraction, are expressed in dpm of [3H] product/10⁶ cells and in percent of total radioactivity recovered in the migration lane. Each sample represents the pool of quadruplicates. Data are from a typical experiment that has been repeated twice. Standard [3H]AA was migrated in parallel in order to determine the nonenzymatic breakdown of AA. HETE, hydroxyeicosatetraenoic acid.

| Metabolites                      | Basal conditions | 10 μM AACOCF₃ | Migration of standard [3H]AA |
|---------------------------------|-----------------|---------------|-----------------------------|
|                                 | Control | Zintoler | Control | Zintoler | Control | Zintoler | Control | Zintoler |
| Arachidonic acid                | 1,833    | (75)   | 3,788   | (79)   | 2,053   | (55)   | 2,798   | (61)   | 89       |
| Lipoxigenase products (HETE₁₁, HETE₁₃, and HETE₁₅) | 188     | (8)    | 225     | (5)    | 750     | (20)   | 433     | (9)    | 3        |
| Cyclooxygenase products (PGE₂ and PGE₃) | 203     | (8)    | 255     | (5)    | 383     | (10)   | 588     | (13)   | 2        |
| Non-identified products         | 231     | (9)    | 543     | (11)   | 568     | (15)   | 780     | (17)   | 6        |

**Fig. 6.** AA mimics β₂-AR action on [Ca²⁺], cycling and 8-Br-cAMP potentiates AA effect. Embryonic chick ventricular cardiomyocytes, loaded with Fura-2, were electrically stimulated at 0.5 Hz, as described under “Experimental Procedures,” and perfused with increasing concentrations of AA, in the absence or in the presence of 75 μM 8-Br-cAMP. Values are means ± S.E. of the effects observed on at least 10 cells, obtained from two different isolations.

efficiency of PTX treatment was checked by the blockade of Gₛ-mediated acetylcholine inhibition of isoproterenol effect on Ca²⁺ cycling (Fig. 9B). It should be noted that treatment with pertussis toxin was without detectable impact on basal or isoproterenol-stimulated [Ca²⁺], transients suggesting the absence of a tonic control by Gₛ, in particular over Gₛ₂.

**DISCUSSION**

In the present study, we show that β₁- and β₂-ARs are both expressed in embryonic chick ventricular cardiomyocytes, and this model allowed us to demonstrate the following: (i) β₂-ARs are specifically coupled to cPLA₂ via a Gₛ protein; (ii) cAMP exerts a dual tuning on cell responses to β₂-AR stimulation. In electrically stimulated embryonic chick cardiomyocytes, 30–100 nM zinterol, a specific partial β₂-AR agonist, elicited a 40–50% increase over basal in the amplitude of [Ca²⁺], transients (Fig. 2), which correlated with increases in twitch amplitude and twitch velocity (Fig. 3). Such a positive inotropic effect of β₂-AR agonists is undisputed. Nevertheless, in contrast to β₁-AR-mediated positive inotropic effect, which definitely relies on a rise in intracellular cAMP, the contribution of cAMP to the positive inotropic effect of β₂-AR agonists, and the possible coupling of β₂-AR to cAMP-independent pathways, are still a matter of debate. According to Bristow et al. (6), β₂-ARs in the non-failing human heart are tightly coupled to adenylyl cyclase since a numerically small β₂-AR fraction (19% of the total β₁- and β₂-ARs) accounts for the majority of adenylyl cyclase stimulation. Such an inherent efficacy for the human β₂-AR in activating adenylyl cyclase, compared with that of its β₁ counterpart, has been confirmed by expression of those receptors in fibroblast cell lines (9, 10). However, Kaumann and Lemoine (7) have compared the relative contribution of β₁- and β₂-ARs to adenylyl cyclase stimulation and positive inotropic effects of adrenaline and noradrenaline in pathologic human heart. They concluded that the positive inotropic response was not straightforwardly correlated to adenylyl cyclase stimulation. These authors were also the first to suggest compartmentation of cAMP since cAMP produced upon β₂-AR stimulation was less efficiently used than cAMP produced upon β₁-AR stimulation by cellular effectors involved in contractility. More recently, the group of Lakatta (12) suggested that, in addition to coupling to adenylyl cyclase, β₂-AR stimulation activates other signal transduction pathways to produce changes in [Ca²⁺] and contraction. This proposal relies on two observations. First, in rat ventricular cells β₂-AR stimulation elicits a positive inotropic response that is dissociated from cAMP increase (12). Evidence for the involvement of cAMP is given only for high β₂-agonist concentrations; indeed, activation by 10 μM zinterol of both contraction (32) and L-type Ca²⁺ current (2) is blocked by (Rp)-cAMPS, the specific inhibitory cAMP analog. Second, in electrically stimulated dog myocytes, β₂-AR activation is ineffective in stimulating adenylyl cyclase, whereas it produces increases in [Ca²⁺], transient and twitch amplitudes (13). In this regard, we show here that, in embryonic chick heart cells, β₂-AR stimulation by zinterol triggers a positive inotropic effect, independent of adenylyl cyclase activation (Figs. 2, 3, and 4A). The absence of participation of cAMP in this effect of zinterol is further confirmed by the fact that, in contrast to the actions of β₁-AR agonists, it is not blocked by either (Rp)-cAMPS (Figs. 4B and 8B) or the PKA inhibitor, H89 (Fig. 8C). Thus, we conclude that cAMP does not support the inotropic effect of low β₂-AR agonist concentrations although it could contribute in the effects of high β₂-AR agonist concentrations.

Glucagon action in heart relies on the synergistic actions of glucagon itself and its metabolite (19–29), mini-glucagon (15, 22). We have demonstrated that cAMP mediates glucagon action and that AA is the second messenger of mini-glucagon (15). In the present study, several lines of evidence support the proposal that AA is also the second messenger in response to
stimulations by β2-AR agonists: 1) zinterol increases AA release from [3H]AA-prelabeled myocytes in a dose-dependent manner from 3 to 100 nM (Fig. 5A); 2) AA, added to the cell medium at concentrations as low as 1–3 μM, reproduces the effect of zinterol on [Ca2+]i cycling in electrically stimulated cardiomyocytes (Fig. 6). AA release results from β2-AR activation of the cPLA2 via a pertussis toxin-sensitive G protein (Fig. 8). Such a coupling of β2-ARs to pertussis toxin-sensitive G protein(s) has been already reported in rat cardiomyocytes (30) and in cells sur-expressing β2-ARs (31).

cAMP exerts a dual tuning on cell responses to β2-AR stimulation. On the one hand, we show that cAMP, produced upon β1-AR stimulation, evokes a quenching of cell responses to β2-AR stimulation; thus, after 3 min exposure to prenalterol, cells did not respond further to β2-AR stimulation, whereas following complete desensitization of β1-ARs, β2-AR stimulation was restored (Fig. 7). The negative constraint exerted by cAMP is likely to rely on PKA stimulation since H89, the PKA inhibitor, hampers it. It could be due to phosphorylation, and inhibition, by protein kinase A of the PTX-sensitive G protein coupling cPLA2 to β2-AR (33). On the other hand, downstream cPLA2 activation, cAMP potentiates AA-mediated stimulation of [Ca2+]i cycling (Fig. 6). Those synergistic actions of cAMP and AA would rely on the ability of AA to accumulate Ca2+ into the sarcoplasmic reticulum stores and that of cAMP to induce “Ca2+-induced Ca2+-release” from these stores (15).

In conclusion, we show that, at low concentrations, β2-AR agonists elicit a positive inotropic effect via cPLA2 activation and AA release. Contrary to the β1-AR/cAMP pathway, β2-AR/cPLA2 pathway involves a pertussis toxin-sensitive G protein. cAMP exerts a dual regulation on the β2-AR/AA pathway; it inhibits the cell response to β2-AR stimulation but potentiates AA-mediated stimulation of [Ca2+]i, cycling.

There is now accumulating evidence that hydrolytic products of cAMP, such as 1,2-diacylglycerol or IP3, could also participate in the regulation of AA release from myocytes.
derived from membrane phospholipids play important roles in cardiovascular signaling (34). Originally, attention mainly focused on diacylglycerol and eicosanoids (prostaglandins, prostacyclins, thromboxanes, leukotrienes, etc.) (35, 36). However, the list of bioactive lipidic molecules now includes AA, the precursor of eicosanoids. Studies on mice deficient in cPLA2 have demonstrated the major role of this enzyme in allergic responses, reproductive physiology, and pathophysiology of

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neuronal death (37, 38). The participation of cPLA2 and/or AA in mediating positive inotropic response to various agents was suspected (14–18). Our results unequivocally establish that, at low concentrations of agonist, the \( \beta_2 \)-AR-mediated inotropic effect relies on the selective activation of cPLA2 and AA release. Although this remains to be demonstrated, it is tempting to speculate that the \( \beta_2 \)-AR/cPLA2/AA pathway could be determinant in failing hearts that have lost 50% of \( \beta_1 \)-ARs and show a parallel decrease in agonist-stimulated adenylyl cyclase activity (4, 6, 7).

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