Arachidonic Acid Drives Mini-glucagon Action in Cardiac Cells*

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Anne Sauvadet, Troy Rohn, Françoise Pecker, and Catherine Pavoine
From INSERM Unité 99, Hôpital Henri Mondor, 94010 Créteil, France

Recent studies have shown that glucagon is processed by cardiac cells into its COOH-terminal (19–29) fragment, mini-glucagon, and that this metabolite is an essential component of the contractile positive inotropic effect of glucagon (Sauvadet, A., Rohn, T., Pecker, F. and Pavoine, C. (1996) Circ. Res. 78, 102–109). We now show that mini-glucagon triggers arachidonic acid (AA) release from [3H]AA-loaded embryonic chick ventricular myocytes via the activation of a phospholipase A2 sensitive to submicromolar Ca2+ concentrations. The phospholipase A2 inhibitor, AACOCF3, prevented mini-glucagon-induced [45Ca2+] accumulation into the sarcoplasmic reticulum, but inhibitors of lipoxygenase, cytochrome P450, or epoxygenase pathways were ineffective. AA applied exogenously, at 0.3 μM, reproduced the effects of mini-glucagon on Ca2+ homeostasis and contraction. Thus AA: (i) caused [45Ca2+] accumulation into a sarcoplasmic reticulum compartment sensitive to caffeine; 2) potentiated caffeine-induced Ca2+ mobilization from cells loaded with Fura-2; 3) acted synergistically with glucagon or cAMP to increase both the amplitude of Ca2+ transients and contraction of electrically stimulated cells. AA action was dose-dependent and specific since it was mimicked by its non-hydrolyzable analog 5,8,11,14-eicosatetraynoic acid but not reproduced by other lipids such as, arachidonic acid, linolenic acid, cis-5,8,11,14,17-eicosapentaenoic acid, cis-4,7,10,13,16,19-docosahexaenoic acid, or arachidonoyl-CoA, even in the micromolar range. We conclude that AA drives mini-glucagon action in the heart and that the positive inotropic effect of glucagon on heart contraction relies on both second messengers, cAMP and AA.

Data from the past few years have shed a new light on the physiological pathways of glucagon action (for review, see Ref. 1). Thus, the interaction of glucagon with cardiac tissue leads to its processing by a specific ectoenzymepeptidase and to the liberation of the COOH-terminal (19–29) fragment, mini-glu-
cagon. This metabolite plays a key role in the positive inotropic effect of the hormone (2–5) since glucagon alone, under minimal degradation conditions, has no effect on heart cell contraction (5). We have shown that both peptides act synergistically on Ca2+ cycling in heart cells (6). Mini-glucagon and glucagon actions can be summarized as the ability of the former to accumulate Ca2+ into sarcoplasmic reticulum stores (SR stores)1 and that of the latter to induce Ca2+-induced-Ca2+-release from the same stores (6). Glucagon action is mediated by cAMP produced from either stimulation of adenyl cyclase or inhibition of the cyclic GMP-inhibited phosphodiesterase (CGI-PDE or PDE III), depending on the species (6–8). In contrast, the action of mini-glucagon does not rely on classical transduction pathways. In fact, mini-glucagon does not evoke any detectable change in either cAMP or cGMP or inositol 1,4,5-trisphosphate production and its second messenger remained to be identified (5, 6).

Recently, considerable evidence has accumulated to suggest a role for arachidonic acid (AA) and/or its oxidized metabolites in signal transduction processes (9). AA release in response to cell receptor activation is considered as a key step in the positive inotropic response of angiotensin II, bradykinin, and endothelin (10–13).

Arachidonic acid is stored in esterified form in cell membrane phospholipids, from which it can be liberated through multiple enzymatic pathways (for review, see Ref. 14). PLA2 catalyzes the hydrolysis of phospholipids at the sn-2 position. Therefore, this enzyme can release arachidonate in a single step reaction. By contrast, PLC and PLD do not release free arachidonic acid directly, but generate lipid products containing arachidonate (diacylglycerol and phosphatidic acid, respectively), which can be released subsequently by diacylglycerol and monoaoylglycerol lipases. Once released, free arachidonate may diffuse out of the cell, be reincorporated into phospholipids, or metabolized. In addition, several reports have focused on a signaling role for AA in heart cells. One of the most thoroughly characterized targets of AA is protein kinase C (15), and AA has also been reported to modulate sarcolemmal ion channels, including K+ channels (10, 16, 17), and voltage-dependent Ca2+ channels (18), as well as to increase Ca2+ release from the sarcoplasmic reticulum in heart (19).

Several hormones which exert positive inotropic responses evoke release of AA. Thus, in neonatal rat ventricular myocytes, angiotensin II stimulates AA release via both PLC and PLA2 activation through AT1 and AT2 receptors, respectively (11). In isolated hearts, bradykinin and endothelin, which also activate PLC, stimulate release of AA (12, 13). Thus, in addition to representing an important signaling molecule under pathological circumstances, such as ischemia (20), AA, in the normal physiological setting may modulate important steps in excitation-contraction coupling.

The aim of the present study was to evaluate the role of AA in mediating the actions of mini-glucagon on Ca2+ homeostasis and cell contraction in embryonic chick heart cells. The main findings are that mini-glucagon triggers a Ca2+-dependent release of AA and that AA added exogenously, at a submicromolar concentration (0.3 μM), mimics mini-glucagon actions. Our data suggest that free AA, and not products of AA metabolism, release, PLA2, phospholipase A2; ETYA, 5,8,11,14-eicosatetraynoic acid; 8-BrcAMP, 8-bromo-cAMP; PLD, phospholipase D; PLC, phospholipase C; NDGA, nordihydroguaiaretic acid; OAG, 1-oleoyl-2-acetyl-sn-glycerol.

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1 The abbreviations used are: SR, sarcoplasmic reticulum; AA, arachidonic acid (cis-5,8,11,14-eicosatetraynoic acid); [Ca2+]i, intracellular free Ca2+ concentration; [Ca2+]o, extracellular free Ca2+ concentration; PLA2, phospholipase A2; ETYA, 5,8,11,14-eicosatetraynoic acid; 8-BrcAMP, 8-bromo-cAMP; PLD, phospholipase D; PLC, phospholipase C; NDGA, nordihydroguaiaretic acid; OAG, 1-oleoyl-2-acetyl-sn-glycerol.

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is the primary mediator of mini-glucagon action in ventricular myocytes.

**EXPERIMENTAL PROCEDURES**

**Materials**

Mini-glucagon was obtained from ICN (Orsay, France), and glucagon from Novo Nordisk Laboratories ( Bagsvaerd, Denmark). Penicillinstreptomycin antibiotic solution, trypsin, nucleotide, bovine serum albumin, arachidonic acid, cis-4,7,10,13,16,19-docosahexaenoic acid, and cis-5,8,11,14,17-eicosapentaenoic acid were purchased from Sigma (Saint Quentin Fallavier, France). AOCOCF3 and ETYA were from Biomol (Plymouth Meeting, PA). Fura-2, Fura-2/AM are from Molecular Probes (Interchim, Montluçon, France). Fetal calf serum was from Life Technologies Inc. (Cergy Pontoise, France). Phosphate-buffered saline 2040 and M199 media were obtained from Eurobio (Les Ulis, France). Digitonin from Merck has been recrystallized and dissolved as a 8 mg/ml solution in Me2SO. [45Ca2+] (Saint Quentin Fallavier, France). AACOCF3 and ETYA were from DuPont NEN (Les Ulis, France). Antibodies from rabbit anti-mouse IgG-conjugated peroxydase were purchased from BIOSYST (Compiègne, France). Mouse monoclonal antibodies against human cytoplasmic phospholipase A2 were from Santa Cruz.

**Methods**

**Primary Culture of Chick Embryo Ventricular Cells—**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 (6, 21). 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% (v/v) l-glutamine, 0.1% penicillin-streptomycin antibiotic solution) until used, up to 5 days.

**Rat Ventricular Myocytes—**Ventricular myocytes were enzymatically dispersed from adult male Wistar rat (250–300 g) as described previously (7).

**Measurements of [45Ca] Accumulation into Intracellular Compartments—**Myocytes (5×10^6 cells/ml), suspended in buffer A and supplemented with 5% (v/v) fetal calf serum, were plated on glass coverslips in multwell plates and kept at 37 °C in humidified 5% CO2, 95% air for 17–24 h. All tracings of cell length are representative of at least 5 cells and percent change 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 in Ref. 25. 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 (1:10:2:2, v/v) as the solvent system. Standard concentrations of AA, prostaglandin Es, 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.

**Measurements of [3H]Arachidonic Acid Release on Cells Permeabilized with Digitonin—**[3H]Iodinated cells were incubated for 10 min at 37 °C in the presence of 0.1 nm mini-glucagon or vehicle. Cells were next subjected to digitonin lysis, which selectively disruptive the sarcolemmal membranes. This procedure, previously described by Altschuld (22), consisted of an incubation for 45 s at 25 °C in Ca-free saline buffer B, containing 2 mM MgCl2 and 10 mM ATP, 5 εM ruthenium red and digitonin (16 εg/ml of protein). Mg-ATP was added to protect against hypercontracture and ruthenium red to block Ca2+-efflux from the SR. Intracellular Ca2+ accumulation was estimated from [45Ca] recovered in digitonin-resistant structures attached to coverslips after the addition of 0.2 M NaOH for 2 h at room temperature. Samples were diluted in 10 ml of Beckman Ready Safe and counted in a scintillation counter. Data are expressed as mean ± S.E. To determine significant differences from control values, results were analyzed by employing the Student’s t test.

**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 εg/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 and incubated for 20 min at 25 °C with 1.5 εM Fura-2/AM (3 εM of 1 ml Fura-2/AM in Me2SO), in the presence of 1 mg/ml bovine serum albumin to improve Fura-2 dispersion and facilitate cell loading. Cells were then washed three times in buffer B (12×2 ml) to remove any buffer B [12×2 ml added for 15 min at 25 °C] to facilitate hydrolysis of intracellular Fura-2/AM. The concentration of Fura-2 in myocytes was estimated as described previously (6, 21), according to the procedure of Donnadieu et al. (23). Under usual loading conditions, the average intracellular concentration of Fura-2 was 15 εM.

Ca2+ imaging, developed by A. Trautmann in collaboration with the IMSTAR CO (Paris, France), was essentially as described by Sauvadet et al. (6, 21). All tracings of fluorescence ratio are representative of at least 10 cells, and were performed on at least two different cell isolations. Imaging studies were performed on cells in which no spontaneous rise in [Ca2+]i was observed prior to experimental manipulation.

Blood electrical stimulation was performed on the left ventricle at 1 Hz, with an amplitude 20% above threshold, 0.5 Hz) was supplied through a pair of platinum electrodes connected to the output of a HAMEG stimulator (Paris, France). Cells were perfused with saline buffer B containing 1.27 mM CaCl2 and stimulated until a steady-state level of the Ca2+ transients was achieved, before each protocol, as described previously by Do and Sauvadet (24). To evaluate the buffer-releasable [Ca2+]i pool, drugs and peptides were added to the perfusion medium a few seconds after interruption of electrical stimulation. In a second series of experiments, performed to measure variations in the amplitude of Ca2+ transients, electrical stimulation of the cells was maintained throughout the experiment. Drugs and peptides were added to the perfusion medium at time 0. To evaluate the effect of glucagon, mini-glucagon, 8-Br-cAMP, and/or arachidonic acid quantitatively, we used two parameters: the percentage increase in diastolic Fura-2 ratio (360:380) and the percentage -increase in the amplitude of Ca2+ transient.

**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, 0.847 cm high sensitivity) connected to the sideport of the microscope. 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 Hz. Contractility measurements were determined by assessing changes in cell length using the Morphostar II software, developed by the IMSTAR CO (Paris, France). All tracings of cell length are representative of at least 5 cells and performed on at least two different cell isolations.

**[3H]Arachidonic Acid Labeling—**Embryonic chick ventricular myocytes (5×10^5 cells/ml), suspended in buffer A, were plated in multwell plates and incubated with 1.5 εCi/ml [3H]Arachidonic Acid (6.75 nm) in humidified 5% CO2, 95% air, at 37 °C. After 24 h, the cells were washed twice in saline buffer B containing 0.2% fatty acid-free bovine serum albumin and reseeded in saline buffer B, 30% fetal bovine serum, 10% horse serum, and 0.1% (v/v) penicillin-streptomycin antibiotic solution.

**[3H]Arachidonic Acid Release in Intact Cells—**At time 0 of the experiment, [3H]Iodinated cells were exposed to various peptides and/or enzymatic inhibitors, and incubated for various periods at time 0 of 37 °C. Incubation was terminated by the addition of ice-cold EGTA (2 mM final), and the media were immediately transferred to microcentrifuge tubes. Centrifugation at 17,600 × g for 20 min in a Sigma T10 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.

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amount of radioactivity in the supernatant was quantitated by standard liquid scintillation procedures.

**Immunoblot Analysis of cPLA<sub>2</sub>**—Embryonic chick ventricular cells or rat ventricular myocytes were disrupted by sonication in buffer C (40 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.25% sarcosinate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 50 mM sodium fluoride, 100 μM sodium orthovanadate). After dilution (v/v) with sample buffer (250 mM Tris-HCl, pH 6.8, 8% SDS, 20% glycerol, 10% β-mercaptoethanol), samples were boiled for 5 min, and the proteins (25 μg/lane) were separated on 7.5% SDS-polyacrylamide gel electrophoresis (25 mA/gel). The proteins were transferred to Hybond C super nitrocellulose membrane (Amersham), by electrobrightening using Tris glycine buffer containing 20% methanol. The transfer was performed at 90 mA for 60 min. Protein transfer was evaluated by staining the gel with Coomassie Blue. The nitrocellulose blots were agitated for 1 h at room temperature in TBST buffer (10 mM Tris-HCl, pH 8, 150 mM NaCl, 0.15% Tween 20) supplemented with 5% nonfat dry milk, washed three times with TBST, and incubated with primary monoclonal antibodies against cPLA<sub>2</sub> (1: 500 dilution in TBST supplemented with 5% nonfat dry milk) for 180 min. Membranes were washed three times with TBST and incubated with peroxidase-conjugated rabbit anti-mouse IgG (1:1000 dilution) for 30 min. Membranes were washed three times with TBST and the peroxidase activity was determined using the enhanced chemiluminescence Western blotting detection system (ECL (Amersham Corp.).

**RESULTS**

**Mini-glucagon Stimulates Arachidonic Acid Release from Embryonic Chick Ventricular Myocytes**—The action of mini-glucagon on AA release was assessed on embryonic chick ventricular myocytes labeled for 24 h with [3H]AA before the addition of mini-glucagon, to allow steady state labeling of the cellular AA pool. As shown in Fig. 1A, 0.1 nM mini-glucagon evoked a sustained release of AA from cells over the 30-min period examined. Under similar experimental conditions, the level of AA release in the absence of mini-glucagon remained constant (Fig. 1A). The action of mini-glucagon was dose-dependent, with a maximal (164 ± 7%) increase of [3H]AA released after 30 min observed at 0.1 nM mini-glucagon and a half-maximal response occurring at 0.01 nM mini-glucagon (Fig. 1B). Under the same experimental conditions, angiotensin II, at 0.3 μM, evoked a similar increase (173 ± 14%) in [3H]AA release (Fig. 2). The action of mini-glucagon was specific since glucagon itself did not affect [3H]AA release significantly, even at micromolar concentrations (Fig. 1B, inset).

It was important to determine if the [H]-labeled material that was released was authentic AA. Accordingly, cell supernatants were analyzed by extraction and resolution on thin layer chromatography (TLC). As shown in Table I, the [3H]-labeled material in the supernatants of control and mini-glucagon-treated cells was mainly identified as [3H]AA (67 and 54%, respectively) but also consisted of [3H]lipoxygenase products (11 and 14%) in [3H]AA release (Fig. 1A).

**Evidence Supporting PLA<sub>2</sub> Activation in Mini-glucagon Stimulated AA Release**—In heart, AA formation is potentially due to PLA<sub>2</sub> stimulation but may also occur upon activation of either PLC or PLD. The next series of experiments were performed to examine whether the release of AA elicited by mini-glucagon relied on PLA<sub>2</sub> activation. Results shown in Fig. 2 indicated that the addition of PLA<sub>2</sub> inhibitors, quinacrine, or the analogue of AA, AACOCF<sub>3</sub>, resulted in complete inhibition of mini-glucagon-promoted release of AA (Fig. 2). Quinacrine and AACOCF<sub>3</sub>, also blocked angiotensin II-induced AA release. In contrast, these PLA<sub>2</sub> inhibitors were partially effective against endothelin elicited AA release (Fig. 2), suggesting that part of release of AA derived from the PLC pathway (26).

The hormone-activated PLA<sub>2</sub> (cPLA<sub>2</sub>) is considered to be a Ca<sup>2+</sup>-dependent enzyme. The Ca<sup>2+</sup> dependence of mini-glucagon-stimulated AA release was tested by two ways. First, in intact cells, lowering the extracellular free Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]), from 2 mM to 500 μM, considerably reduced mini-glucagon-stimulated AA release, while basal AA release remained unchanged (Fig. 3). The presence of Ca<sup>2+</sup> in the extracellular medium was therefore a requirement for mini-glucagon action. Second, we examined AA release under controlled free Ca<sup>2+</sup> concentration conditions. Myocytes were permeabilized with digitonin and incubated in Ca<sup>2+</sup>-EGTA buffers with varying free Ca<sup>2+</sup>-. Those cells were still responsive to mini-glucagon, however, substantial variations in the degree of stimulation by mini-glucagon were observed. These variations could be overcome if cells were preincubated for 10 min with mini-glucagon before lysis with digitonin and if micromolar concentrations of GTP were added to the assay medium. This GTP requirement for the activation of AA release by mini-glucagon would suggest the involvement of a G protein. As shown in the Fig. 3B, under these conditions, a highly reproducible increase in arachidonic acid release was observed at 700 nM [Ca<sup>2+</sup>], with 0.1 nM mini-glucagon in the presence of...
10 μM GTP. The half-maximal stimulation occurred at 450 nM [Ca\(^{2+}\)]. It is noteworthy that in the absence of mini-glucagon, the basal release of AA had a similar Ca\(^{2+}\) dependence (Fig. 3B), but was not improved by the additional presence of 10 μM GTP in the incubation medium (not shown). Taken together these data are consistent with the mini-glucagon activation of a Ca\(^{2+}\)-dependent PLA\(_2\).

The presence of a cPLA\(_2\) in embryonic chick ventricular cells was evidenced by immunoblotting analysis using a mouse monoclonal antibody directed against the amino-terminal domain of human cPLA\(_2\) (amino acids 1–216) (Fig. 4). The major immunoreactive protein in embryonic chick ventricular cells had an apparent molecular mass of 80 kDa. Under the same experimental conditions, two bands were detected in rat ventricular myocytes, a major band of 105 kDa and an additional band of 85 kDa (Fig. 4).

Mini-glucagon Causes \(^{45}\)Ca Accumulation into SR Stores via AA—We have previously shown that mini-glucagon increases \(^{45}\)Ca accumulation into caffeine-sensitive SR stores. Our data raised the question as to the role of AA in mediating mini-glucagon-induced increase in SR Ca\(^{2+}\) content. Quiescent embryonic chick heart cells were incubated for 5 min at 37 °C, in a medium containing 2 mM \(^{45}\)Ca, with or without 0.1 mM mini-glucagon, and the influence of inhibitors of either PLA\(_2\) activity or AA metabolism was examined. After incubation, the cells were washed and subjected to digitonin lysis. \(^{45}\)Ca accumulation was then evaluated, both in the digitonin-sensitive structures, taken as cytosolic fractions, and in digitonin-resistant structures. The SR Ca\(^{2+}\) pool was identified among digito-

### TABLE I

| Metabolites                        | Basal conditions | Inhibitors of AA metabolism | Migration of standard \(^{3}H\)AA |
|-----------------------------------|-----------------|-----------------------------|----------------------------------|
|                                   | Control         | Mini-glucagon               | Inhibitor Add (dpm)               | dpm (%) | dpm (%) | S.E. of two different experiments |
| Arachidonic acid                  |                 |                             |                                   | Control | Mini-glucagon | AACOCF\(_3\) |
|                                   | 2,950 (67)      | 4,754 (54)                  | 8,879 (84)                        | 20,388 (81) | 81 |
| Lipoxynogenase products (HETE\(_1\); HETE\(_2\); HETE\(_3\)) | 484 (11)        | 2,113 (24)                  | 423 (4)                           | 1,258 (5) | 7 |
| Cyclooxygenase products (PGE\(_1\); PGE\(_2\)) | 352 (8)        | 440 (5)                     | 211 (2)                           | 252 (1) | 5 |
| Nonidentified products            | 176 (4)         | 1,497 (17)                  | 1,057 (10)                        | 3,272 (9) | 7 |

FIG. 2. Mini-glucagon-induced \(^{3}H\) AA release relies on PLA\(_2\) activation. Embryonic chick ventricular cells were labeled for 24 h with 1.5 μCi/ml \(^{3}H\)AA as described under “Experimental Procedures.” Radiolabeled cells were washed twice in saline buffer containing 0.2% fatty acid-free bovine serum albumin and incubated for 30 min in the presence or absence of various peptides (mini-glucagon, angiotensin, or endothelin) and with or without a PLA\(_2\) inhibitor, quinacrine or AACOCF\(_3\). The amount of \(^{3}H\)AA released was expressed as percentage of control value (zero time: 148 ± 7 disintegrations/min/μg of protein). Values are means ± S.E. of two different experiments done in triplicate.

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Arachidonic Acid Mediates Mini-glucagon Action on Ca\(^{2+}\) Cycling

Embryonic chick heart cells were labeled with 1.5 μCi/ml \(^{3}H\)AA as described under “Experimental Procedures.” After two washings in saline buffer containing 0.2% fatty acid free bovine serum albumin, \(^{3}H\)AA-labeled cells were incubated for 30 min in the presence or absence of 0.1 mM mini-glucagon, and with or without a mixture of inhibitors of AA metabolism including: 30 μM NDGA, 1 μM indomethacin, and 100 μM SKF 250A. Analysis of the \(^{3}H\)-lipids 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 disintegrations/min of \(^{3}H\)-product/10\(^4\) cells and in % of total radioactivity recovered in the migration lane. Each sample represents the pool of quadruplicates. Data are from a typical experiment which has been repeated twice. Standard \(^{3}H\)AA was migrated in parallel in order to determine the nonenzymatic breakdown of AA.

Exogenous application of AA, at a concentration of 0.3 μM, mimicked the effect of mini-glucagon, inducing a 270 ± 51% increase in \(^{45}\)Ca accumulation into the digitonin-resistant structures which was abolished in the presence of caffeine. PLA\(_2\) inhibitors markedly decreased (5 μM quinacrine) or completely prevented (10 μM AACOCF\(_3\)) the \(^{45}\)Ca accumulation caused by mini-glucagon. It should be noted that quinacrine and AACOCF\(_3\) had no significant effect on \(^{45}\)Ca accumulation, when added alone.

To check the efficiency of the mixture of AA metabolism inhibitors, the supernatants of \(^{3}H\)AA-labeled cells were analyzed by TLC. As shown in Table I, in the absence or presence of mini-glucagon, the radioactivity of the supernatants recovered was 84–81% AA, 4–5% lipoxynogenase products, and 2–1% cyclooxygenase products. This pattern was similar to that of standard \(^{3}H\)AA (Table I), proving an efficient blockade by the
heart cells were labeled for 24 h with 1.5 mM [3H]AA according to the protocol previously described (6). As previously reported (6), the application of 10 mM caffeine produced a unique [Ca2+]i transient, longer than those observed during electrical stimulation. AA added alone, at 0.3 μM, had no effect (Fig. 6B).

In contrast, when cells were perifused with 30 nM AA plus 0.3 mM ETYA, the C20 nonhydrolyzable analogue of AA, elicited a response identical to that of AA, a maximal effect (11.3 ± 0.1 spikes of Ca2+) being observed in the presence of 3 mM ETYA. In contrast, exposure of myocytes to arachidonic acid (C20:4), linolenic acid (C18:3), cis-5,8,11,14,17-eicosapentaenoic acid (C20:5), cis-4,7,10,13,16,19-docosahexaenoic acid (C22:6), or arachidonic acid (C20:4), in the concentration range of 0.3 to 3 μM, failed to mimic the action of AA on caffeine contractures (Fig. 7).

AA Mimics the Mini-glucagon Action on [Ca2+]i Cycling in Electrically Stimulated Cells—As previously reported (6) and as shown in Fig. 8A, perfusion of myocytes with 30 nM glucagon alone evoked a small increase in the amplitude of electrically stimulated Ca2+ transients (1.19 ± 2% of control amplitude, n = 58). Mini-glucagon added alone at 0.1 nM had no effect (n = 82, Fig. 8B). In contrast, when cells were perfused with 30 nM glucagon plus 0.1 nM mini-glucagon, a marked rise in the amplitude of Ca2+ transients was observed (200 ± 6% of control amplitude, n = 75) together with a 2–3-fold increase in diastolic [Ca2+]i, estimated as described previously in Refs. 6 and 21 (Fig. 8C).

AA added alone had no effect on Ca2+ cycling in electrically stimulated cells (n = 75, Fig. 8D). However, when 0.3 μM AA was added in combination with 30 nM glucagon, marked increases in both the amplitude of Ca2+ transients (183 ± 7% of control amplitude, n = 69) and diastolic [Ca2+]i, were observed (Fig. 8E). Similar results were obtained with 0.3 μM AA plus 75 μM 8-Br-cAMP (Fig. 8F). Under the same experimental conditions, 100 nM isoproterenol produced a 220 ± 9% increase over control in the amplitude of the Ca2+ transients (n = 40) along with a 3-fold increase in diastolic [Ca2+]i (not shown). These data clearly demonstrate the ability of AA to mimic the action of mini-glucagon.

The Synergistic Action of AA and 8-Br-cAMP on Cell [Ca2+]i Cycling Produces a Positive Inotropic Effect—Fig. 9A shows parallel measurements of Ca2+ and contraction in isolated

FIG. 3. Ca2+-dependent stimulation of AA release by mini-glucagon: dependence on [Ca2+]i in intact cells (A) and dependence on [Ca2+]i in digitonin-permeabilized cells (B). Embryonic chick heart cells were labeled for 24 h with 1.5 μCi/ml [3H]AA as described under “Experimental Procedures.” Radiolabeled cells were washed twice at 37°C in saline buffer containing 0.2% fatty acid-free bovine serum albumin. In a first series of experiments, intact cells were incubated for 30 min in the absence or presence of 0.1 nM mini-glucagon, in a medium containing 0.5 or 2 mM Ca2+ (A). In a second series of experiments, cells were preincubated for 10 min with or without (control) 0.1 nM mini-glucagon, then permeabilized with digitonin, and incubated for 30 min with 10 μM GTP and increasing [Ca2+]i (B) [Ca2+]i was determined both by the EQUIV program and the Fura-2 procedure as described under “Experimental Procedures.” The amount of [3H]AA released was expressed as percentage of control value (139 ± 6% of control). Values are means ± S.E. of triplicate determinations from two different experiments (A) or means ± S.E. of quadruplicate determinations from three to six different experiments (B).

inhibitors of the enzymatic degradation of AA. It has to be noted that exposure of the cells to inhibitors of AA metabolism induced a marked increase of [3H]AA release, both in control and mini-glucagon-treated cells. This denoted an active metabolism of AA into eicosanoids in embryonic chick heart cells. Taken together, these results suggest that AA, and not a product of its metabolism, triggers [3H]Ca2+ accumulation into the SR stores.

Arachidonic Acid Potentiates Caffeine-induced Ca2+ Mobilization—Previous imaging studies (6) have shown that mini-glucagon potentiates caffeine-induced Ca2+ mobilization from the SR. Thus, we next examined the effect of AA on Ca2+ transients triggered by caffeine in Fura-2 loaded cells. Fig. 6A shows Ca2+ transients during electrical stimulation, and after caffeine application under steady state conditions, i.e. within a few seconds after interruption of electrical stimulation, according to the protocol previously described (6). As previously reported (6), the application of 10 mM caffeine produced a unique [Ca2+]i transient, longer than those observed during electrical stimulation. AA added alone, at 0.3 μM, had no effect (Fig. 6B). In contrast, the application of 10 mM caffeine together with 0.3 μM AA resulted in a train of Ca2+ transients (Fig. 6C). These experiments confirmed that AA mimics mini-glucagon action and leads to the Ca2+ loading of caffeine-sensitive SR stores.

An additional series of experiments were performed to examine the dose dependence and the specificity of AA action on caffeine-induced Ca2+-mobilization (Fig. 7). The action of AA (C20:4) was dose-dependent, with a maximal (9.5 ± 0.1) number of Ca2+ spikes detected over a period of 40 s at 3 μM AA and a half-maximal response occurring at 100 nM AA (Fig. 7). ETYA, the C20 nonhydrolyzable analogue of AA, elicited a response identical to that of AA, a maximal effect (11.3 ± 0.1 spikes of Ca2+) being observed in the presence of 3 μM ETYA. In contrast, exposure of myocytes to arachidonic acid (C20:4), linolenic acid (C18:3), cis-5,8,11,14,17-eicosapentaenoic acid (C20:5), cis-4,7,10,13,16,19-docosahexaenoic acid (C22:6), or arachidonic acid (C20:4), in the concentration range of 0.3 to 3 μM, failed to mimic the action of AA on caffeine contractures (Fig. 7).
myocytes stimulated at 0.5 Hz. Exposure of myocytes to 30 nM glucagon plus 0.1 nM mini-glucagon resulted in both an increase in the amplitude of Ca$^{2+}$ transients (113% over control) and an increase in the amplitude of contraction (70% over control) (Fig. 9B). The addition of AA (0.3 mM) plus 8-Br-cAMP (75 μM) evoked 61 and 77% increases over control amplitude of both the contraction and Ca$^{2+}$ transients, respectively (Fig. 9C), reproducing the effect of glucagon plus mini-glucagon.

Perfusion of the cells with 0.3 mM AA was without effect on either [Ca$^{2+}$]$_i$ transient or cell contraction (not shown) while 75 μM 8-Br-cAMP added alone evoked a 10% increase in the amplitude of the Ca$^{2+}$ transients, and a small (4% over control) increase in contraction (not shown).

**DISCUSSION**

The goal of the present study was to examine the role of AA in mediating the actions of mini-glucagon in ventricular myo-
FIG. 8. AA mimics mini-glucagon action on \([\text{Ca}^{2+}]_i\) cycling in electrically stimulated cells. Embryonic chick ventricular cells were loaded with Fura-2 as described under "Experimental Procedures." Cells were electrically stimulated at 0.5 Hz and perfused in the presence of 30 nM glucagon alone (A), 0.1 nM mini-glucagon alone (B), 30 nM glucagon plus 0.1 nM mini-glucagon (C), 0.3 \(\mu\text{M}\) AA alone (D), 0.3 \(\mu\text{M}\) AA plus 30 nM glucagon (E), or 0.3 \(\mu\text{M}\) AA plus 75 \(\mu\text{M}\) 8-Br-cAMP (F). These data are typical tracings representative of at least 40 cells obtained from at least three different isolations.

Arachidonic Acid Mediates Mini-glucagon Action on \([\text{Ca}^{2+}]_i\) Cycling

FIG. 9. Effects of AA added with 8-Br-cAMP, and mini-glucagon added with glucagon on \([\text{Ca}^{2+}]_i\) transients and contraction. Embryonic chick ventricular cells were loaded with Fura-2 as described under "Experimental Procedures." Cells were electrically stimulated at 0.5 Hz. Each trace is an average of five steady-state beats in a single cell. Data are representative of at least 5 cells obtained from two different isolations. Control trace (A); 0.1 nM mini-glucagon plus 30 nM glucagon (B); 0.3 \(\mu\text{M}\) AA plus 75 \(\mu\text{M}\) 8-Br-cAMP (C).
cytes. Several lines of evidence support the conclusion that AA is indeed the second messenger of mini-glucagon: 1) mini-glucagon increases AA release from \(^{[3]H}\)AA prelabeled myocytes, in a dose-dependent manner (0.001–0.1 nM), most likely via the activation of a Ca\(^{2+}\)-dependent PLA\(_2\); 2) Ca\(^{2+}\) accumulation into the SR stores of intact cells following exposure of myocytes to mini-glucagon (6) is prevented by PLA\(_2\) inhibitors, quinacrine, and AACOCF\(_3\); 3) the Ca\(^{2+}\) accumulation into the SR stores is mimicked by AA as well as by its nonhydrolyzable analogue, ETYA; 4) AA acts synergistically with either glucagon or 8-Br-cAMP in increasing Ca\(^{2+}\), cycling in electrically stimulated myocytes; 5) finally, perfusion of ventricular myocytes with both AA and 8-Br-cAMP leads to a positive inotropic contractile response identical to that produced by the mixture, glucagon plus mini-glucagon.

It is known that the formation of AA may occur independently of PLA\(_2\) activity. For example, diacylglycerol can serve as a precursor for AA via activation of diacylglycerol lipase. Diacylglycerol can be produced upon activation of PLC or from phosphatidic acid, following PLD activation. However, in the present study, the cell permeant analogue of diacylglycerol, OAG, failed to reproduce mini-glucagon actions (not shown), making it unlikely that mini-glucagon stimulated AA release through a diacylglycerol pathway. In addition, mini-glucagon, in contrast with other hormones inducing AA release (i.e. endothelin, angiotensin or bradykinin (10–13, 19)), did not activate PLC (6). Taken together, these data support the proposal that mini-glucagon causes AA release via the activation of a PLA\(_2\).

Activation of PLA\(_2\) may lead to a PKC-dependent phosphorylation event (9, 20). However, such a mechanism is not likely here since ETYA, which is structurally similar to AA, but not a known activator of PKC, mimicked mini-glucagon action.

AA release evoked by mini-glucagon requires submicromolar concentrations of Ca\(^{2+}\). Angiotensin has been reported to stimulate the release of AA in heart cells through activation of a Ca\(^{2+}\)-dependent PLA\(_2\) (11). However, the precise range of sensitivity of the enzyme toward Ca\(^{2+}\) has not been established. Three types of PLA\(_2\) have been identified in the cardiac tissue (20). A large part of the PLA\(_2\) activity in myocardium relies on a Ca\(^{2+}\)-independent, 40-kDa PLA\(_2\) (27) responsible for the accelerated phospholipid catabolism during myocardial ischemia (27). Recently, a low molecular mass PLA\(_2\) (14 kDa), which is almost exclusively expressed in heart and placenta, has been cloned (28). This enzyme contains 16 cysteines, is sensitive to Ca\(^{2+}\), but only in the millimolar range and it remains to establish whether it is secreted or is located intracellularly. Finally, a PLA\(_2\) of high molecular mass, referred to as “cytosolic PLA\(_2\)” (cPLA\(_2\)), which has been purified (29) and cloned (30), is expressed in various tissues, including the heart (31, 32). The amino acid sequence inferred from the human cDNA encodes an 85.2 kDa protein, which, however, migrates as a 100–110 kDa protein on SDS-polyacrylamide gel electrophoresis (31). This reduced rate of migration has been suggested to be inherent to the sequence and not to glycosylation (31). Amino acid sequence of the chicken cPLA\(_2\) differs from the sequences of human and mouse enzymes by 20–30% (33). Therefore, the difference in the rates of migration (80 versus 105 kDa) that we observe between the chicken and the rat cPLA\(_2\) may be due to species variations in the amino acid sequence of the enzyme. It may be noted that a cPLA\(_2\) with an apparent molecular mass of 80 kDa has recently been detected in rabbit vascular smooth muscle cells (34). Thus, our results indicate that cPLA\(_2\) is present in chick embryonic ventricular cells. As reviewed by Van Bilsen and Van der Vusse (20), the biochemical properties of this enzyme, including translocation to the membrane in a Ca\(^{2+}\)-dependent manner and requisite for submicromolar concentrations of Ca\(^{2+}\) for optimal activity, make it an ideal candidate as a target for mini-glucagon action. Although the role of G proteins in the modulation of the cardiac PLA\(_2\) activity has not been subject of investigation, G proteins have been implicated in the regulation of the cytosolic PLA\(_2\) activity in other tissues (9, 35). We observed that the release of AA stimulated by mini-glucagon required GTP. Although further characterization is needed, these results suggest that mini-glucagon, through a G protein, activates a Ca\(^{2+}\)-dependent PLA\(_2\).

The major role of AA as the precursor of the extended family of bioactive metabolites, the eicosanoids, is undisputed (15, 36). Our results suggest that the action of mini-glucagon is not dependent upon the production of eicosanoids from AA (Fig. 4 and Table I). More debated is the role of AA as a direct second messenger. Our data support the view that AA itself is an important signaling molecule in heart since, physiologically relevant (15), submicromolar concentrations of AA mimic mini-glucagon action. The most thoroughly characterized target of AA is protein kinase C (15). In heart cells, it has been shown that activation of protein kinase C by AA leads to the phosphorylation of troponin I and myosin light chain 2 (37). However, in our study, ETYA, which does not stimulate protein kinase C activity (19), mimics AA action and the target of AA remains to be identified.

In conclusion, we demonstrate that mini-glucagon action is mediated via AA and that AA together with 8-Br-cAMP triggers a positive inotropic response. Thus, the action of glucagon in heart does not only rely on a cAMP pathway but also requires the synergistic support of AA pathway. It is interesting to speculate whether such a synergism could also be involved in other positive inotropic responses to hormonal stimuli.

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Anne Sauvadet, Troy Rohn, Françoise Pecker and Catherine Pavoine

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