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Dual action of arachidonic acid on calcium mobilization in avian granulosa cells

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Summary

The primary aim of this study was to evaluate the effects of arachidonic acid (AA) on calcium mobilization from intracellular compartments in digitonin-permeabilized granulosa cells isolated from the largest preovulatory follicles of laying hens. At low concentrations (ED_{50} 0.2 μM) AA released 35% ^{45}Ca from the endoplasmic reticulum (ER), whereas at higher concentrations (ED_{50} 16 μM) it stimulated ^{45}Ca efflux from mitochondria. These effects of AA were mimicked at 10–20 times lower concentration by the calcium ionophore A23187. Inositol 1,4,5-trisphosphate (IP_3) also stimulated ^{45}Ca efflux from the ER, with a markedly lower potency than AA (ED_{50} 6.2 μM), as well as exhibiting a biphasic response. Heparin abolished the effect of IP_3 and luteinizing hormone (LH), but it had no influence on AA-promoted ^{45}Ca efflux. Moreover, the actions of IP_3 and AA were additive, indicating that AA and IP_3 access different Ca pools in the ER by different mechanisms. Several other unsaturated fatty acids also stimulated ^{45}Ca mobilization from both ER and mitochondria but, with the exception of eicosapentaenoic acid, were significantly less effective than AA. It is concluded that free AA, at submicromolar concentrations that might be viewed as physiological, is a potent calcium mobilizing agent and thus may play an important role in signal transduction in avian granulosa cells, akin to that of IP_3. At high (> 10 μM) concentrations AA removes Ca^{2+} from the mitochondria, an action that may be responsible for its reported inhibitory effects on steroidogenesis and other cellular functions.

Introduction

Calcium plays an important, though still poorly understood, role in hormone-induced steroidogenesis. Previous studies from this laboratory have found that omission of extracellular Ca^{2+} from the incubation medium reduced by about 50% luteinizing hormone (LH)-promoted progesterone production in chicken granulosa cells (Asem et al., 1984). Moreover, LH was found not only to activate the adenylate cyclase/cyclic AMP system (Takáts and Hertelendy, 1982), but also to promote ^{45}Ca efflux from extramitochondrial stores (Asem et al., 1987), as well as raising intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) via the activation of the inositol lipid signaling pathway (Hertelendy et al., 1989).

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There is growing evidence that arachidonic acid (AA) may also fulfil the role of an intracellular signaling molecule not unlike that played by inositol 1,4,5-trisphosphate (IP_3). For example, AA can be released as a result of a G protein-coupled activation of phospholipase A_2 (Axelrod et al., 1988) or derived from diacylglycerol produced from phospholipids by receptor-G protein-coupled activation of phospholipase C (Lapetina, 1982) and D (Exton, 1990). Whatever the immediate source of AA may be, it has been shown that exogenous AA can stimulate Ca^{2+} mobilization in a variety of permeabilized cells (Whiting and Barritt, 1982; Kolesnick et al., 1984; Wolf et al., 1986; Chan and Turk, 1987), as well as raising $[Ca^{2+}]_i$ in intact neutrophils (Naccache et al., 1989) and bovine luteal cells (Alila et al., 1990). Arachidonic acid has also been implicated as an intracellular modulator of steroidogenesis in ovarian cells of the rat (Wang and Leung, 1988), chicken (Johnson and Tilly, 1990) and fish (Van Der Kraak and Chang, 1990), as well as in rat Leydig cells (Abayasekara et al., 1990).

The aim of the present investigation was to evaluate the effects of AA on ^{45}Ca efflux from permeabilized avian granulosa cells. Our study has revealed a dual effect of AA. At low concentration it released ^{45}Ca from the endoplasmic reticulum (ER), whereas at higher concentrations it mobilized ^{45}Ca from the mitochondria as well, in a fashion that was similar, though less effective than the action of the calcium ionophore A23187.

Materials and methods

Hormones and chemicals

Ovine LH (oLH; NIAMDD-LH 22;.2–3 NIH LH Si U/mg) was kindly provided by the NIH Pituitary Hormone Distribution Program. Medium 199 with Hanks' salts was a product of Gibco (Grand Island, NY, USA). Sodium vanadate was obtained from Fisher Scientific Co. (Pittsburgh, PA, USA). ^{45}Ca (spec. act. 8.62 Ci/g) was obtained from ICN Radiochemicals (Irvine, CA, USA). 5,8,11,14-Eicosatetraynoic acid (ETYA) was a product of Hoffmann-La Roche (Nutley, NJ, USA). 1-[6[[17β-3-Methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione (U-73,122) was obtained from the Upjohn Co. (Kalamazoo, MI, USA) through the courtesy of Dr. John Bleasdale. 2-Nitro-4-carboxyphenyl-*N*,*N*-diphenyl-carbamate (NCDC), nordihydroguaiaretic acid (NDGA) and all other reagents and chemicals were from Sigma Chemical Co. (St. Louis, MO, USA).

Experimental animals

White Leghorn hens (Agri Foods, Hawk Point, MO, USA) in their first year of reproductive activity were caged individually, in a dark windowless, air-conditioned room, with a 14 h light and 10 h dark cycle. The birds had free access to a pelleted commercial laying ration (Purina Layena, Ralston-Purina, St. Louis, MO, USA) and tap water. The time of oviposition was monitored with an electric device and birds with at least five consecutive laying days were selected for the experiments. Granulosa cells used in the present investigation were obtained from the largest preovulatory follicle (F_1) 3 h before expected ovulation.

Preparation of granulosa cells

The birds were killed by cervical fracture and the follicles removed and placed in ice-cold saline. The granulosa layer was separated and the cells dissociated in Medium 199 containing collagenase and soybean trypsin inhibitor (Zakár and Hertelendy, 1980a). The viability of the cells was routinely higher than 90% as determined by the trypan blue dye exclusion method.

Measurement of ^{45}Ca mobilization in permeabilized cells

^{45}Ca efflux was determined as described previously (Asem et al., 1987) with minor modifications. Briefly, the dispersed cells were treated with digitonin (20 μM) in a 20 mM Pipes buffer (pH 7.0) containing 5 mM KCl, 150 mM NaCl, 2 mM $MgCl_2$, 1 mM EGTA for 8 min at 37°C. After washing rapidly with the same cold buffer, the cells were suspended in 'intracellular' buffer containing 20 mM Pipes (pH 7.0), 140 mM KCl, 5 mM $MgCl_2$, and 0.1% bovine serum albumin (BSA) (essentially fatty acid free) and loaded with ^{45}Ca (5 μCi) for 30 min at 37°C in the presence of 5 mM ATP and 6.5 μM ruthenium red, to prevent the Ca uptake by mitochondria or 1.3 mM

vanadate, to inhibit Ca uptake by the ER (Wolf et al., 1986; Asem et al., 1987). The cells were then stimulated with agonists dissolved in either 'intracellular' buffer or dimethyl sulfoxide (DMSO) and the incubation was allowed to continue. At denoted times, aliquots (100 μl) of the cell suspension were rapidly removed and washed on Millipore filters (pore size 0.45 μm, Millipore Corp., Bedford, MA, USA) with a buffer containing 140 mM KCl buffered with 10 mM Tris-HCl (pH 7.0). The radioactivity in the cells retained by the filter was determined by liquid scintillation spectrometry. The free ^{45}Ca concentration, adjusted with EGTA, was 0.1 μM when cells were loaded in the presence of ruthenium red (ER loading) or 100 μM when cells were loaded in the presence of vanadate. Results are expressed as percent ^{45}Ca efflux, which was estimated as follows:

$$\%\ ^{45}\text{Ca efflux} = \frac{T - Y}{T - Z} \times 100$$

where T is the total radioactivity in the cells before the addition of agonist, Y is radioactivity retained in cells after time t (at the end of incubation), and Z is the radioactivity after A23187 (2 μM) treatment. $(T-Y)$ thus represents the amount of ^{45}Ca mobilized at time t and $(T-Z)$ is the total amount of mobilizeable ^{45}Ca. Each data point represents percent ^{45}Ca efflux corrected by the value of the vehicle-treated control.

Measurement of the mitochondrial ^{45}Ca content

Collagenase-dispersed cells were washed and resuspended in a Ca-free Krebs-Ringer bicarbonate buffer (KRBG) containing 120 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 2.3 mM $MgSO_4$, 25 mM $NaHCO_3$, 5.6 mM glucose, 0.1% BSA and 25 mM Hepes (pH 7.4). The cells were preloaded with ^{45}Ca (10 μCi/ml) for 3 h. After washing, the cells were suspended in KRBG containing 0.1 μM free Ca^{2+} buffered with EGTA or 1 mM Ca^{2+} and incubated with or without AA for 5 min. The reaction was stopped by placing the samples into an ice-bath and the mitochondria were isolated from the granulosa membranes using the procedure described by Asem et al. (1985). Briefly, the cells were suspended in 5–7 volumes of medium containing 250 mM sucrose, 1 mM dithiothreitol (DTT), 0.1% BSA and 25 mM Tris-HCl (pH 7.4) and were sonicated while kept on ice, using a Sonifier sonicator (Branson Instruments, Stanford, CA, USA), equipped with a microprobe, at setting 6 tuned to 5 A of direct current for 3×10 s.

The homogenates were centrifuged at $600 \times g$ for 10 min and the resulting supernatants were centrifuged at $15{,}000 \times g$ for 15 min using a Beckman airfuge ultracentrifuge (Beckman Instruments, Palo Alto, CA, USA). The pellet was dissolved in formic acid and the ^{45}Ca content was determined by liquid scintillation spectrometry. Results are expressed as percent of ^{45}Ca content of the vehicle-treated control cells.

Statistical analysis

Data are expressed as mean $\pm$ SE of n observations. Statistical analysis was performed by one-way repeated measures analysis of variance (ANOVA) and post hoc Dunnett's test or by Student's t-test. Significance was set at $P < 0.05$.

Results

Arachidonic acid evoked a dose-dependent release of ^{45}Ca from permeabilized granulosa cells prelabeled with ^{45}Ca in the presence of ruthenium red. Maximal efflux of 35% of total releasable extramitochondrial ^{45}Ca was obtained at 1 μM AA (ED_{50} 205 $\pm$ 35 nM) (Fig. 1). Calcium mobilization was rapid, peaking at 60 s, after which it declined, but remained significantly above vehicle-treated controls during the 20 min exposure to 1 μM AA (inset, Fig. 1). When granulosa cells were loaded with ^{45}Ca in a medium containing 100 μM Ca^{2+} (to promote mitochondrial uptake) and vanadate (to block uptake by ER), AA caused a rapid and dose-dependent release of ^{45}Ca (Fig. 2). However, both the kinetics and dose dependence of mitochondrial efflux were distinctly different from that originating from the ER. The lowest effective dose of AA which provoked a significant ($P < 0.05$) efflux of ^{45}Ca was obtained at 10 μM (ED_{50} 16 $\pm$ 3.5 μM) and near-maximal responses (50% of total mobilizeable fraction) were measured at 100 μM.

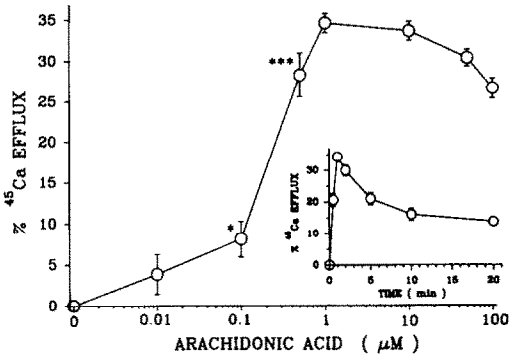

Fig. 1. Dose–response of arachidonic acid-promoted ^{45}Ca efflux from the endoplasmic reticulum. Digitonin-permeabilized granulosa cells were preloaded with ^{45}Ca for 30 min in the presence of 6.5 μM ruthenium red to block mitochondrial uptake of ^{45}Ca before the addition of arachidonic acid. The incubation was stopped 1 min later. Inset shows the time-course of ^{45}Ca efflux in response to 1 μM arachidonic acid. Results are mean ± SEM of four separate experiments performed in triplicate. * $P < 0.05$; *** $P < 0.005$.

To corroborate the observed effects of AA on the release of ^{45}Ca from mitochondria in permeabilized cells, we attempted to assess the ^{45}Ca content of mitochondria after a brief exposure of intact cells to AA. When such cells were prelabeled for 3 h with ^{45}Ca, and after washing incubated with AA in high (1 mM) and low (0.1 μM)

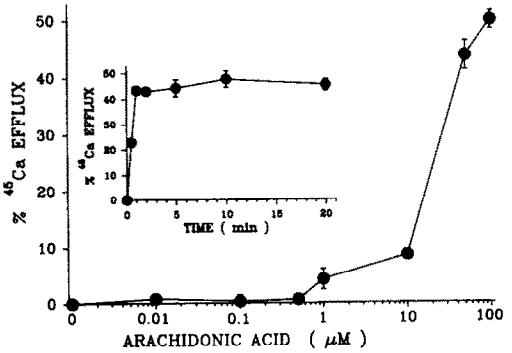

Fig. 2. Dose–response of arachidonic acid-promoted ^{45}Ca efflux from mitochondria of digitonin-permeabilized granulosa cells. Cells were preloaded with ^{45}Ca for 30 min in the presence of 1.3 mM sodium orthovanadate, to prevent ^{45}Ca uptake by endoplasmic reticulum, before the addition of arachidonic acid. The incubation was stopped 5 min later. Inset shows the time-course of 50 μM arachidonic acid-promoted ^{45}Ca mobilization. Results are mean ± SEM of four separate experiments performed in triplicate. Values were significantly different from control at 10 μM arachidonate ($P < 0.05$) and 50 and 100 μM ($P < 0.001$).

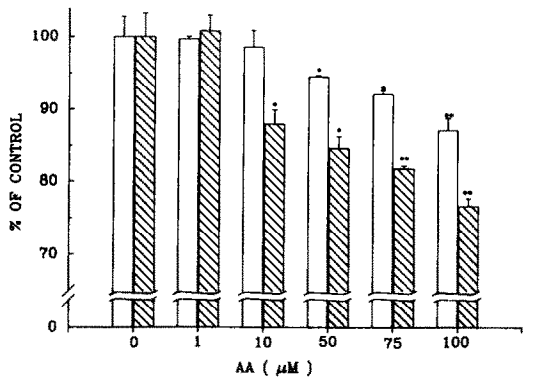

Fig. 3. Effect of arachidonic acid on ^{45}Ca content of isolated mitochondria. Intact granulosa cells were preloaded with ^{45}Ca for 3 h. After washing, the cells were resuspended in buffer containing 1 mM (open bars), or 0.1 μM $CaCl_2$ (hatched bars) without ^{45}Ca, and the reaction was started by the addition of arachidonic acid (0–100 μM). After 5 min, the reaction mixtures were rapidly chilled and the ^{45}Ca content of the mitochondria estimated as described in Materials and methods. Results (mean ± SEM) are expressed as percent of vehicle-treated control values.

calcium-containing buffer, a significant and dose-dependent decrease in ^{45}Ca content of isolated mitochondria was observed at 10–100 μM AA in low Ca^{2+} buffer (Fig. 3). In the presence of 1 mM extracellular Ca^{2+}, AA was less effective in lowering mitochondrial calcium content, reducing it by 5%, 7%, and 14% at 50, 75 and 100 μM, respectively ($P < 0.05$; Fig. 3). Exposure of permeabilized granulosa cells to the ionophore A23187 mimicked the action of AA (Fig. 4). As in the case of AA, the ionophore was also found to be more effective in mobilizing ^{45}Ca from the ER (ED_{50} 22 ± 3 nM), compared to mitochondria (ED_{50} 590 ± 150 nM). However, unlike AA, 1 μM A23187 released the total (100%) mobilizeable ^{45}Ca from either mitochondrial or extramitochondrial pools. (The nature of the methodology is such that agonist-promoted release is expressed as percent of total, i.e. 2 μM A23187 releasable ^{45}Ca, which we have found to amount to 86 ± 0.8% of the total cellular ^{45}Ca.)

To ascertain that the Ca-mobilizing action of AA was not due to one or more of its metabolites, we evaluated the effects of AA in the presence of indomethacin, which inhibits AA metabolism via the cyclooxygenase pathway, or NDGA, which blocks lipoxygenases, or ETYA,

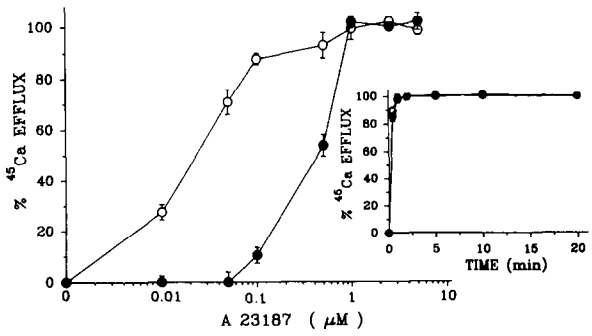

Fig. 4. Dose–response of A23187-promoted ^{45}Ca efflux in digitonin-permeabilized avian granulosa cells. The cells were preloaded with ^{45}Ca in the presence of 6.5 μM ruthenium red (open symbols), or in the presence of 1.3 mM vanadate (closed symbols) for 30 min before the addition of ionophore and incubated for 5 min before aliquots were taken for ^{45}Ca measurement. Data represent mean ± SEM of four experiments. Values were significantly different from control at 10 nM and up (open symbols) and at 100 nM and up (closed symbols). Inset shows the time-course of ^{45}Ca release from endoplasmic reticulum and mitochondria in response to 1 μM A23187. The two curves overlap.

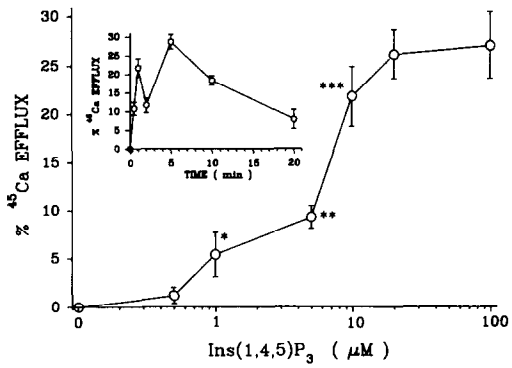

Fig. 5. Dose–response of inositol 1,4,5-trisphosphate-promoted ^{45}Ca mobilization in digitonin-permeabilized granulosa cells loaded with ^{45}Ca for 30 min in the presence of ruthenium red. Reaction was stopped 1 min after the addition of agonist. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$. Inset shows the kinetics of 10 μM IP$_3$-stimulated ^{45}Ca efflux.

which, in addition, inhibits the *P*-450 epoxygenase pathway, at concentrations that have been shown to be effective in a variety of cells (for references, see Needleman et al., 1986). Accordingly, permeabilized granulosa cells were loaded with ^{45}Ca either in the presence of vanadate or ruthenium red for 30 min. 10 min before the end of the loading period and the addition of AA, indomethacin (10 μM), NDGA (10 μM), or ETYA (50 μM) were added to the cell suspension. The release of ^{45}Ca from the ER was measured 1 min, and from the mitochondria 2 min after the addition of AA. None of the inhibitors had any influence on AA-generated ^{45}Ca efflux from either the ER or mitochondria (results not shown).

To learn more about the mode of AA-promoted ^{45}Ca mobilization, in the next experiment we compared the action of AA with that of IP$_3$, which was shown previously to stimulate ^{45}Ca efflux from extramitochondrial compartment(s) of digitonin-permeabilized hen granulosa cells (Hertelendy et al., 1989). The dose response and time course of IP$_3$-induced ^{45}Ca efflux from the ER are shown in Fig. 5. The minimum dose causing a significant release of ^{45}Ca was 1 μM IP$_3$, whereas maximal responses were obtained at 20 μM IP$_3$ (ED_{50} 6.2 ± 0.9 μM). However, unlike AA-promoted ^{45}Ca efflux, the kinetics of IP$_3$ evoked response was biphasic. After a rapid initial rise peaking at 60 s, the rate of efflux declined, exhibiting a second peak at 5 min. Further, when maximally stimulating doses of both AA and IP$_3$ were combined, a significant additive effect on ^{45}Ca efflux was obtained compared to the responses elicited by either agonist applied singly (Fig. 6).

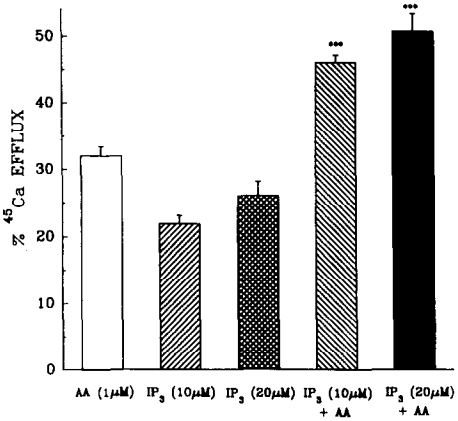

Fig. 6. Additive effects of arachidonic acid and inositol 1,4,5-trisphosphate on ^{45}Ca efflux from permeabilized granulosa cells. ^{45}Ca labeling was done for 30 min in the presence of 6.5 μM ruthenium red, and efflux was measured after 1 min stimulation. Results are mean ± SEM of four experiments. *** $P < 0.005$ vs. IP$_3$ alone (Student's *t*-test).

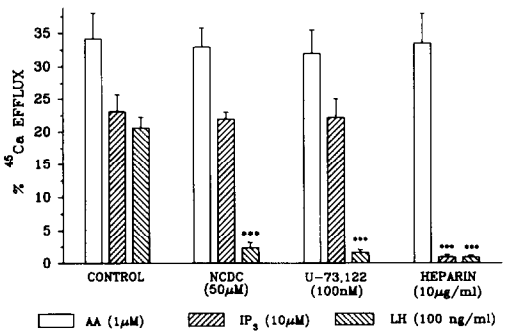

Fig. 7. Effect of phospholipase C inhibitors (NCDC and U-73,122) and heparin on arachidonic acid (AA)-, inositol 1,4,5-trisphosphate (IP_3)- and luteinizing hormone (LH)-stimulated ^{45}Ca efflux in digitonin-permeabilized granulosa cells loaded with ^{45}Ca for 30 min in the presence of 6.5 μM ruthenium red. Data are from five experiments (mean ± SEM). *** $P < 0.005$ by ANOVA.

Previous studies from this laboratory have demonstrated a rapid mobilization of nonmitochondrial ^{45}Ca in mature chicken granulosa stimulated with LH (Asem et al., 1987). To compare the nature of LH-promoted ^{45}Ca release with that elicited by AA and IP_3, we pretreated the cells for 10 min with two inhibitors of phospholipase C to prevent endogenous IP_3 generation, or heparin to block IP_3-mediated Ca^{2+} release from the ER. As illustrated in Fig. 7, the two enzyme inhibitors, NCDC (Clark and Garland, 1991) and U-73,122 (Bleasdale et al., 1990), suppressed LH, but not AA- or IP_3-promoted ^{45}Ca efflux, whereas heparin blocked both LH and IP_3 effects, while having no influence on AA-induced ^{45}Ca release. The specificity of AA on promoting ^{45}Ca efflux was evaluated by comparing its action to that of several other fatty acids of various chain length and unsaturation. In addition to AA, five fatty acids tested stimulated ^{45}Ca efflux from both ER and mitochondria, with the following rank order of potency: AA > pentaenoic ≫ tetraenoic > linoleic > linolenic > oleic acid (Fig. 8).

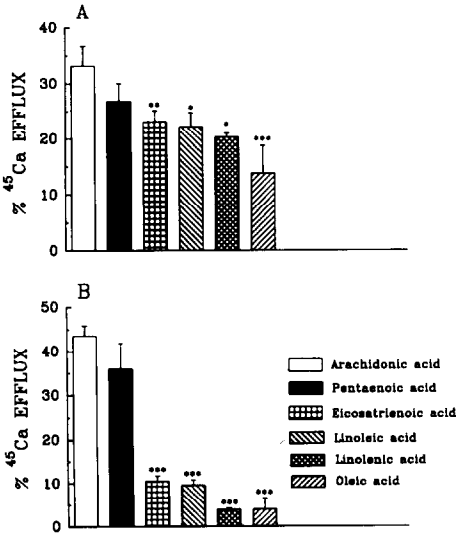

Fig. 8. Effects of various fatty acids on ^{45}Ca efflux from the endoplasmic reticulum (*A*) and mitochondria (*B*) of digitonin-permeabilized granulosa cells. Efflux measurements were done 1 min after the addition of fatty acids (1 μM: *A* and 50 μM: *B*). Data illustrate the results of three experiments each done in triplicate. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$ vs. arachidonic acid.

Discussion

Permeabilized cells have been frequently used to study various aspects of signal transduction (Hersey and Perez, 1990). It allows the introduction of key components of these mechanisms such as ATP, GTP-γ-S and their analogs, as well as inositol phosphates. Using the digitonin-permeabilized cell model, we have examined previously some aspects of Ca^{2+} mobilization in ovarian granulosa cells (Asem et al., 1987; Hertelendy et al., 1989), as well as smooth muscle cells of the hen oviduct (Molnár et al., 1987; Molnár and Hertelendy, 1990). In the present study we extended our observations to the action of AA, in view of its putative role in signal transduction in general, and specifically in the regulation of steroidogenesis in ovarian cells of several vertebrate species, including the domestic fowl (see Introduction). By selectively labeling the two main Ca^{2+}-sequestering intracellular compartments (the ER and mitochondria) with ^{45}Ca (Wolf et al., 1988), we have been able to demonstrate that AA exerts a dual effect on Ca^{2+} mobilization from these compartments. At low concentrations (0.1–1.0 μM), that could be considered physiological (although no values for free intracellular levels of AA are available), AA was found to cause a rapid release of ^{45}Ca from the ER, representing about one-third of the total releasable ^{45}Ca. This is similar to the amount which can be released by IP_3, although the ^{45}Ca-mobilizing potency of AA

is about 30 times greater than that of IP_3 (ED_{50} 0.2 μM vs. 6.2 μM). However, it would appear from the results of this study that the two Ca-mobilizing agonists access different Ca pools in the ER by discrete mechanisms. This interpretation is supported by the following observations. First, when heparin, which blocks IP_3-mediated ^{45}Ca release from the ER (Kobayashi et al., 1988; Chopra et al., 1989), was incorporated in the incubation medium, IP_3-, but not AA-releasable ^{45}Ca was inhibited; second, phospholipase C inhibitors had no effect on the action of AA, while inhibiting LH-promoted ^{45}Ca release believed to result from phospholipase C-mediated generation of IP_3; third, the kinetics of AA- and IP_3-promoted ^{45}Ca efflux were found to be different. The former generated a rapid single phase, whereas the response to IP_3 was biphasic. Finally, and perhaps most significantly, the effects of maximally stimulating doses of AA and IP_3 were additive. Such an additive effect on ^{45}Ca release has also been observed both from digitonin-permeabilized pancreatic islets (Wolf et al., 1986), and from human platelets (Nozawa et al., 1991).

The implication of this phenomenon, with respect to signal transduction, could be potentially significant. A Ca-mobilizing signal generated by a hormone receptor-G-protein-coupled activation of phospholipase C, producing IP_3, would be amplified by AA, released not only from glycerides derived from phosphoinositides, but perhaps more importantly, via the activation of phospholipase A_2 by a rise in $[Ca^{2+}]_i$, as a result of IP_3-triggered Ca^{2+} release and increased Ca^{2+} influx (Berridge and Irvine, 1989). Alternately, or in addition to the above mechanism, protein kinase C, activated by diacylglycerol (derived from phospholipase C-catalyzed hydrolysis of phosphoinositides) could lead to stimulation of phospholipase A_2 and AA release (Chang et al., 1987; Zor et al., 1991). Protein kinase C activation may further increase free AA levels by inhibiting key enzymes involved in reacylation (Fuse et al., 1989).

Although AA is rapidly converted to eicosanoids in many different types of cells, it seems unlikely that the observed effect of AA on ^{45}Ca efflux was due to prostaglandins or other metabolites of AA. We found that indomethacin, which blocks the cyclooxygenase pathway, NDGA, which inhibits lipoxygenase, and the AA analog ETYA, which inhibits in addition epoxygenase-catalyzed oxygenation of AA (Needleman et al., 1986), had no effect on ^{45}Ca release in response to AA stimulation. Moreover, chicken granulosa cells prelabeled with [^{3}H]AA produce only trace amounts of [^{3}H]eicosanoids (F. Hertelendy and M. Molnár, unpublished observations). Other investigators have also found that the ability of AA to stimulate Ca^{2+} release from extramitochondrial Ca pools of permeabilized neutrophils (Beaumier et al., 1987) or rat liver microsomes (Chan and Turk, 1987) was independent of its metabolism to lipoxygenase or cyclooxygenase products.

The second noteworthy aspect of this study was the demonstration that by raising AA levels above 1 μM a rapid, sustained, and dose-dependent release of ^{45}Ca from the mitochondrial pool was obtained. This observation was corroborated by the demonstration that AA (10–100 μM) reduced in a dose-related fashion the ^{45}Ca content of mitochondria isolated after exposure to AA. Although the role of mitochondrial calcium in signal transduction may be of less importance compared to calcium derived from the ER, agonist-induced mobilization of mitochondrial calcium has been observed in a variety of cells (Wolf et al., 1988). In agreement with published reports, the present observations have shown that the release of mitochondrial calcium is observed at concentrations of AA which may be higher than those occurring under physiological conditions. However, in response to a massive stimulus, several pathways of AA release may be activated (Burgoyne and Morgan, 1990), leading to AA concentrations sufficiently high, particularly in ovarian cells where total AA content is very high (Alila and Hansel, 1990), to affect mitochondrial calcium egress. The consequences of such 'decalcification' of mitochondria may represent a negative feedback mechanism, due possibly to inhibition of key calcium-requiring mitochondrial enzymes involved in ATP production (Denton and McCormack, 1985), and hence impair various cellular functions such as protein synthesis, which has been shown to be essential for LH-promoted progesterone production in hen granulosa cells (Zakár and Hertelendy, 1980b). Indeed, AA in

the concentration range that was found in the present study to cause ^{45}Ca release from mitochondria, has also been reported to inhibit LH- and forskolin-stimulated cAMP production, as well as steroidogenesis in hen ovarian cells (Johnson and Tilly, 1990; Johnson et al., 1991).

The mechanism of AA-promoted ^{45}Ca release from the mitochondria is unknown. However, the similarity between the action of AA and that of A23187 (i.e. stimulation of ^{45}Ca release from the ER at submicromolar concentrations and from the mitochondria at higher concentrations) suggests that the mode of action of AA involves an ionophoric mechanism. This notion is supported by the observations that at high concentrations both calcium ionophores and AA inhibit progesterone production in hen granulosa cells in response to steroidogenic agonists (Zobell et al., 1987; Johnson and Tilly, 1990). Because inhibitors of AA metabolism failed to affect AA-promoted ^{45}Ca efflux from the mitochondria, it seems unlikely that a metabolite or an oxidative product was responsible for the observed effect. Fischer et al. (1990) have also found that AA itself and not its metabolites was responsible for Ca^{2+} mobilization from human platelets and have provided evidence that AA-induced Ca^{2+} efflux involves a carrier-type ionophoric mechanism analogous to that described for A23187.

In summary, the present study has demonstrated that in hen granulosa cells AA is a potent mobilizer of Ca^{2+} from intracellular stores. At submicromolar concentrations, which could presumably occur in response to agonist-induced stimulation of phospholipases, AA acts in a fashion similar to that of IP_3, although by a discrete mechanism, releasing Ca^{2+} from the ER. At a concentration of $> 10\ \mu M$ (which is less likely to occur under physiological conditions), AA releases Ca^{2+} from the mitochondria and inhibits LH-supported steroidogenesis and possibly other energy-dependent cellular functions.

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