

# Arachidonic acid release from rat Leydig cells: the involvement of G protein, phospholipase A<sub>2</sub> and regulation of cAMP production

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## Abstract

We have previously demonstrated that the release of arachidonic acid (AA) from human chorionic gonadotropin (hCG)-stimulated Leydig cells occurs in a dose- and time-dependent manner. In addition, the amount of AA released was dependent on the hormone-receptor interaction and the concentration of LH-hCG binding sites on the cell surface. The present study was conducted to evaluate the involvement of phospholipase A<sub>2</sub> (PLA<sub>2</sub>) and G proteins in AA release from hormonally stimulated rat Leydig cells, and the possible role of this fatty acid in cAMP production. Cells were first prelabelled with [<sup>14</sup>C]AA to incorporate the fatty acid into cell phospholipids, and then treated in different ways to evaluate AA release. hCG (25 mIU) increased the release of AA to 180 ± 12% when compared with AA released from control cells, arbitrarily set as 100%. Mepacrine and parabromophenacyl bromide (pBpB), two PLA<sub>2</sub> inhibitors, decreased the hormone-stimulated AA release to 85 ± 9 and 70 ± 24% respectively. Conversely, melittin, a PLA<sub>2</sub> stimulator, increased the release of AA up to 200% over control. The inhibitory effect of mepacrine on the release

of AA was evident in hCG-treated Leydig cells, but not in the melittin-treated cells. To determine if the release of AA was also mediated through a G protein, cells were first permeabilized and subsequently treated with pertussis toxin or GTPγS, a non-hydrolyzable analog of GTP. Results demonstrate that GTPγS was able to induce a similar level of the release of AA as hCG. In addition, pertussis toxin completely abolished the stimulatory effect of hCG on the release of AA, indicating that a member of the G<sub>i</sub> family was involved in the hCG-dependent release of AA. Cells treated with PLA<sub>2</sub> inhibitors did not modify cAMP production, but exogenously added AA significantly reduced cAMP production from hCG-treated Leydig cells, in a manner dependent on the concentration of AA and hCG. Results presented here suggest an involvement of PLA<sub>2</sub> and G proteins in the release of AA from hCG-stimulated Leydig cells, and under particular conditions, regulation of cAMP production by this fatty acid in these cells.

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## Introduction

Activation and regulation of Leydig cell function is exerted primarily by luteinizing hormone (LH) or its analogue, human chorionic gonadotropin (hCG). Both molecules interact with membrane LH-hCG receptors, activating the classic second messenger, cAMP (Catt *et al.* 1980). However, additional second messengers such as calcium and chloride ions, and arachidonic acid (AA) or its metabolites, have also been involved in the regulation of steroidogenesis (Sullivan & Cooke 1986, Choi & Cooke 1990, Cooke *et al.* 1991). Several reports have indicated an important role for AA in the modulation of hormone-induced steroid production in rat Leydig cells (Cooke *et al.* 1991, Moraga *et al.* 1997, Wang *et al.* 1999). In these cells, a rapid release of AA has been detected after stimulation by LH (Cooke *et al.* 1991). We have demonstrated that AA release occurred in a dose- and time-dependent manner in

hCG-stimulated Leydig cells. Furthermore, the release of AA was dependent on the hormone-receptor interaction and the concentration of LH-hCG binding sites on the cell surface (Moraga *et al.* 1997). AA may be released after hormone-receptor interaction, mainly by the action of phospholipase A<sub>2</sub> (PLA<sub>2</sub>) or phospholipase C (PLC) and diacylglycerol lipase. In addition, AA can be metabolized by three independent pathways (cyclo-oxygenase, lipoxygenase and the cytochrome P450-dependent epoxygenase; Needleman *et al.* 1986), producing metabolites able to regulate LH and LH releasing hormone (LHRH)-induced testosterone production by rat Leydig cells (Didolkar & Sundaram 1987, Romanelli *et al.* 1995, Mele *et al.* 1997).

The role of AA in trophic hormone-stimulated steroid production in various steroidogenic cells has been well documented (Yamazaki *et al.* 1996, Abayasekara *et al.* 1990, Cooke *et al.* 1991, Boone *et al.* 1993, Moraga *et al.* 1997). Recently, in MA-10 mouse Leydig tumor cells, it

was demonstrated that AA is essential for steroidogenic acute regulatory (StAR) protein expression at the level of gene transcription (Wang *et al.* 2000). Moreover, the AA-mediated signal transduction pathway was different from the previously reported cAMP–protein kinase A–phosphorylation pathway (Wang & Stocco 1999). Recent results have shown that AA modulates the GTPase activity of G proteins, maintaining  $G_i$  proteins in their activated state, which in turn inhibits adenylate cyclase, which results in a decrease in cAMP concentrations (Marinero *et al.* 2000). All these results demonstrate that AA is involved in trophic hormone-stimulated steroid production in Leydig cells. At present, however, mechanisms involved in the release of AA from plasma membrane of hormonally stimulated Leydig cells are not completely understood. Therefore, the aim of this study was to evaluate the possible involvement of PLA<sub>2</sub> and G proteins in the release of AA by hCG-stimulated rat Leydig cells, and the possible role of this fatty acid in cAMP production.

The present study provides evidence indicating that molecules able to modulate PLA<sub>2</sub> activity and G proteins also regulate the release of AA from hCG-stimulated Leydig cells. Moreover, when hCG-stimulated release of AA is inhibited in the presence of PLA<sub>2</sub> inhibitors, cAMP production is not modified. Nevertheless, in the presence of exogenously added AA, hCG-stimulated cAMP production is inhibited in a manner dependent on the concentrations of both hCG and AA. These results suggest an involvement of PLA<sub>2</sub> and G proteins in the release of AA from hCG-stimulated Leydig cells. In addition, although cAMP production may occur independently of AA release in hormonally stimulated cells, the fatty acid may be able to modulate cAMP production under conditions exceeding a threshold of intracellular AA concentration. In summary, this study provides evidence for a mechanism of the release of AA in hCG-stimulated rat Leydig cells and the possible involvement of this fatty acid in modulation of cAMP production.

## Materials and Methods

### Materials

Human chorionic gonadotrophin (hCG, CR127) was generously donated by the NIHPP (USA). Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco (Paisley, Strathclyde, UK). Bovine serum albumin (fraction V BSA and fatty acid-free BSA), arachidonic acid (free acid, sealed ampoule), GDP, GTP $\gamma$ S, pertussis toxin (PTX), mepacrine, parabromophenacyl bromide (pBpB), melittin and Percoll were obtained from Sigma Chemical Co. (St Louis, MO, USA). The cAMP detection kit was purchased from Amersham International (Amersham, Bucks, UK). [<sup>14</sup>C]Arachidonic acid ([<sup>14</sup>C]AA, specific activity 55 mCi/mmol) was purchased from NEN

(Boston, MA, USA). Collagenase was from Worthington Biochemical Co. (Freehold, NJ, USA). All other chemicals were of analytical grade.

### Cell isolation and purification

Leydig cells were isolated from 200–300 g Wistar rats from our colony. All animals were cared for according to the guidelines from International Animal Care. Animals were anesthetized and killed by decapitation. The testes were then removed, decapsulated and subjected to enzymatic dispersion with collagenase (0.25 mg/ml) at 37 °C for 15 min in a shaking bath to obtain interstitial cells, as previously described (Ronco & Valladares 1993). Interstitial cells were then filtered through gauze to remove fragments of seminiferous tubules and purified through a density gradient as described before (Ronco & Valladares 1993). Briefly, interstitial cells were applied to a 26 ml linear 10–80% Percoll gradient in HEPES buffer, pH 7.4, and centrifuged at 800 g for 30 min. Gradient fractions (1 ml) were collected and fractions 20–26 were pooled (Hedger & Eddy 1986). Leydig cell number was established by the histochemical reaction for 3 $\beta$ -hydroxysteroid dehydrogenase (3 $\beta$ -HSD) (Steinberger *et al.* 1966). Cell viability was assessed by trypan blue exclusion and histochemical staining for diaphorase, as previously described (Aldred & Cooke 1983). Leydig cell purity and viability were at least 90%. Data obtained from experiments were normalized and expressed per 10<sup>6</sup> Leydig cells.

### Incorporation of AA to membrane phospholipids

Approximately 2 × 10<sup>6</sup> Leydig cells were incubated with 0.2  $\mu$ Ci [<sup>14</sup>C]AA in 1 ml DMEM–0.1% BSA, pH 7.3, at 34 °C, under an atmosphere of 95% air–5% CO<sub>2</sub>, for 4 h. Cells were then collected and centrifuged at 800 g for 10 min. The cellular pellet was washed twice and incubated under different experimental conditions to assess [<sup>14</sup>C]AA release.

### Release of AA from membrane phospholipids

AA release was measured from prelabelled cells, essentially as already described (Moraga *et al.* 1997). Aliquots of resuspended Leydig cells (2 × 10<sup>6</sup> cells/ml) were preincubated with 0.2  $\mu$ Ci/tube [<sup>14</sup>C]AA as described before. After 4 h, cells were collected and washed twice with 4 ml DMEM–0.5% fatty acid-free BSA to remove free [<sup>14</sup>C]AA, and incubated for 2 h with different treatments. Total [<sup>14</sup>C]AA release (intracellular and from the incubation medium) was determined by adding 2 ml cold methanol to the cell suspension, and samples were stored under N<sub>2</sub> at –20 °C until required for analysis. [<sup>14</sup>C]AA was extracted from the methanol phase with a solvent mixture, added in the following order: 1 ml chloroform,

32  $\mu\text{l}$  HCl (12.06 M), 20  $\mu\text{g}$  cold AA (as a tracer), 1 ml chloroform, 1 ml KCl 0.5 M (Kamel & Kubajac 1988). The phases were separated by low-speed centrifugation, the final chloroform phase dried under  $\text{N}_2$ , and [ $^{14}\text{C}$ ]AA separated from other lipids by thin-layer chromatography (TLC) on silica-gel plates. The ascending chromatography was developed with a mixture of hexane–diethylether–acetic acid (65:35:4, v/v/v). The [ $^{14}\text{C}$ ]AA band was detected with iodine, scraped, and analyzed by liquid scintillation counting in a Beckman LS 5000 TD liquid scintillation spectrometer.

#### Treatments with inhibitors and activators of $\text{PLA}_2$

To determine if  $\text{PLA}_2$  was involved in the release of AA, prelabelled cells were preincubated for 30 min in DMEM–0.5% fatty acid-free BSA in the presence of different inhibitors (10  $\mu\text{M}$  mepacrine or pBpB) or activators (melittin, 10  $\mu\text{M}$ ) of  $\text{PLA}_2$ . After that period, cells were treated with hCG (25 mIU) and incubated for 2 h. To assess if the activator melittin was able to reverse the inhibition induced by mepacrine, cells were preincubated with the inhibitor for 30 min, and then melittin or hCG was added and the incubation proceeded for 2 h. Total [ $^{14}\text{C}$ ]AA released was determined as described.

#### GTP $\gamma$ S assays and toxin treatments

For GTP assays, [ $^{14}\text{C}$ ]AA prelabelled cells were permeabilized with saponin (25  $\mu\text{g}/\text{ml}$ ) for 15 min at 37 °C. In multiple repetitions, this procedure resulted in 85–94% of permeabilized Leydig cells, as determined by trypan blue inclusion. Permeabilized cells were incubated in the presence of GTP $\gamma$ S (100  $\mu\text{M}$ ), hCG (25 mIU) or GDP (100  $\mu\text{M}$ ) as previously described (Gaurisankar & Fox 1994). For PTX treatment, prelabelled and permeabilized cells were preincubated for 40 min with PTX (20 ng/ml), then hCG (25 mIU) was added and incubation continued for an additional 2 h. Total [ $^{14}\text{C}$ ]AA released was determined as described.

#### cAMP assays

Purified Leydig cells ( $2 \times 10^6$ ) were preincubated for 30 min at 34 °C with DMEM–0.1% BSA under an atmosphere of 95% air–5%  $\text{CO}_2$ , then incubated with isobutylmethylxanthine (IBMX, 25  $\mu\text{M}$ ) in the presence or absence of hCG (5 or 25 mIU) and different concentrations of AA (1–100  $\mu\text{M}$ ). In additional experiments, cells were preincubated for 30 min in the presence of different concentrations of mepacrine or pBpB (0.1–10  $\mu\text{M}$ ) under an atmosphere of 95% air–5%  $\text{CO}_2$ . After 3 h of incubation, aliquots from the cell suspension were extracted for cAMP determination according to the manufacturer's instructions. In brief, 200  $\mu\text{l}$  of the reaction mixtures were mixed with 1 ml ethanol and centrifuged.

The pellets were washed with a mixture of ethanol– $\text{H}_2\text{O}$  (2:1, v/v), and the successive supernatants were collected, mixed, and finally evaporated under  $\text{N}_2$  at 55 °C. Residues were analysed for cAMP content.

#### Data analysis

Results are presented as mean  $\pm$  s.e.m. Statistical analyses were made with the non-parametric Friedman ANOVA and Kendall coefficient of concordance test (Statistica for Windows, Release 4.5 Statsoft, Inc. 1993). Significance was assumed at  $P \leq 0.05$ .

## Results

#### Effect of $\text{PLA}_2$ inhibitors and activators on the release of AA

To determine the role of  $\text{PLA}_2$  on the release of AA, Leydig cells prelabelled with [ $^{14}\text{C}$ ]AA were treated with inhibitors of  $\text{PLA}_2$  and then stimulated with hCG (25 mIU).

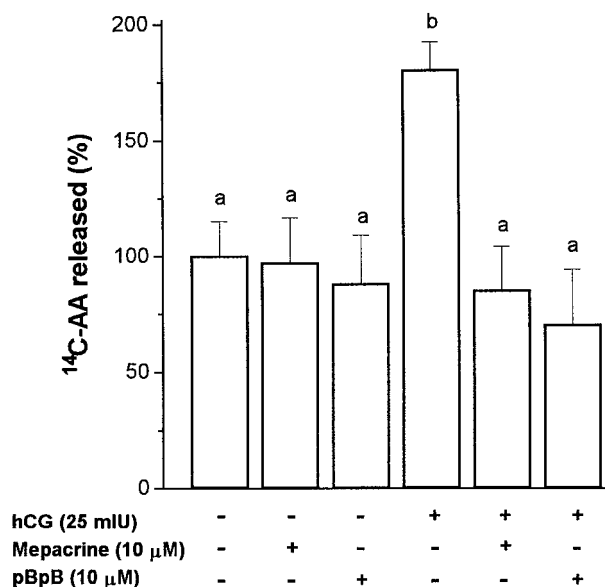
Generally, after 4 h of incubation with 0.2  $\mu\text{Ci}$  [ $^{14}\text{C}$ ]AA, a mean value of 0.054  $\mu\text{Ci}$  (s.e.m. < 10%), equivalent to 982 pmol, was incorporated into Leydig cell phospholipids. Radioactivity released from control untreated Leydig cells after 2 h of incubation reached a value of  $6895 \pm 659$  d.p.m., equivalent to a mass of  $56.5 \pm 5.4$  pmol of [ $^{14}\text{C}$ ]AA. Usually, the amount of AA released from control cells was arbitrarily set as 100  $\pm$  s.e.m.%. In the presence of hCG, the release of AA significantly increased, to  $101.7 \pm 12.4$  pmol, equivalent to 180  $\pm$  22% of the non-stimulated AA release (Fig. 1). However, hCG-stimulated AA release was completely inhibited by mepacrine and pBpB ( $85 \pm 9\%$  and  $70 \pm 24\%$  respectively; Fig. 1). In addition, mepacrine and pBpB did not significantly affect the release of AA from non-stimulated cells (Fig. 1).

The effect of melittin, an activator of  $\text{PLA}_2$ , was also evaluated. A dose–response curve of AA release is depicted in Fig. 2, where it can be observed that concentrations greater than 0.5  $\mu\text{M}$  melittin significantly stimulated the release of AA over that in control cells.

The effect of mepacrine on hCG or melittin-stimulated AA release was also examined. In the presence of 10  $\mu\text{M}$  melittin, the release of AA was increased threefold ( $310 \pm 21\%$ ). As previously shown, hCG was able to stimulate the release of AA significantly, but the percentage stimulation was lower than that obtained with melittin ( $198 \pm 18\%$ ). Although mepacrine completely inhibited the stimulatory effect of hCG on the release of AA, no inhibitory effect on the release of AA was observed in melittin-treated cells (Fig. 3).

#### GTP $\gamma$ S and PTX

To determine if the release of AA was mediated through a G protein, prelabelled Leydig cells were permeabilized



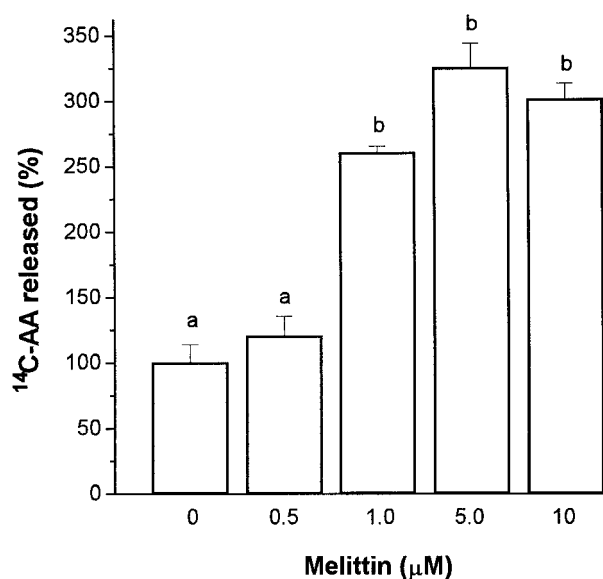
**Figure 1** Effect of the PLA<sub>2</sub> inhibitors mepacrine and pBpB on the release of AA from Leydig cells. Leydig cells ( $2 \times 10^6$ ) were incubated with  $0.2 \mu\text{Ci}$  [ $^{14}\text{C}$ ]AA for 4 h, then cells were collected, centrifuged at  $800 g$  for 10 min, and washed twice. Prelabelled Leydig cells were preincubated for 30 min in DMEM–0.5% fatty acid-free BSA under an atmosphere of 95% air–5%CO<sub>2</sub> in the presence of mepacrine (10 μM) or pBpB (10 μM), then incubated in the presence or absence of 25 mIU hCG for 2 h. After that, total [ $^{14}\text{C}$ ]AA was extracted, separated by TLC and quantified by liquid scintillation spectrometry as indicated in Materials and Methods. [ $^{14}\text{C}$ ]AA released from cells without any treatment was arbitrarily set as  $100 \pm \text{s.e.m.}\%$ . Different letters indicate significantly different values ( $P \leq 0.05$ , mean  $\pm$  s.e.m.;  $n=3$  independent experiments performed in triplicate).

with saponin to allow entry of GTPγS, a non-hydrolyzable analog of GTP. It was shown that GTPγS induces the release of AA to a similar extent as hCG (Fig. 4). Furthermore, the effects of these stimuli were not additive. As expected, GDP was not able to stimulate the release of AA.

To evaluate the type of G protein involved in AA release, the effect of PTX on the release of AA was determined. It was observed that PTX-treated cells showed a marked inhibition of the hCG-stimulated release of AA (Fig. 5).

#### AA and cAMP production

The effects of exogenous AA on cAMP production stimulated by two doses of hCG are shown in Fig. 6. The exogenous addition of AA to suspensions of Leydig cells did not affect cAMP production in the absence of hCG. As expected, a low dose of hCG (5 mIU) increased the cAMP production slightly, but significantly. Under these conditions, the additional presence of exogenous AA (1–50 μM) did not modify cAMP production. Although



**Figure 2** Effect of the PLA<sub>2</sub> activator melittin on the release of AA from Leydig cells. Leydig cells ( $2 \times 10^6$ ) were incubated with  $0.2 \mu\text{Ci}$  [ $^{14}\text{C}$ ]AA for 4 h, then cells were collected, centrifuged at  $800 g$  for 10 min and washed twice. Prelabelled Leydig cells were preincubated for 30 min under an atmosphere of 95% air–5%CO<sub>2</sub> in DMEM–0.5% fatty acid-free BSA and then treated with different doses of melittin (0, 0.5, 1.0, 5.0 and 10 μM) for 2 h. After that, total [ $^{14}\text{C}$ ]AA was extracted, separated by TLC and quantified by liquid scintillation spectrometry as indicated in Materials and Methods. [ $^{14}\text{C}$ ]AA released from control cells was arbitrarily set as  $100 \pm \text{s.e.m.}\%$ . Different letters indicate significantly different values ( $P \leq 0.05$ , mean  $\pm$  s.e.m.;  $n=3$  independent experiments performed in triplicate).

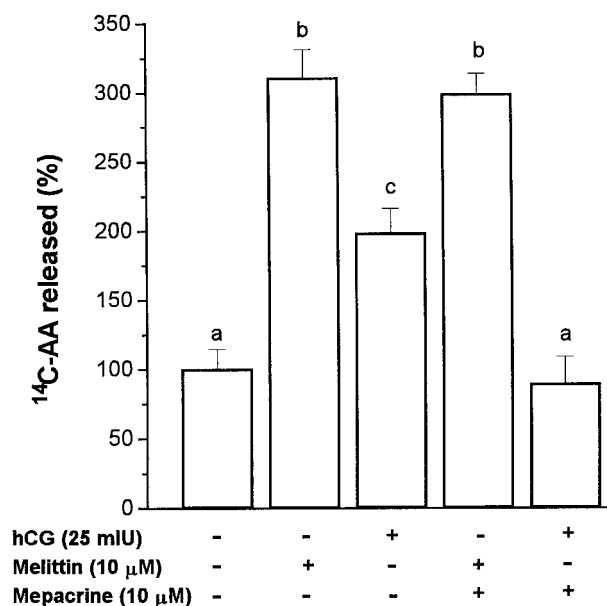
100 μM AA reduced the hCG-stimulated cAMP production, the amount of cAMP was still significantly greater than that produced by control cells (Fig. 6A).

Addition of a higher dose of hCG (25 mIU) approximately doubled the amount of cAMP produced by Leydig cells (Fig. 6B). However, when cAMP production was stimulated with the higher dose of hCG, the response to exogenous AA was clearly different. Doses of 25 μM or more of AA very significantly inhibited the amount of cAMP produced in those hCG-stimulated Leydig cells (Fig. 6B).

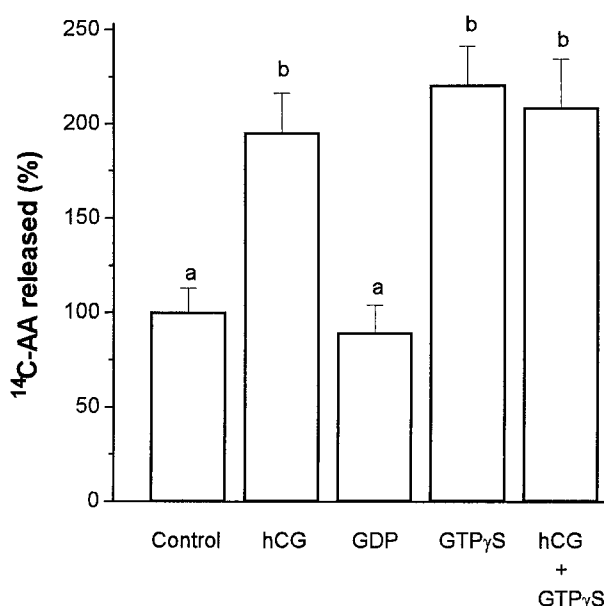
To study the effect of PLA<sub>2</sub> inhibitors on cAMP production stimulated by hCG, Leydig cells were independently treated with mepacrine and pBpB. Cells treated with mepacrine (0.1–10 μM; Fig. 7A) or pBpB (0.1–10 μM; Fig. 7B) did not inhibit cAMP production in cells stimulated with a dose of 25 mIU hCG.

#### Discussion

AA is a known component of cell membrane phospholipids. Because it is released in response to a hormonal



**Figure 3** Effect of the PLA<sub>2</sub> inhibitor mepacrine on the release of AA from melittin- and hCG-stimulated Leydig cells. Leydig cells ( $2 \times 10^6$ ) were incubated with  $0.2 \mu\text{Ci } [^{14}\text{C}]\text{AA}$  for 4 h, then cells were collected, centrifuged at  $800 \text{ g}$  for 10 min, and washed twice. Prelabelled Leydig cells were preincubated for 30 min in DMEM–0.5% fatty acid-free BSA under an atmosphere of 95% air–5%CO<sub>2</sub> in the presence of mepacrine ( $10 \mu\text{M}$ ), then incubated in the presence or absence of hCG ( $25 \text{ mIU}$ ) or melittin ( $10 \mu\text{M}$ ) for 2 h. After that, total [<sup>14</sup>C]AA was extracted, separated by TLC and quantified by liquid scintillation spectrometry, as indicated in Materials and Methods. [<sup>14</sup>C]AA released from cells without any treatment was arbitrarily set as  $100 \pm \text{s.e.m.}\%$ . Different letters indicate significantly different values ( $P \leq 0.05$ , mean  $\pm$  s.e.m.;  $n=3$  independent experiments performed in triplicate).



**Figure 4** Effect of GTP $\gamma$ S on the release of AA from Leydig cells. Leydig cells ( $2 \times 10^6$ ) were incubated with  $0.2 \mu\text{Ci } [^{14}\text{C}]\text{AA}$  for 4 h, then collected, centrifuged at  $800 \text{ g}$  for 10 min, and washed twice. Prelabelled Leydig cells were permeabilized with saponin ( $25 \mu\text{g/ml}$ ) for 15 min to allow entry of GTP $\gamma$ S, then incubated in DMEM–0.5% fatty acid-free BSA under an atmosphere of 95% air–5%CO<sub>2</sub> in the presence of GTP $\gamma$ S ( $100 \mu\text{M}$ ), GDP ( $100 \mu\text{M}$ ), hCG ( $25 \text{ mIU}$ ) or GTP $\gamma$ S plus hCG for 2 h. After that, total [<sup>14</sup>C]AA was extracted, separated by TLC and quantified by liquid scintillation spectrometry as indicated in Materials and Methods. [<sup>14</sup>C]AA released from control cells was arbitrarily set as  $100 \pm \text{s.e.m.}\%$ . Different letters indicate significantly different values ( $P \leq 0.05$ , mean  $\pm$  s.e.m.;  $n=3$  independent experiments performed in triplicate).

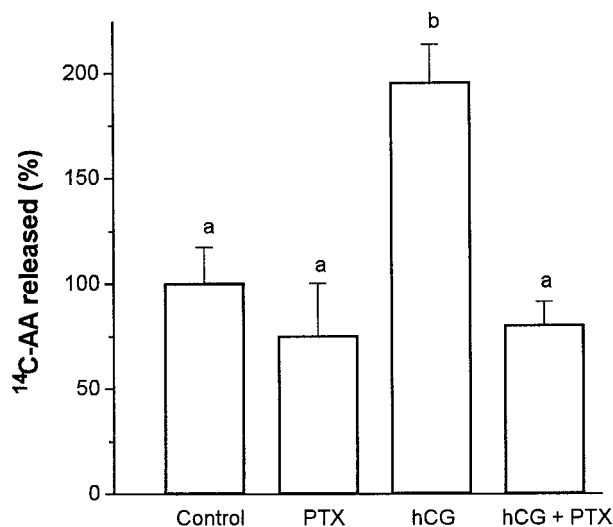
stimulus, it is now included in the list of fatty acids having a role as a signalling molecule in different somatic cells (Yamazaki *et al.* 1996, Cooke *et al.* 1991, Naor 1991, Boone *et al.* 1993, Gaurisankar & Fox 1994, Xing *et al.* 1995, Moraga *et al.* 1997). AA, which is esterified to the sn-2 position of membrane phospholipids, can be preferentially released from phospholipids by activation of PLA<sub>2</sub>. It can also be produced by activation of phospholipase C followed by diacylglycerol lipase, phospholipase D followed by phosphatidic acid phosphohydrolase and diacylglycerol lipase, and finally, PLA<sub>1</sub> followed by lysophospholipase (Graber *et al.* 1994). Previous reports have suggested that AA mediated the positive effect of PLA<sub>2</sub> on testosterone production in Leydig cells (Romanelli *et al.* 1995, Yamazaki *et al.* 1996), indicating that AA, in addition to cAMP, could be regulating LH–hCG-stimulated steroidogenesis.

At present, however, mechanisms involved in the release of AA by Leydig cells are not completely understood. Previous studies have indicated a direct effect of LH (Cooke *et al.* 1991) and hCG (Moraga *et al.* 1997) on the

release of AA in these cells. Specifically, our previous results demonstrated that the release of AA in hCG-treated rat Leydig cells occurred in a dose- and time-dependent manner (Moraga *et al.* 1997). In addition, the amount of AA released was dependent on the hormone–receptor interaction and the concentration of LH–hCG binding sites present on the cell surface (Moraga *et al.* 1997).

Results of the present study performed in the presence of inhibitors or activators of PLA<sub>2</sub> indicate that PLA<sub>2</sub> could be involved in hCG-induced AA release. Two different inhibitors of PLA<sub>2</sub> having different modes of action were used: mepacrine, which interferes with the substrate–enzyme interface, preventing the PLA<sub>2</sub> from hydrolysing the phospholipid substrate (Chang *et al.* 1987), and pBpB, which binds covalently to a histidine residue on PLA<sub>2</sub>, reducing calcium binding and enzyme activity (Drenth *et al.* 1976). Both compounds consistently inhibited the hCG-stimulated release of AA. Conversely, melittin, an activator of PLA<sub>2</sub>, increased the release of AA (Fig. 2). Melittin, a bee venom polypeptide, forms a complex with the phospholipid substrate, which appears to be closely

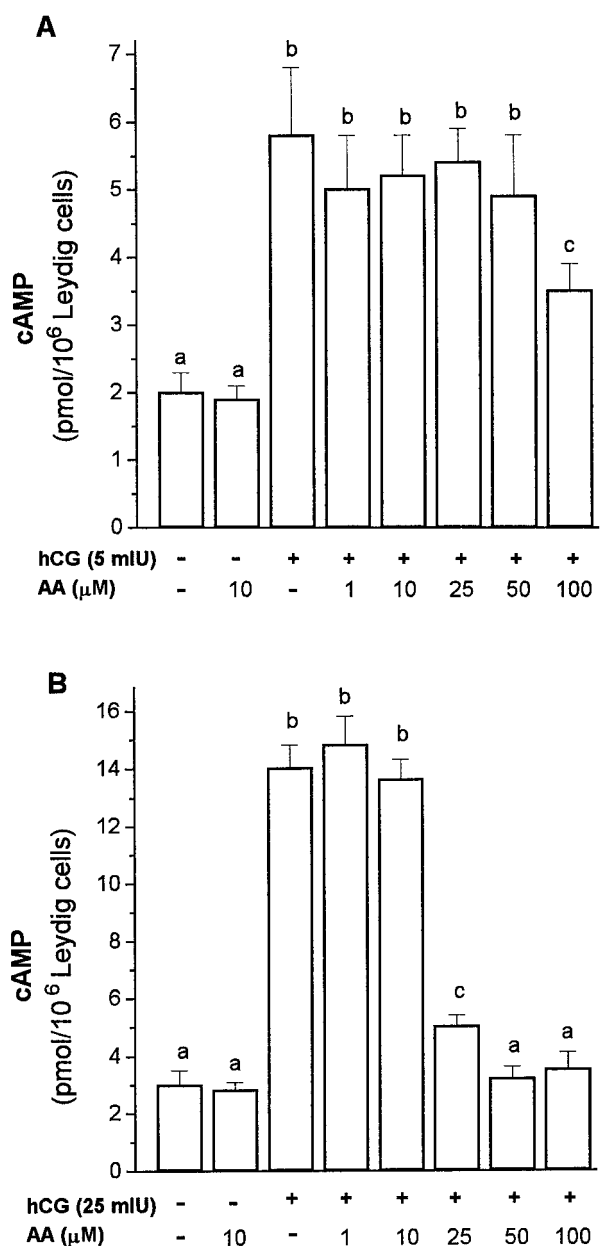




**Figure 5** Effect of PTX on the release of AA from Leydig cells. Leydig cells ( $2 \times 10^6$ ) were incubated with  $0.2 \mu\text{Ci}$  [ $^{14}\text{C}$ ]AA for 4 h, then cells were collected, centrifuged at  $800 g$  for 10 min, and washed twice. Prelabelled Leydig cells were permeabilized with saponin to allow entry of PTX, and subsequently incubated in DMEM–0.5% fatty acid-free BSA under an atmosphere of 95% air–5% $\text{CO}_2$  with PTX (20 ng/ml) for 40 min. hCG (25 mIU) was added to some tubes and incubation continued for an additional 2 h. After that, total [ $^{14}\text{C}$ ]AA was extracted, separated by TLC and quantified by liquid scintillation spectrometry as indicated in Materials and Methods. [ $^{14}\text{C}$ ]AA released from control cells was arbitrarily set as  $100 \pm \text{S.E.M.}\%$ . Different letters indicate significantly different values ( $P \leq 0.05$ , mean  $\pm$  S.E.M.;  $n=3$  independent experiments performed in triplicate).

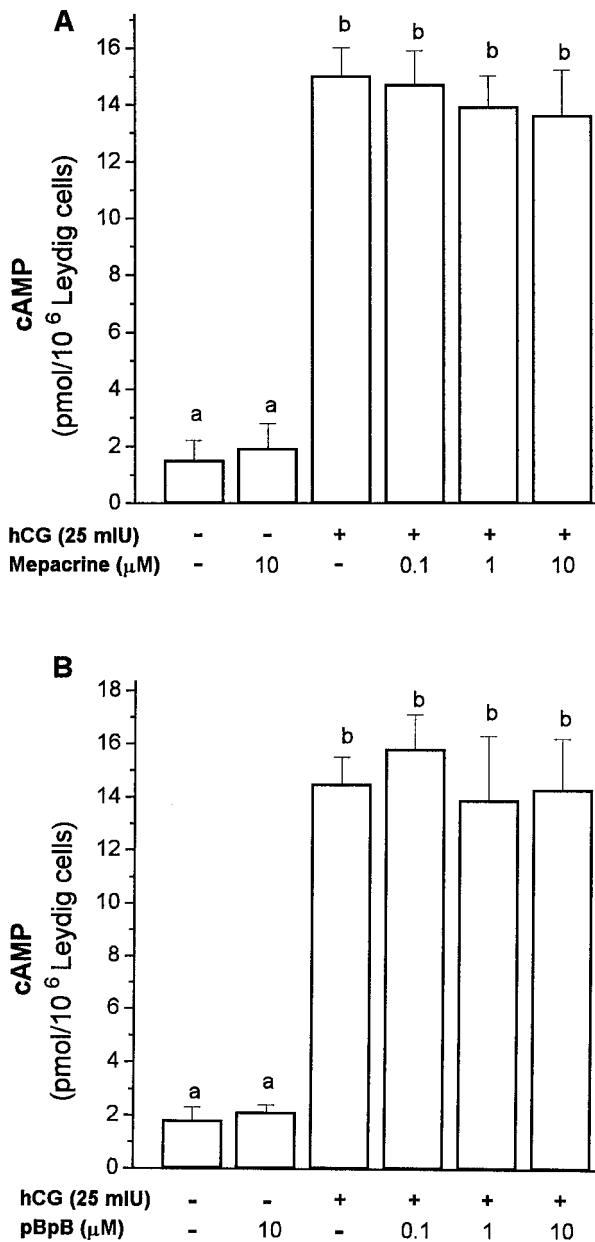
linked with the ability to enhance the action of  $\text{PLA}_2$  (Mollay & Kreil 1974). Mepacrine was able to inhibit the hCG-stimulated release of AA, but not the melittin-induced release, indicating that melittin was able to bypass the effect of mepacrine on  $\text{PLA}_2$  (Fig. 3). A possible explanation for this unexpected result may be that, in the presence of melittin, mepacrine was unable to be affecting the lipid–water interface. Therefore, the positive effects of melittin at the interface might prevail over the negative effects of mepacrine at this level.

In Leydig cells, several reports have indicated that AA may regulate both basal and hormone-stimulated steroidogenesis (Didolkar & Sundaram 1987, Lopez-Ruiz *et al.* 1992, Marinero *et al.* 1996). This suggests a link between intracellular release of AA and steroidogenesis. Experiments with  $\text{PLA}_2$  inhibitors (to inhibit the release of AA) have demonstrated an inhibition of  $\text{Bt}_2\text{cAMP}$  and LH-stimulated testosterone production in rat Leydig cells (Abayasekara *et al.* 1990, Cooke *et al.* 1991, Mele *et al.* 1997). Conversely, melittin, the activator of  $\text{PLA}_2$ , had positive effects on testosterone production (Mukhopadhyay *et al.* 1985). Although these results provide evidence for a stimulatory role of AA on steroidogenesis, it was also shown that exogenous AA might exert



**Figure 6** Effect of exogenous AA on cAMP production in Leydig cells. Approximately  $2 \times 10^6$  Leydig cells were preincubated for 30 min at  $34^\circ\text{C}$  with DMEM–0.1% BSA under an atmosphere of 95% air–5% $\text{CO}_2$ , then incubated with IBMX (25  $\mu\text{M}$ ) and different concentrations of AA (0, 0.1, 1.0, 10, 50 and 100  $\mu\text{M}$ ) in the presence or absence of 5 mIU hCG (A) or 25 mIU hCG (B). After 3 h, cAMP was determined by RIA as described in Materials and Methods. Different letters indicate significantly different values ( $P \leq 0.05$ , mean  $\pm$  S.E.M.;  $n=3$  independent experiments performed in triplicate).

a dose- and time-dependent biphasic effect on LH-stimulated Leydig cells (Lopez-Ruiz *et al.* 1992). In cells incubated with sub-maximal LH concentrations, AA



**Figure 7** Effect of mepacrine and pBpB on cAMP production in Leydig cells. Approximately  $2 \times 10^6$  Leydig cells were preincubated for 30 min at  $34^\circ\text{C}$  with DMEM-0.1% BSA under an atmosphere of 95% air-5% $\text{CO}_2$  with different concentrations of mepacrine (A) and pBpB (B), and then incubated with IBMX (25  $\mu\text{M}$ ) in the presence or absence of hCG (25 mIU). After 3 h, cAMP was determined by RIA as described in Materials and Methods. Different letters indicate significantly different values ( $P \leq 0.05$ , mean  $\pm$  S.E.M.;  $n=3$  independent experiments performed in triplicate).

did not modify testosterone production. Conversely, in the presence of a maximal dose of LH, AA inhibited testosterone production in a dose-dependent fashion (Lopez-Ruiz *et al.* 1992).

The function of AA in steroidogenesis involves the regulation of the steroidogenic acute regulatory protein (StAR; Wang *et al.* 1999), which mediates the transfer of cholesterol to the inner mitochondrial membrane (Lin *et al.* 1995). Specifically, in MA-10 Leydig tumor cells, AA regulates StAR production at the level of gene transcription (Wang *et al.* 2000). This AA-mediated signal transduction pathway is clearly different from the reported cAMP-protein kinase A-phosphorylation pathway, the classical mechanism by which trophic hormones stimulate steroidogenesis (Wang & Stocco 1999).

In the present study, in addition to  $\text{PLA}_2$ , a G protein was also found to be involved in the release of AA, as GTP $\gamma$ S, a non-hydrolyzable analogue of GTP, was also able to increase AA release in permeabilized cells (Fig. 4). In the absence of hCG, exogenous GTP $\gamma$ S stimulated similar degrees of AA release as with the hormone alone. In addition, the stimulatory effect of GTP $\gamma$ S was not additive to that of hCG (Fig. 4). This observation suggests that hCG stimulates the release of AA through a mechanism involving activation of G proteins. In this regard, it has been shown that, after LH-hCG receptor activation, a family of G proteins present in Leydig and ovarian follicular cell membranes are activated. These G proteins include  $G_{\alpha_s}$ ,  $G_{\alpha_{q/11}}$ ,  $G_{\alpha_{13}}$  and  $G_{\alpha_i}$  (Rajagopalan-Gupta *et al.* 1998). The authors postulated that activated G proteins could be related to the PLC pathway. Nevertheless, it cannot be completely ruled out that at least one of the multiple LH-hCG-activated G proteins could be related to a different effector, such as  $\text{PLA}_2$ . This type of interaction has previously been reported for other somatic cells (Fain *et al.* 1988, Axelrod *et al.* 1988, Chen & Chen 1998).

Experiments performed in the presence of PTX showed that this compound was able to inhibit hCG-stimulated release of AA, suggesting the involvement of a member of the  $G_i$  proteins family in this event (Fig. 5). PTX catalyses the ADP-ribosylation of a specific cysteine residue in the  $G_{\alpha_i}$  family, inactivating the  $\alpha_i$ -subunits, such that they cannot exchange GDP for GTP (Nestler & Duman 1994). We therefore hypothesize that a G protein, presumably from the  $G_i$  family, may be part of the mechanism involved in the release of AA. Early studies have shown that PTX can stimulate cAMP production in Leydig cells (Platts *et al.* 1988). In addition, in whole testicular cells, PTX potentiated hCG-stimulated cAMP and testosterone production (Adashi *et al.* 1984). Nevertheless, it is worth noting that the effects reported by these authors were not observed when cells were incubated with PTX during periods of incubation less than 24 h.

In our previous work, we suggested that AA released from rat Leydig cells was independent of cAMP, because the stimulation of cAMP production and the release of AA were dependent on different hCG concentrations (Moraga *et al.* 1997). However, in MA-10 mouse Leydig tumor cells,  $\text{Bt}_2\text{cAMP}$ -induced steroid production was inhibited in the presence of  $\text{PLA}_2$  inhibitors – an effect that was

reversed by the addition of exogenous AA to the medium (Wang *et al.* 1999, 2000). These results, although not directly, would indicate that AA release may occur downstream of cAMP production, and that cAMP could regulate PLA<sub>2</sub> activity and the release of AA. At present, however, reported data suggest that LH-hCG-stimulated release of AA might occur either dependently or independently of cAMP (Moraga *et al.* 1997, Wang & Stocco 1999).

In contrast, results from the present study indicate that exogenous AA could modulate cAMP production in a manner dependent on the amount of AA and the concentration of hCG (Fig. 6). It was shown that, in the presence of 5 mIU hCG, an exogenous concentration of AA as high as 100 µM inhibits cAMP production by Leydig cells (Fig. 6A). However, in the presence of 25 mIU hCG, a lower concentration of exogenous AA (25 µM) is able to inhibit cAMP production (Fig. 6B). These results can be explained in the light of our previous findings (Moraga *et al.* 1997) of a clearly demonstrated dose-dependent effect of hCG on the release of AA. Higher concentrations of AA should therefore be released with 25 mIU than with 5 mIU of hCG. Thus, when Leydig cells are treated with 25 mIU hCG, 25 µM exogenous AA will be sufficient to contribute to attaining a total amount of AA (entry of exogenous AA+intracellular AA released by hCG) that is able to modulate cAMP production. Conversely, lower concentrations of intracellular AA will be released in the presence of 5 mIU hCG, therefore more exogenous AA (100 µM) will be required to reach the necessary total amount of AA needed to modulate cAMP. These findings are in accordance with results shown in Fig. 7, where it can be observed that, in the presence of PLA<sub>2</sub> inhibitors, cAMP production was not modified. Under these conditions, concentrations of AA released were similar to those found in untreated cells (Fig. 1), and therefore not sufficient to modulate the hCG-stimulated cAMP. The inhibitory effect of AA on cAMP production in Leydig cells has been described before (Marinero *et al.* 1996). These authors demonstrated that AA inhibited, in a dose-dependent manner, LH-induced cAMP and testosterone production. In addition, they concluded that AA maintained G<sub>i</sub> proteins in their activated state, which in turn inhibited adenylate cyclase and decreased cAMP concentrations (Marinero *et al.* 2000).

Our results and others from the literature provide evidence that attainment of an intracellular AA concentration able to modulate cAMP clearly depends on exogenously added fatty acid. Nevertheless, we would like to speculate that, inside a hormonally stimulated Leydig cell, AA may reach a transient concentration high enough to modulate cAMP in a specific confined location, and thus may constitute a physiologically relevant event. This kind of compartmentalization might be similar to that reported for cAMP (Dufau 1988). All these results indicate that both pathways, albeit independent, are related, and

suggest that modulation of intracellular concentrations of cAMP could constitute one of the rapid non-genomic actions of the AA released in response to the hormonal stimulus.

As AA and cAMP are essential for maximal steroidogenesis, neither pathway alone being sufficient for trophic hormone-induced steroid production, the finding that AA was able to inhibit cAMP production could be considered contradictory. However, it has long been recognized that very small increases in intracellular cAMP, not necessarily detected under typical experimental conditions, are sufficient to trigger maximal steroidogenic responses (Catt & Dufau 1973). Thus the regulatory effect of AA on cAMP production not only does not impair optimal steroid production, but also helps to co-regulate optimal concentrations of both messengers, when needed inside the cell to assure the steroidogenic function.

In summary, results presented in this study provide evidence to suggest that, in Leydig cells: (1) PLA<sub>2</sub> is involved in the release of AA stimulated by hCG; (2) a G protein from the PTX-sensitive family is involved in PLA<sub>2</sub>-mediated AA release, and (3) AA may be involved in the regulation of cAMP production.

Collectively, our present and previous results (Moraga *et al.* 1997), and those reported in the literature (Axelrod *et al.* 1988, Marinero *et al.* 1996, 2000, Wang *et al.* 1999, 2000), lead us to suggest the following mechanism of LH-hCG action in Leydig cells. The LH-hCG-receptor interaction promotes the release of AA through the activation of a G protein, presumably a G<sub>i</sub>, which in turn activates PLA<sub>2</sub> activity and releases AA from membrane phospholipids. AA then acts at the plasma membrane level, possibly by activation of additional G proteins (G<sub>i</sub>) involved in adenylate cyclase inhibition, and thus decreases cAMP production. Alternatively, the LH-hCG-receptor complex could be simultaneously coupled to different G protein families, modulating both the adenylate cyclase and PLA<sub>2</sub> activities. These events could be occurring synchronously at different plasma membrane microdomains, after hormone-receptor interaction. Thus each intracellular signal, cAMP or AA, could temporarily modify the concentration of the other, constituting a transient and spatially confined cross-talk between the two molecules. This interaction would allow a co-ordinated and regulated biological response at different levels, leading to optimal steroidogenesis in Leydig cells.

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## References

- Abayasekara DRE, Band AM & Cooke BA 1990 Evidence for the involvement of phospholipase A<sub>2</sub> in the regulation of luteinizing hormone-stimulated steroidogenesis in rat testis Leydig cells. *Molecular and Cellular Endocrinology* **70** 147–153.
- Adashi EY, Resnick CE, Cronin MJ & Hewlett EL 1984 'Antigonadal' activity of the neurohypophysial hormones in cultured rat testicular cells: abolition by pertussis toxin. *Endocrinology* **115** 839–841.
- Aldred LF & Cooke BA 1983 The effect of cell damage on the density and steroidogenic capacity of rat testis Leydig cells using an NADH exclusion test for determination of viability. *Journal of Steroid Biochemistry* **18** 411–414.
- Axelrod J, Burch M & Jelsema C 1988 Receptor-mediated activation of phospholipase A<sub>2</sub> via GTP-binding proteins: arachidonic acid and its metabolites as second messengers. *Trends in Neurosciences* **11** 117–123.
- Boone DL, Currie WD & Leung PCK 1993 Arachidonic acid and cell signaling in the ovary and placenta. *Prostaglandins Leukotrienes and Essential Fatty Acids* **48** 79–87.
- Catt KJ & Dufau ML 1973 Spare gonadotropin receptors in rat testis. *Nature (New Biology)* **244** 219–221.
- Catt KJ, Harwood JP, Clayton RN, Davies TF, Chan V, Katikineni M, Nozu K & Dufau ML 1980 Regulation of peptide hormone receptors and gonadal steroidogenesis. *Recent Progress in Hormone Research* **36** 557–622.
- Chang J, Musser JH & McGregor H 1987 Phospholipase A<sub>2</sub>: function and pharmacological regulation. *Biochemical Pharmacology* **36** 2429–2436.
- Chen WC & Chen CC 1998 ATP-induced arachidonic acid release in cultured astrocytes is mediated by G<sub>i</sub> proteins coupled P2Y1 and P2Y2 receptors. *Glia* **22** 360–370.
- Choi MSK & Cooke BA 1990 Evidence for two independent pathways in the stimulation of steroidogenesis by luteinizing hormone involving chloride channels and cyclic AMP. *FEBS Letters* **261** 402–404.
- Cooke BA, Dirami L, Chaudry L, Choi MSK, Abayasekara DRE & Phipp L 1991 Release of arachidonic acid and the effects of corticosteroids on steroidogenesis in rat testis Leydig cells. *Journal of Steroid Biochemistry and Molecular Biology* **40** 465–471.
- Didolkar AK & Sundaram K 1987 Arachidonic acid is involved in the regulation of hCG induced steroidogenesis in rat Leydig cells. *Life Sciences* **41** 471–477.
- Drenth J, Enzing CM, Halk KH & Vessies JCA 1976 Structure of porcine pancreatic phospholipase A<sub>2</sub>. *Nature* **264** 373–377.
- Dufau ML 1988 Endocrine regulation and communicating functions of the Leydig cell. *Annual Review of Physiology* **50** 483–508.
- Fain JN, Wallace MA & Wojcikiewicz RJ 1988 Evidence for involvement of guanine nucleotide-binding regulatory proteins in the activation of phospholipases by hormones. *FASEB Journal* **2** 2569–2574.
- Gaurisankar S & Fox PL 1994 Basic fibroblast growth factor-stimulated endothelial cell movement is mediated by a pertussis toxin-sensitive pathway regulating phospholipase A<sub>2</sub> activity. *Journal of Biological Chemistry* **269** 3219–3225.
- Graber R, Sumida C & Nunez EA 1994 Fatty acids and cell signal transduction. *Journal of Lipid Mediators and Cell Signalling* **9** 91–116.
- Hedger MP & Eddy EM 1986 Monoclonal antibodies against rat Leydig cell surface antigens. *Biology of Reproduction* **35** 1309–1319.
- Kamel F & Kubajac C 1988 Gonadal steroids effects on LH response to arachidonic acid and protein kinase C. *American Journal of Physiology* **255** E314–E321.
- Lin D, Sugawara T, Strauss III JF, Clark BJ, Stocco DM, Saenger P, Rogol A & Miller WL 1995 Role of steroidogenic acute regulatory protein in adrenal and gonadal steroidogenesis. *Science* **267** 1828–1833.
- Lopez-Ruiz MP, Choi MSK, Rose MP, West AP & Cooke BA 1992 Direct effect of arachidonic acid on protein kinase C and LH-stimulated steroidogenesis in rat Leydig cells; evidence for tonic inhibitory control of steroidogenesis by protein kinase C. *Endocrinology* **130** 1122–1130.
- Marinero MJ, Colás B, Prieto JC & López-Ruiz MP 1996 Different sites of action of arachidonic acid on steroidogenesis in rat Leydig cell. *Molecular and Cellular Endocrinology* **118** 193–200.
- Marinero MJ, Ropero S, Colás B, Prieto JC & López-Ruiz MP 2000 Modulation of guanosine triphosphatase activity of G proteins by arachidonic acid in rat Leydig cell membranes. *Endocrinology* **141** 1093–1099.
- Mele, PG, Dada LA, Paz C, Neuman I, Cymeryng CB, Mendez CF, Finkelstein CV, Maciel FC & Podesta EJ 1997 Involvement of arachidonic acid and the lipoxygenase pathway in mediating luteinizing hormone-induced testosterone synthesis in rat Leydig cells. *Endocrine Research* **23** 15–26.
- Mollay C & Kreil G 1974 Enhancement of bee venom phospholipase A<sub>2</sub> activity by melittin direct lytic factor from cobra venom and polymyxin B. *FEBS Letters* **46** 141–144.
- Moraga P, Llanos M & Ronco AM 1997 Arachidonic acid release from rat Leydig cells depends on the presence of luteinizing hormone/human chorionic gonadotrophin receptors. *Journal of Endocrinology* **154** 231–239.
- Mukhopadhyay AK, Kistler M & Dasselaar J 1985 Phospholipase A<sub>2</sub> and hormone-stimulated steroidogenesis by isolated rat Leydig cells. *Acta Endocrinologica* **108** (Suppl 267) 94–95.
- Naor Z 1991 At the cutting edge. Is arachidonic acid a second messenger in signal transduction. *Molecular and Cellular Endocrinology* **80** C181–C186.
- Needleman P, Turk J, Jakshicck B, Morrison A & Lefkowitz J 1986 Arachidonic acid metabolism. *Annual Review of Biochemistry* **55** 69–102.
- Nestler EJ & Duman RS 1994 G proteins and cyclic nucleotides in the nervous system. In: *Basic Neurochemistry: Molecular, Cellular and Medical Aspects*, vol 21, pp 429–447. Ed. GJ Siegel. New York: Raven Press.
- Platts EA, Schulster D & Cooke BA 1988 The inhibitory GTP-binding protein (G<sub>i</sub>) occurs in rat Leydig cells and is differentially modified by lutropin and 12-O-tetradecanoylphorbol 13-acetate. *Biochemical Journal* **253** 895–899.
- Rajagopalan-Gupta RM, Lamm MLG, Mukherjee SM, Rasenick MM & Hunzicker-Dunn M 1998 Luteinizing hormone-choriogonadotropin receptor-mediated activation of heterodimeric guanine nucleotide binding proteins in ovarian follicular membranes. *Endocrinology* **139** 4547–4555.
- Romanelli F, Valenca M, Conte D, Isidori A & Negro-Vilar A 1995 Arachidonic acid and its metabolites effects on testosterone production by rat Leydig cells. *Journal of Endocrinological Investigation* **18** 186–193.
- Ronco AM & Valladares LE 1993 Phospholipid methylation decreases in human chorionic gonadotrophin-induced desensitized rat Leydig cells. *Journal of Endocrinology* **138** 65–71.
- Steinberger E, Steinberger A & Vilar O 1966 Cytochemical study of Δ<sup>3</sup>-3β-hydroxysteroid dehydrogenase in testicular cells grown in vitro. *Endocrinology* **79** 406–410.
- Sullivan M, Cooke B 1986 The role of Ca<sup>2+</sup> in steroidogenesis in Leydig cells. *Biochemical Journal* **236** 45–51.
- Wang X & Stocco DM 1999 Cyclic AMP and arachidonic acid: a tale of two pathways. *Molecular and Cellular Endocrinology* **158** 7–12.
- Wang X, Walsh LP & Stocco DM 1999 The role of arachidonic acid on LH-stimulated steroidogenesis and steroidogenic acute regulatory protein accumulation in MA-10 mouse Leydig tumor cells. *Endocrine* **10** 7–12.

Wang XJ, Walsh LP, Reinhart AJ & Stocco DM 2000 The role of arachidonic acid in steroidogenesis and steroidogenic acute regulatory (StAR) gene and protein expression. *Journal of Biological Chemistry* **275** 20204–20209.

Xing M, Miele L & Mukherjee AB 1995 Arachidonic acid release from NIH 3T3 cells by group-I phospholipase A<sub>2</sub>: Involvement of a receptor-mediated mechanism. *Journal of Cellular Physiology* **165** 566–575.

Yamazaki T, Higuchi K, Kominami S & Takemori S 1996 15-lipoxygenase metabolite(s) of arachidonic acid mediates adrenocorticotropin action in bovine adrenal steroidogenesis. *Endocrinology* **137** 2670–2675.

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