Inhibition of Gonadotropin-Stimulated Ovarian Steroid Production by Polyunsaturated Fatty Acids in Teleost Fish

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ABSTRACT: The effects of the polyunsaturated fatty acids (PUFAs)—eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and arachidonic acid (AA)—on *in vitro* steroid production by full-grown prematurational ovarian follicles from goldfish and rainbow trout were investigated. EPA and DHA inhibited gonadotropin-stimulated testosterone production in a dose-related manner, but AA was inhibitory only at the highest dose tested (400 μM). AA alone stimulated testosterone production by increasing cAMP production, but the effects of other PUFAs alone were marginal. The inhibitory actions by PUFAs were not restricted to long-chain PUFAs, as linoleic and linolenic acids had similar actions in the goldfish. The inhibitory action of EPA on testosterone production was reversible upon removal of the PUFA from medium. Testosterone production stimulated by the addition of the cAMP analogues, dibutyryl cAMP, and 8-bromo cAMP, was attenuated by PUFAs, suggesting that they act at a site distal to cAMP formation. A post-cAMP site regulating cholesterol availability may be involved as testosterone production induced by addition of 25OH-cholesterol was not affected by the PUFAs in either fish species. Together, these findings underscore the importance of lipids in ovarian physiology and suggest that PUFAs may participate in the regulation of ovarian steroidogenesis in teleost fish.
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Environmental influences on long-chain polyunsaturated fatty acids' (PUFAs) composition and metabolism in fish have been considered by many (1–4). However, even though lipid composition in fish is seasonal and may be related to reproductive status (5), little is known of how PUFAs affect ovarian physiology. In freshwater fish, arachidonic acid (AA) (20:4n-6) is usually the most abundant tetraenoic fatty acid, eicosapentaenoic acid (EPA) (C20:5n-3) is often the predominant pentaenoic acid, and docosahexaenoic acid (DHA) (C22:6n-3) is, without exception, the main hexaenoic acid (5). The physiological significance of AA as an eicosanoid precursor and in signal transduction is well established (6–9), as is the importance of eicosanoids, such as prostaglandins, as regulators of ovarian physiology in fish and in many vertebrates (10). However, the roles of EPA and DHA in fish and in mammals remain relatively unclear, even though they are the focus of much research in human health (11,12).

In reproduction, lipids are most often considered from an energetic point of view, and their possible involvement in other physiological processes are often overlooked. Selective lipid depletion has been observed in spawning salmonids (13), and selective PUFA utilization has been reported during gonadal development of salmonids and goldfish (14–16). AA, EPA, and DHA were preferentially released from arctic char ovarian follicles in the presence of melittin, a phospholipase A_2 activator (Geritts, M., Van Der Kraak, G., and Ballantyne, J., unpublished data), and from goldfish testicular fragments upon stimulation with a protein kinase C activator and a calcium ionophore (17). AA, *via* its conversion to eicosanoids, stimulates testosterone production in goldfish ovaries (18), and also in testes (19). By comparison, EPA and DHA inhibit gonadotropin-stimulated testosterone production by goldfish testis (17). PUFAs also modulate gonadal steroidogenesis in avian and in mammalian species (20–25).

This study examines the effects of unesterified long-chain PUFAs including AA, EPA, and DHA on goldfish and rainbow trout ovarian steroidogenesis *in vitro*. Testosterone production was used as an index of steroidogenic activity, as it is a major steroid secreted by full-grown, prematurational ovarian follicles of teleost fish (26,27).

MATERIALS AND METHODS

Fish. Goldfish (*Carassius auratus*), common or comet varieties, were purchased from Grassyfork Fisheries Co. (Martinsville, IN). Fish were held in 1.2-m diameter tanks with flow-through water at 15–18°C under a constant photoperiod (14 light/10 dark). Fish were fed a commercial trout diet once daily to satiation. Prespawning female rainbow trout (*Oncorhynchus mykiss*) were obtained from the Alma Aquaculture Research Station (Alma, Ontario, Canada). Trout were held at the University of Guelph for a period no longer than a week, at 8–11°C.

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Abbreviations: AA, arachidonic acid; 25-OH Chol, 25-hydroxycholesterol; dbcAMP, dibutyryl cAMP; DHA, docosahexaenoic acid; DMSO, dimethyl sulfoxide; EPA, eicosapentaenoic acid; hCG, human chorionic gonadotropin; IBMX, 3-isobutyl-1-methylxanthine; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PUFA, polyunsaturated fatty acid; SG-G100, partially purified salmon gonadotropin.

Ovarian follicle incubations. The follicle incubations followed the general methods described by Van Der Kraak and Chang (18). Fish were sacrificed by spinal transection, and ovaries were immediately placed in a modified Cortland's medium consisting of (g/L): NaCl, 7.25; KCl, 0.38; $CaCl_2 \cdot 2H_2O$, 0.23; $NaH_2PO_4 \cdot H_2O$, 0.41; $MgCl_2 \cdot 6H_2O$, 0.20; $MgSO_4 \cdot 7H_2O$, 0.23; $NaHCO_3$, 1.0; bovine serum albumin, 1.0; glucose, 1.0; and streptomycin sulfate, 0.1; pH 7.6. Each experiment used full-grown, prematurational follicles from a single fish. Due to the asynchronous development of goldfish follicles, full-grown follicles (0.9–1.1 mm in diameter) were manually sorted from the smaller vitellogenic follicles using a Pasteur pipette before placing them in polystyrene (24-well) tissue culture plates (Falcon 3047; Toronto, Ontario, Canada) containing incubation medium. Each treatment consisted of four replicate incubations of 20 follicles for goldfish or 10 full-grown follicles (4–6 mm in diameter) for rainbow trout. Immediately prior to the addition of test chemicals and/or hormones, the medium was replaced with fresh medium. Goldfish follicles incubations routinely included the phosphodiesterase inhibitor 3isobutyl-1-methylxanthine (IBMX) at 1 mM to ensure detectable basal steroid levels and to facilitate measurement of cAMP. IBMX was not added to trout follicle incubations, as basal levels were often higher in this species. Treatment chemicals and hormones were added from concentrated stocks and diluted to working concentrations in the incubation medium. Final incubation volume was 1 mL. Fresh aliquots of fatty acids were opened immediately prior to each experiment to ensure their integrity. Incubations were in air at 18°C in the dark. Incubation periods were routinely for 18 h or as indicated in the figure legends. Following incubation, 0.8 mL of medium was removed from each well and placed in separate borosilicate test tubes to be stored at –20°C for subsequent radioimmunoassay. Media collected for cAMP measurement was boiled immediately for 15 min to destroy phosphodiesterase activity prior to freezing.

The protocols for the testosterone and cAMP assays were described by Van Der Kraak and Chang (18), and by Van Der Kraak (28), respectively. Medium from each replicate was assayed in duplicate.

Chemicals and hormones. Unesterified free fatty acids, human chorionic gonadotropin (hCG), forskolin, IBMX, dibutyryl cAMP (dbcAMP), 8-bromo cAMP, and 25-hydroxycholesterol (25-OH Chol) were obtained from Sigma Chemical Co. (St. Louis, MO). Partially purified salmon gonadotropin (SG-G100) was a gift from Dr. E.M. Donaldson (Department of Fisheries and Oceans, West Vancouver, British Columbia, Canada). hCG, SG-G100, IBMX, and dbcAMP were dissolved directly in incubation medium; and forskolin, free fatty acids, and 25-OH Chol were dissolved in absolute ethanol. 8-Bromo cAMP was solubilized in dimethyl sulfoxide (DMSO). The final volume of ethanol never exceeded 1.0% of final incubation volume, and this concentration has no effect on basal or gonadotropin-stimulated testosterone production (18). Similarly, DMSO at final concentration of 0.5% had no effect on steroid production (data not shown).

Statistical analysis. Following logarithmic transformation (Log_{10}), differences between means were assessed by one-way analysis of variance and by Duncan's Multiple Range test. A P value of ≤ 0.05 was considered significant. Values reported in all figures represent the mean ± SEM of four replicate incubations, and representations lacking error bars indicate a smaller error than the symbol depicting the mean. Data presented are representative of at least two experiments yielding similar results.

RESULTS

Effects of PUFAs on steroidogenesis. Experiments were conducted to test the effects of graded dosages of PUFAs (6.25–400 µM) on ovarian steroidogenesis of goldfish and rainbow trout. Consistent with earlier studies, hCG caused a marked increase in testosterone production by goldfish prematurational follicles (Fig. 1). Addition of EPA and DHA caused similar inhibitions of hCG-stimulated testosterone production, with the effects being significant at the higher doses (100–400 µM). AA was also inhibitory, but only at the highest dose tested. AA alone stimulated testosterone production in a dose-dependent manner with maximum stimulation achieved at 100 µM. EPA and DHA caused marginal increase in testosterone production at the higher dosages. In the trout, DHA inhibited SG-G100-stimulated testosterone production in a dose-related manner, but the inhibition by AA was significant only at the highest dose (Fig. 2). AA at 6.25 µM, but not at higher dosages, stimulated testosterone production.

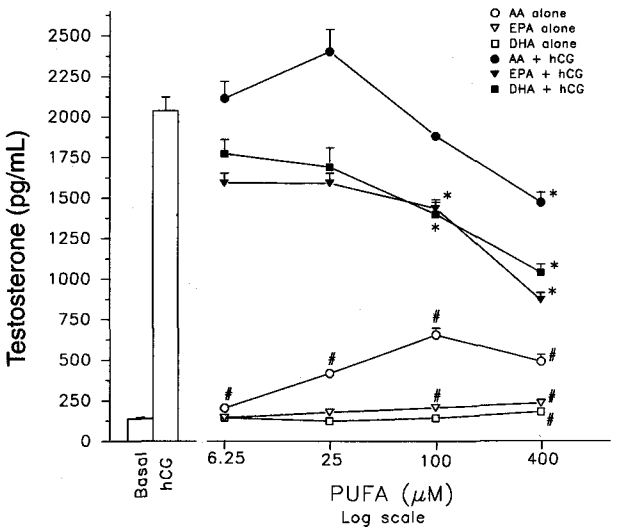

FIG. 1. Effects of graded dosages of arachidonic acid (AA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) on basal- and on human chorionic gonadotropin (hCG)-stimulated (10 IU) testosterone production by goldfish full-grown ovarian follicles incubated for 18 h at 18°C. Values represent the mean ± SEM of four replicate incubations. * Indicates a significant difference from hCG alone, and # indicates a significant difference from basal level.

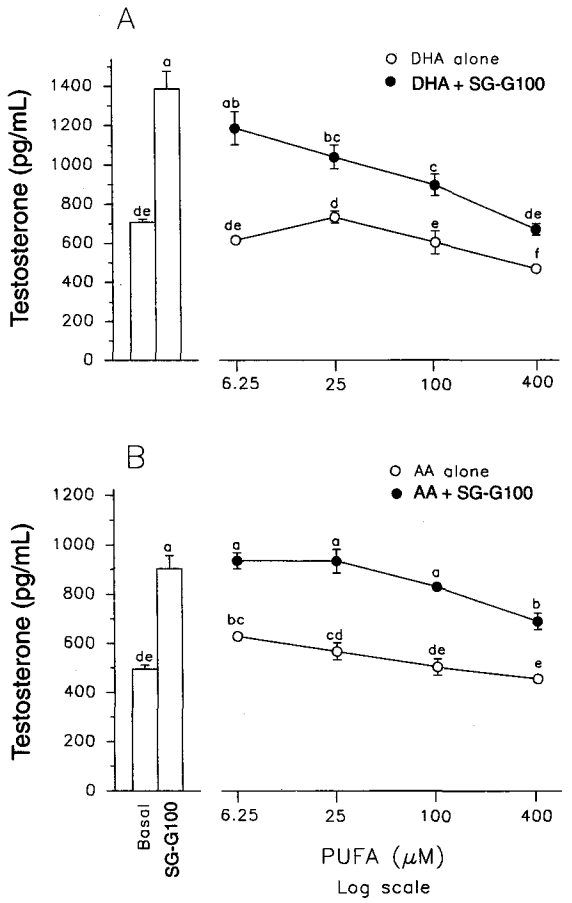

FIG. 2. Effects of graded dosages of DHA (A) and AA (B) on basal- and on partially purified salmon gonadotropin (SG-G100) (1 μg/mL) stimulated testosterone production by rainbow trout full-grown ovarian follicles incubated for 18 h at 18°C. Values represent the mean ± SEM of four replicate incubations. Means with different letters are significantly different. Abbreviations as in Figure 1.

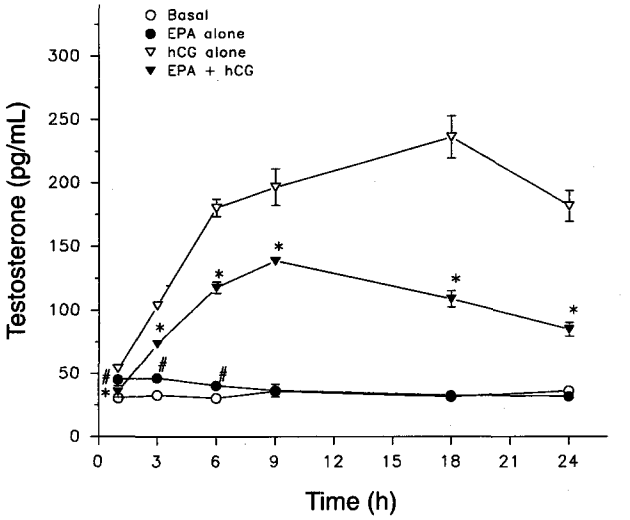

FIG. 3. Time course response of EPA (400 μM) effects on basal- and hCG-stimulated (10 IU) testosterone production by goldfish full-grown ovarian follicles incubated at 18°C. Values represent the mean ± SEM of four replicate incubations. * Indicates a significant inhibition by EPA from hCG alone, and # indicates a significant difference from basal level at each time interval. Abbreviations as in Figure 1.

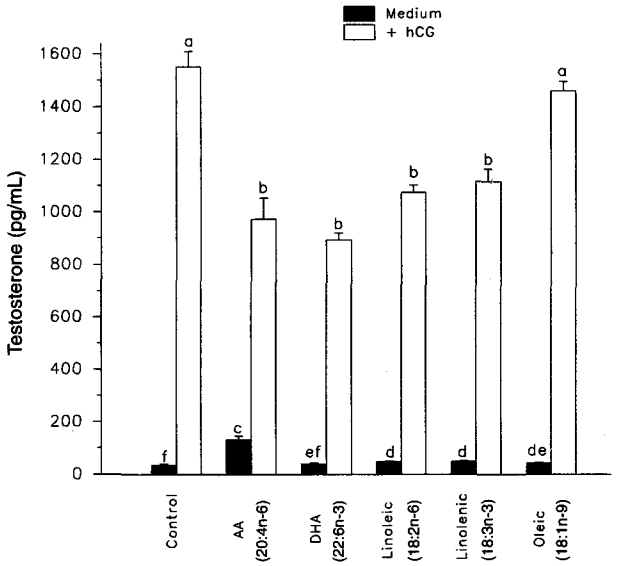

FIG. 4. Effects of AA, EPA, linoleic acid, linolenic acid, and oleic acid at 400 μM on basal- and on hCG-stimulated (10 IU) testosterone production by goldfish full-grown ovarian follicles incubated for 18 h at 18°C. Values represent the mean ± SEM of four replicate incubations. Means with different letters are significantly different. Abbreviations as in Figure 1.

EPA was found to have rapid effects in time course experiments with goldfish follicles (Fig. 3). EPA attenuated hCG actions, but increased basal testosterone production after the first hour on its own. Maximal stimulation by hCG was found after 18 h, where inhibition by EPA was also greatest. The initial stimulation of testosterone production by EPA alone subsequently disappeared after six hours of incubation.

Experiments were conducted to test the specificity of the effects found with the long-chain PUFAs. In addition to AA and DHA, linoleic, and linolenic acids at 400 μM attenuated hCG-stimulated testosterone production by goldfish ovarian follicles, although oleic acid had no significant effect (Fig. 4). Basally, AA, linoleic, linolenic, and oleic acids had stimulatory effects, with AA being most effective.

Effects of PUFA removal. Given that relatively high dosages of fatty acids were routinely used to inhibit gonadotropin-stimulated testosterone production, experiments were conducted with goldfish to establish whether the decrease in steroidogenesis was due to effects on follicular viability. The approach was to conduct an initial 15-h incubation testing the actions of graded dosages of EPA and, following removal of EPA by washing several times with fresh medium, to measure the follicular response to hCG during a subsequent 15-h incubation. Media were removed and frozen until assayed for testosterone after each 15-h incubation. In initial in-

cubation, follicles responded with the typical stimulation by hCG, and EPA attenuated hCG actions (Fig. 5A). In the subsequent incubation, basal testosterone production was maintained: 63 and 64 pg/mL in initial and in subsequent incubations, respectively. However, the overall responsiveness of follicles to hCG was much less than in initial incubation (Fig 5B). Most importantly, the follicles initially exposed to hCG alone and hCG in combination with EPA were equally responsive during a second incubation with hCG. These data demonstrate that although responsiveness of follicles to hCG was affected after 15 h of incubation, the inhibition by EPA was reversible.

Sites of inhibition. Because gonadotropin stimulates steroid production *via* cAMP, the effects of PUFAs on cAMP and testosterone production were assessed in goldfish. As expected, addition of hCG increased accumulation of cAMP in the media (Fig. 6). Addition of EPA (100 and 400 μM) inhibited hCG-stimulated cAMP production (Fig. 6A). EPA alone caused a slight but significant dose-related accumulation of cAMP in the media, but this effect did not match the effect on testosterone production, as EPA at 100 μM was inhibitory, whereas EPA at 400 μM was stimulatory. AA alone increased cAMP levels in a dose-related manner which corresponded with an increase in testosterone production (Fig. 6B). However, AA potentiated hCG-stimulated cAMP production but did not affect testosterone production. Relatively low levels of testosterone were produced in these experiments compared to previous results, due to the shorter (4-h) incubation period used for cAMP assessment.

Phosphodiesterase activity is an important intracellular regulatory site controlling cAMP levels. Because the phosphodiesterase inhibitor IBMX was necessary to detect cAMP levels, and because it was routinely added to all goldfish follicle incubations, it was important to test whether IBMX affected the actions of the PUFAs. IBMX at dosages of 0.1 and 1.0 mM led to a modest increase in basal testosterone production, but markedly enhanced the stimulatory actions of hCG (Fig. 7). More importantly, EPA attenuated hCG-stimulated testosterone production both in the presence or absence of IBMX.

A series of experiments were conducted to test whether PUFAs may act at other sites post-cAMP formation. The

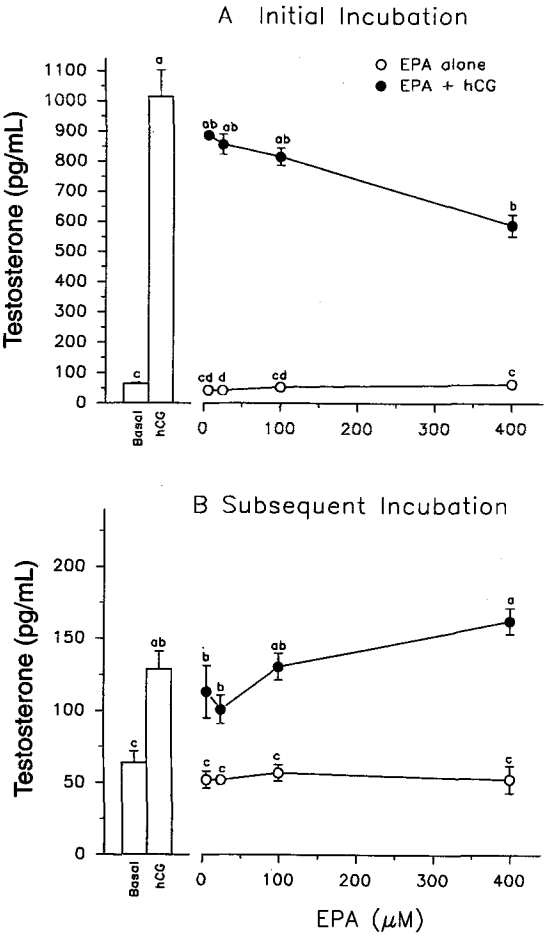

FIG. 5. Assessment of follicular responsiveness to hCG following the removal of EPA. Effects of graded dosages of EPA on basal- and hCG-stimulated testosterone production by goldfish full-grown ovarian follicles after a 15-h initial incubation at 18°C (A). Basal- and hCG-stimulated testosterone production of the same follicles during a subsequent 15-h incubation after removal of EPA and in fresh medium with and without hCG (B). Values represent the mean ± SEM of four replicate incubations. Means with different letters are significantly different. Abbreviations as in Figure 1.

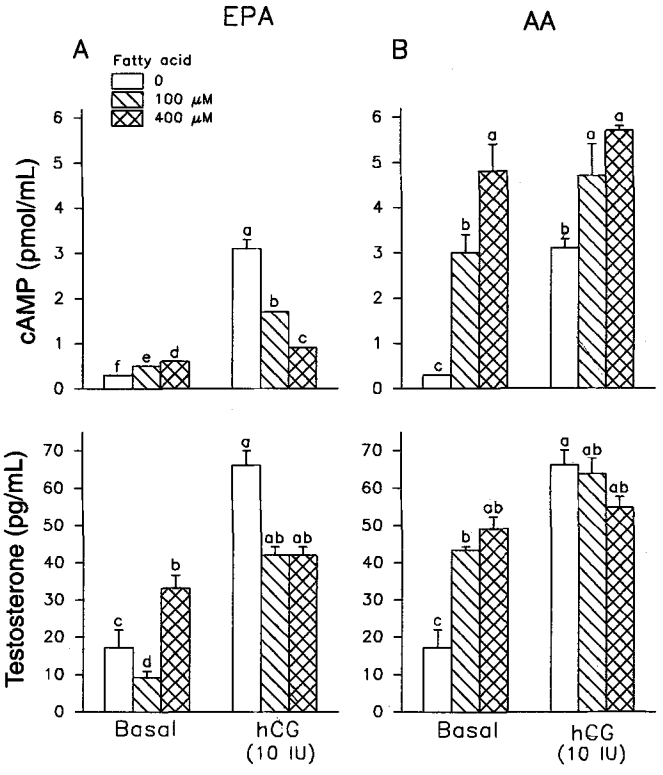

FIG. 6. Effects of EPA (A) and AA (B) on basal- and hCG-stimulated cAMP and testosterone production by goldfish full-grown ovarian follicles incubated for 4 h at 18°C. Values represent the mean ± SEM of four replicate incubations. Means with different letters are significantly different. Abbreviations as in Figure 1.

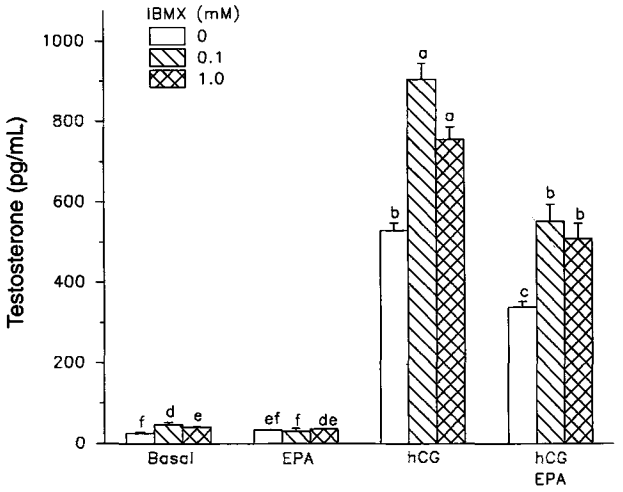

FIG. 7. Dosage effects of 3-isobutyl-1-methylxanthine (IBMX) on basal- and hCG-stimulated (10 IU) testosterone production by goldfish full-grown ovarian follicles incubated for 18 h at 18°C with and without EPA (400 μM). Values represent the mean ± SEM of four replicate incubations. Means with different subscripts are significantly different. Abbreviations as in Figure 1.

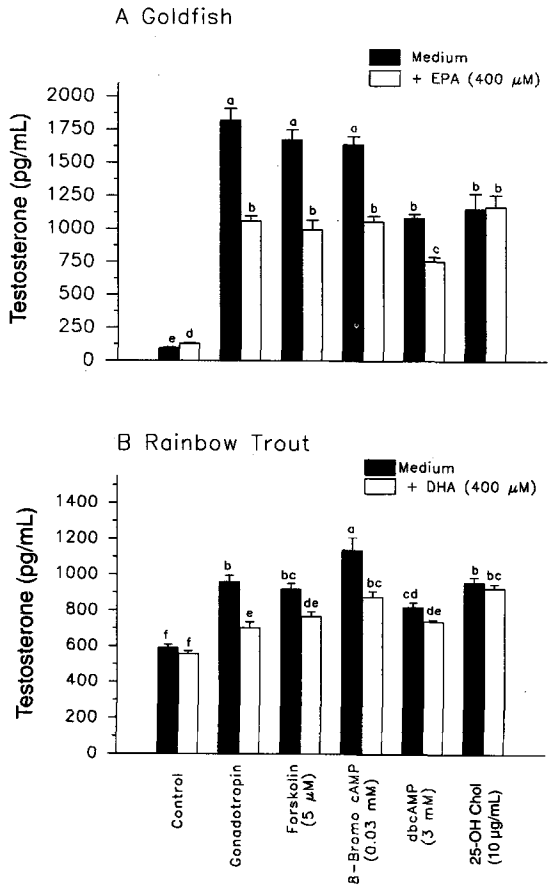

FIG. 8. Effects of polyunsaturated fatty acids on hCG (SG-G100 for trout), forskolin, 8-Bromo cAMP, dibutyryl cAMP (dbcAMP), and 25-OH Cholesterol (25-OH chol) stimulated testosterone production by goldfish (A) and rainbow trout (B) full-grown ovarian follicles incubated for 18 h at 18°C. Values represent the mean ± SEM of four replicate incubations. Means with different letters are significantly different. Abbreviations as in Figures 1 and 2.

stimulatory actions of hCG and forskolin, a direct activator of adenylate cyclase, on testosterone production were inhibited by EPA in the goldfish (Fig. 8A). Testosterone production induced by two cAMP analogues, 8-Bromo cAMP and dbcAMP, which mimic the actions of cAMP, were also inhibited by EPA, indicating an effect distal to cAMP formation. When follicular testosterone production was stimulated by addition of the steroidogenic substrate 25-OH Chol, EPA was without effect. In the rainbow trout, SG-G100, forskolin, and 8-Bromo cAMP actions on testosterone production were inhibited by the addition of DHA (Fig. 8B). However, it is not clear why DHA did not block the dbcAMP-induced increase in testosterone production in the trout. As with the goldfish, the stimulatory effect of 25-OH Chol was not affected by DHA.

DISCUSSION

These studies demonstrate differential actions of PUFAs on basal and on gonadotropin-stimulated steroid production in goldfish and in rainbow trout ovarian follicles. In both species, PUFAs inhibit the stimulatory action of gonadotropin at a site distal to cAMP formation which may regulate cholesterol supply to the steroidogenic pathway. AA alone stimulated testosterone production by increasing cAMP production, but the effects of other PUFAs alone were marginal. These studies support the contention that unesterified long-chain PUFAs, including AA, EPA, and DHA, participate in the regulation of ovarian steroidgenesis in teleosts.

Gonadotropin stimulation of steroidogenesis in teleosts involves the cAMP signal transduction pathway (28–31), and studies with mammals have established that cAMP plays a role in regulating the synthesis, storage, and transport of cholesterol in steroidogenic tissues (32–36). EPA inhibited gonadotropin actions on steroid production by blocking cAMP formation. Given that the phosphodiesterase inhibitor IBMX was added to the incubation medium, the effect of EPA was on cAMP formation rather than on its degradation. However, AA at high dosage inhibited gonadotropin-stimulated testosterone production in spite of its potentiation on cAMP levels, suggesting a second site of action distal to cAMP. This was confirmed in studies showing that PUFAs also inhibited testosterone production stimulated by addition of cAMP analogues. This distal site must affect substrate availability to the steroidogenic enzymes as the PUFAs did not inhibit testosterone production stimulated by 25-OH Chol. It is becoming evident that regulation of substrate availability may be central in controlling gonadal steroidogenesis, as the inhibitory actions of other messengers, such as growth factors and cytokines, on steroid production were also reversible upon addition of exogenous cholesterol (37–39).

As with previous studies with goldfish ovarian follicles (18,28), there was variation in the basal, and gonadotropin-stimulated testosterone production. Although several factors, such as follicular maturity, may affect responsiveness to gonadotropin and steroid production during the preovulatory pe-

riod, PUFAs consistently inhibited gonadotropin-stimulated steroid production. Similarly to vitellogenic goldfish follicles, AA, EPA, and DHA attenuated hCG-stimulated testosterone and 17ß-estradiol production (McFarlane, W., Mercure, F., and Van Der Kraak, G., unpublished).

Because PUFAs can inhibit gonadotropin-stimulated testosterone production independently of their effects on cAMP formation or follicular maturity, intracellular mechanisms other than the cAMP signal transduction pathway may be involved. Several other transduction pathways participate in the regulation of ovarian function in mammals (40,41) and in teleost species (28,42–46). Stimulation of the lipid and calcium dependent phosphoinositol (PI) transduction pathway, with a tumor promoting phorbol ester (phorbol 12-myristate 13-acetate, PMA) and calcium ionophore A23187, was inhibitory to testosterone production in goldfish ovarian follicles (18,45). PMA is known to activate protein kinase C (PKC) (47,48), and in mammalian species PUFAs directly modulate PKC activity (23,24). Therefore, the inhibitory action of PUFAs on testosterone production in teleosts could be mediated by PKC activation. However, in goldfish testicular tissue, in which PUFAs inhibited hCG-stimulated testosterone production, PUFAs failed to activate PKC, suggesting some differences between mammals and fish (49). In chicken ovarian tissue, AA, which also inhibited gonadotropin-stimulated steroid production, failed to activate PKC (21,22). The inhibitory action of AA in hen ovarian granulosa cells appeared to be calcium-dependent (22). The inhibition of steroidogenesis in mouse testicular Leydig cells by nonesterified fatty acids occurs at a step prior to the conversion of cholesterol which was also found to be calcium-dependent (20). Thus, it is plausible that the inhibitory action of PUFAs in fish ovaries may be mediated by a calcium-dependent mechanism. In earlier studies, high dosages of A23187, which would elevate intracellular calcium levels, inhibited gonadotropin-stimulated testosterone production by goldfish prematurational ovarian follicles (28,43).

Since the inhibition of gonadotropin-stimulated testosterone production by PUFAs was not specific to any fatty acid and was found in both species studied, it is unlikely that specific metabolites are responsible for the inhibition. Thus, the inhibitory effect could have been the consequence of some shift in lipid and cholesterol metabolism induced by the PUFAs. Cholesterol metabolism is intimately linked with lipid absorption, transport, and metabolism; and in poikilotherms such as fish, regulation of lipid uptake, membrane integrity, and metabolic pathways must be integrated (50). Recent findings suggest that PUFAs act in a hormone-like manner to regulate the expression of peroxisomal enzymes involved in the metabolism of cholesterol and of long-chain PUFAs (51–56). Peroxisome proliferators are chemicals which induce peroxisomal enzymes by disrupting lipid metabolism, resulting in a fatty acid overload responsible for the induction (57–59). Given the relatively high doses tested and the nonspecificity of the PUFA inhibitory action in this study, it is possible that PUFAs inhibit by way of peroxisomal induction. Preliminary results have shown that recognized peroxisome proliferators inhibit ovarian steroid production of goldfish follicles (Mercure, F., and Van Der Kraak, G., unpublished). Thus, the fatty acid overload model proposed by Lock *et al.* (57), whereby PUFAs induce metabolic changes, warrants more attention in the present experimental model.

The paradoxical effects of AA on basal- and gonadotropin-stimulated testosterone production may relate to differential effects of AA and its metabolites. The finding that AA alone stimulates ovarian steroid production in goldfish and trout was consistent with previous studies showing that the actions of AA were dependent on its conversion to cyclooxygenase metabolites in goldfish ovaries (18) and testes (19), and that prostaglandins stimulate testosterone production *via* cAMP in goldfish testis (19) and in ovaries (Mercure, F., and Van Der Kraak, G., unpublished). However, there were differences in the sensitivity to AA alone between goldfish and trout. Given that temperature affects membrane lipid composition (1–4) and eicosanoid production in the goldfish (Mercure, F., and Van Der Kraak, G., unpublished), important differences may exist with respect to PUFA conversion to eicosanoids in goldfish and trout, which are warm and cold water species, respectively. Although EPA and DHA also affect basal steroid production, these data should be interpreted with caution, as stimulation was modest and not always statistically significant.

We were concerned that the high dose of fatty acids routinely used in this study caused cytotoxicity resulting in lower steroid production. This study demonstrated that PUFAs even at the highest dose did not affect follicular viability, as the inhibition was reversible upon removal of the PUFAs. However, it is unclear why the overall follicular responsiveness to hCG was greatly reduced after 15 h of incubation, and over time. It is likely that the overall follicular steroidogenic capacity diminishes with time under the incubation conditions used. Expression of steroidogenic enzymes, and thus their activity, was found to decline with time in similar incubations of rainbow trout and medaka ovarian follicles (Tanaka, M. and Nagahama, Y., personal communication). Nevertheless, this study demonstrates that PUFAs do not irreversibly affect steroidogenesis, which is consistent with the findings of others. Meikle *et al.* (20) reported, using trypan blue exclusion and chromium-51 release tests, that unesterified fatty acid concentrations up to 2,900 μM had no effect on membrane integrity and cell viability of mouse Leydig cells. Further, they demonstrated that the inhibition by nonesterified fatty acids, such as oleic and linoleic, was reversible upon removal of the fatty acids.

Although the high doses of PUFAs added were not lethal to the cells, the physiological significance of such doses must be addressed. The levels of nonesterified plasma fatty acids in fish are often much higher than in mammals, and large concentrations of nonesterified PUFAs have been reported in the gonads of some fish (5). Plasma levels ranging between 1,200 and 18,500 μM of total nonesterified fatty acids have been measured for several fish species (60), which is significantly

higher than the experimental dosages tested in the present study. As a result, the dosages tested may be regarded as physiological in fish. Due to the hydrophobicity of the fatty acids, dosages in excess of 400 μM could not be tested under the conditions used.

In summary, PUFAs have been found to inhibit gonadotropin-stimulated steroidogenesis in a cold and a warm water fish species. PUFAs have varying effects on cAMP formation, but apparently act in a similar manner at a site distal to cAMP to inhibit testosterone production. Their inhibitory actions were reversible. Intracellular transduction mechanisms other than the cAMP signal transduction pathway, possibly a calcium-dependent mechanism, may mediate the action of the PUFAs. Finally, PUFAs may induce changes in cellular metabolism with consequences on steroidogenic activity. These results implicate PUFAs as putative regulators of ovarian function in teleost fish.

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