J. Steroid Biochem. Molec. Biol. Vol. 43, No. 6, pp. 479–487, 1992
Printed in Great Britain.

0960-0760/92 $5.00 + 0.00

HORMONE STIMULATED STEROID BIOSYNTHESIS IN GRANULOSA CELLS STUDIED WITH A FLUOROGENIC PROBE FOR CYTOCHROME P-450$_{scc}$

ELIKPLIMI K. ASEM,[1]* DANIEL J. SIMPSON[2] and BABETTA L. MARRONE[2]

[1]Department of Physiology and Pharmacology, School of Veterinary Medicine, Purdue University, West Lafayette, IN 47907 and [2]Life Sciences Division, Los Alamos National Laboratory, Los Alamos, NM 87545, U.S.A.

(Received 13 March 1992)

Summary—The regulation of steroidogenesis by luteinizing hormone (LH) was studied in granulosa cells during follicular development using a fluorescent reporter assay based on the metabolism of a fluorescent probe specific for cytochrome P-450$_{scc}$ (cholesterol side-chain cleavage enzyme). Intact granulosa cells or mitochondria were obtained from the first (F_1) second (F_2) and third (F_3) largest preovulatory follicles of the hen ovary and incubated with the fluorogenic substrate. Metabolism of this substrate by cytochrome P-450$_{scc}$ generates the highly fluorescent resorufin anion (the fluorescent reporter). In both mitochondria and intact granulosa cells, incubated with the fluorescent substrate, an increase in resorufin fluorescence was observed and the increase was greater in samples derived from F_1 than in samples from F_2 or F_3. In cells, LH added simultaneously with the P-450$_{scc}$ substrate significantly increased resorufin fluorescence above control values in a time- and dose-dependent manner up to 2–3 h after the incubation was initiated. Forskolin and 8-bromo-cAMP also stimulated metabolism of the P-450$_{scc}$ substrate significantly by 15 min. When granulosa cells were preincubated with LH before exposure to the P-450$_{scc}$ substrate resorufin fluorescence was significantly attenuated compared to controls (not exposed to LH in the preincubation period). The decrease in resorufin fluorescence observed when cells were pretreated with LH, may be due to the release of cholesterol from endogenous pools and its competition with the exogenous fluorogenic for the substrate P-450$_{scc}$ enzyme. In granulosa cells that were preloaded with the P-450$_{scc}$ substrate, the stimulatory effect of LH treatment remained constant from 30 min to 2 h after hormone addition. The results show that this fluorescent probe can be used in a rapid assay for the continuous measurement of the acute effects of hormone agonists on cholesterol conversion to pregnenolone in steroidogenic cells.

INTRODUCTION

The conversion of cholesterol to pregnenolone by the enzyme system cytochrome P-450$_{scc}$ (cholesterol side-chain cleavage) is believed to be the rate-limiting step in steroidogenesis [see reviews 1–4]. Cytochrome P-450$_{scc}$ resides in the inner mitochondrial membrane and is subject to both chronic and acute regulation by trophic hormones mediated through the cAMP-dependent protein kinase second messenger system. In rat granulosa cells, prolonged (hours to days) treatment with gonadotropin has been shown to increase the amount of P-450$_{scc}$ enzyme [5, 6]. This effect on enzyme synthesis is due to an increase in P-450$_{scc}$ mRNA levels in response to gonadotropins, cAMP and forskolin [7–13].

The acute (minutes to hours) steroidogenic response of granulosa cells to gonadotrophic hormones may involve one or more of the following steps: the mobilization of endogenous cholesterol from lipid storage sites [14, 15]; transport of cholesterol to the enzyme site and/or the association of the substrate with the enzyme [16, 17]; and the activation of P-450$_{scc}$ enzyme [18–22].

Elucidation of the acute effects of gonadotropins and second messengers on cholesterol metabolism to pregnenolone has been hindered by the difficulties in isolating each possible site of action for study. In particular, the study of cytochrome P-450$_{scc}$ enzyme activity by luteinizing hormone (LH) and cAMP has been most challenging. A common method for measurement of cytochrome P-450$_{scc}$ activity involves the use of the highly soluble cholesterol analog,

*To whom correspondence should be addressed.

25-hydroxycholesterol (25-OH-Chol). 25-OH-Chol apparently bypasses the steroid transport system and readily enters the mitochondrial membrane [1, 9, 23], where it is cleaved by cytochrome $P\text{-}450_{scc}$ to pregnenolone. The products of cytochrome $P\text{-}450_{scc}$ metabolism of endogenous and exogenous substrate are therefore the same. Regulation of cholesterol metabolism in above-mentioned studies was examined with radioenzymatic assays [23–25] or product formation is measured by radioimmunoassay of total steroid production or of pregnenolone accumulation after pharmacological inhibition of subsequent steroid metabolism [23, 26, 27].

Recently, a fluorogenic substrate (fluorogenic probe) was developed for fluorescence analysis of the cytochrome $P\text{-}450_{scc}$ enzyme and provides means for the continuous measurement of cytochrome $P\text{-}450_{scc}$ activity [28, 29]. In initial studies the fluorogenic probe was demonstrated to be a selective indicator of cytochrome $P\text{-}450_{scc}$ activity. Furthermore, the probe metabolism in granulosa cells appeared to increase with increasing stage of differentiation and be enhanced by concomitant LH treatment. The aim of the present study was to examine in more detail the suitability of the fluorogenic probe as a tool for the study of the hormonal regulation of cytochrome $P\text{-}450_{scc}$ activity in intact cells and tissues using hen ovarian granulosa cells obtained from follicles at different stages of development. The results suggest that spectrofluorometric monitoring of the $P\text{-}450_{scc}$ fluorogenic probe metabolism provides a rapid, specific assay for the presence of cytochrome $P\text{-}450_{scc}$ enzyme and its activity.

EXPERIMENTAL

Hormones and chemicals

Ovine LH (NIAMDD-LH 22; 2.3 × NIH-LH S1 U/mg) was the gift of the Pituitary Hormone Distribution Program of the National Institute of Diabetes, Digestive and Kidney Diseases, National Institute of Health, (Bethesda, MD). *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid (HEPES), trizmabase, collagenase type 1A, soybean trypsin inhibitor bovine serum albumin (BSA; Fraction V) and 8-bromo-cAMP (8-br-cAMP), 25-OH-Chol, cholesterol sulfate and resorufin were from Sigma Chemicals (St Louis, MO). Forskolin was obtained from Calbiochem (La Jolla, CA). (20R)20-phenyl-5-pregnene-3β,20-diol (20-PPD) was a generous gift of Dr Larry Vickery (UC Irvine).

Fluorogenic substrate ($P\text{-}450_{scc}$ probe)

The synthesis and characterization of the $P\text{-}450_{scc}$ probe (*22-phenoxazonoxy-5-cholene-3β-ol*) is reported elsewhere [28]. A saturated stock solution of the $P\text{-}450_{scc}$ probe was made in 95% ethanol and filtered with a 0.2 μm filter before use. The concentration of the stock solution after filtering was determined spectrophotometrically and was 200–250 μM.

Experimental animals

White Leghorn hens in the first year of reproductive activity were obtained from Purdue Baker Farms (West Lafayette, IN), and caged individually in a windowless, air-conditioned room with a 14 h-light/10 h-dark cycle. They had free access to a pelletted laying ration and tap water. The bird colony was patrolled routinely and the time of egg lay was noted to the nearest 30 min.

The animals were killed by cervical dislocation 10–14 h before the expected time of ovulation of the largest preovulatory follicle. The granulosa cell layer was separated from the theca layer as previously described by Gilbert *et al.* [30].

Incubation of mitochondria

Cholesterol side-chain cleavage enzyme (cytochrome $P\text{-}450_{scc}$) activity was measured in mitochondrial preparations obtained from granulosa membranes as described by Asem and Hertelendy [27]. Briefly, the granulosa membranes were homogenized at 4°C in 5–7 vol of medium containing 250 mM sucrose, 1 mM EDTA, 25 mM Tris, pH 7.4 (homogenization buffer). The homogenates were centrifuged at 600 *g* for 10 min and the resulting supernatants were centrifuged at 10,000 *g* for 10 min to sediment mitochondria. The mitochondrial pellets were suspended in incubation buffer which contained 200 mM sucrose, 5 mM $MgCl_2$, 20 mM KCl, 0.2 mM EDTA, 25 mM Tris and 10 mM Na_2HPO_4, pH 7.4 (incubation buffer). Aliquots containing 50–200 μg/ml protein were added to a reaction mixture composed of 10 mM sodium isocitrate 2 ml final volume and incubated at 37°C in incubation buffer with 1–6 μM $P\text{-}450_{scc}$ substrate. The final amount of ethanol added to each sample was 1–3%.

Incubation of intact granulosa cells

The granulosa layer was separated and the cells dissociated in modified Hank's Balanced

Salt Solution [(HBSS); NaCl 140 mM, KCl 5 mM, $CaCl_2$ 1.8 mM, $MgCl_2$ 1.8 mM, glucose 10 mM, Hepes 20 mM, pH 7.4] containing collagenase and trypsin inhibitor [31, 32]. Cell viability as determined by the trypan blue dye exclusion method was routinely better than 95%. Granulosa cells (0.5–1 million/ml) were incubated in a cuvette in modified HBSS (2 ml) at 37°C with 1–6 μM P-450$_{scc}$ substrate with and without agonists. The final amount of ethanol added to each sample was 1–3%.

Fluorescence measurements of cytochrome P-450$_{scc}$ activity

Resorufin, the fluorescent product of the P-450$_{scc}$ enzyme reaction with the fluorogenic substrate was measured in a SLM Aminco 8000/DMX 1000 spectrofluorometer thermostatted at 37°C with constant magnetic stirring. At denoted times the amount of resorufin fluorescence was monitored. The excitation wavelength was 530 nm and emission was observed between 570 and 600 nm. Excitation and emission slit widths were 8 and 16 mm, respectively. Fluorescence observed at emission wavelength of 586 nm was used to estimate the amount of resorufin cleaved from the sterol moiety. Each measurement was corrected for background fluorescence by subtraction of the fluorescence spectra of each sample at its zero time point (immediately after addition of the P-450$_{scc}$ probe) from the spectra collected at each subsequent time point. Fluorescence intensity at 586 nm was converted to femtomole (fmol) resorufin using a standard curve of resorufin in the incubation buffer at 37°C.

Statistical analyses

Mitochondria or granulosa cells used in each experiment were pooled from follicles at the same stage of development from at least two hens. Values for each treatment level were pooled from all experiments with similar design and their mean and SEM calculated. These data were subjected to statistical evaluation by analysis of variance and by *post hoc* Tukey's test. Differences of $P \leqslant 0.05$ were considered significant.

RESULTS

Studies with mitochondria

Incubation of mitochondria isolated from the granulosa layer of the largest preovulatory follicle (F_1) with the fluorogenic substrate resulted in an increase in resorufin fluorescence. The amount of resorufin released was significant within 5 min after addition of the substrate and increased with time (Fig. 1). A significant increase in the release of resorufin could be observed within 5 min of incubation. Mitochondria isolated from the granulosa layer of the second (F_2) and third (F_3) largest follicles also released resorufin from the fluorogenic substrate. However, the amount of release was significantly lower than in F_1 mitochondria (Fig. 1). Although the total amount of resorufin fluorescence increased as the duration of incubation increased, the rate was linear up to 60 min and then plateaued (data not shown).

Studies with substrates of cytochrome P-450$_{scc}$

The following studies were performed with isolated mitochondria in order to minimize any differences in cell permeability and solubility between the fluorogenic substrate and the competitive sterol substrates of the enzyme. Incubation of isolated mitochondria with the fluorogenic probe and 1 μM 25-OH-Chol (a well known substrate of cytochrome P-450$_{scc}$) resulted in a suppression of the amount of resorufin release (Fig. 2). It is noteworthy that the extent of 25-OH-Chol's inhibitory effect was greater when it was preincubated with mitochondria prior to the addition of the fluorogenic substrate. Coincubation of mitochondria with 1 or 10 μM cholesterol sulfate, another substrate of cytochrome P-450$_{scc}$, also completely suppressed the ability of mitochondria to release resorufin from the fluorogenic probe (Fig. 3).

Studies with intact cells

Incubation of freshly isolated granulosa cells with the fluorogenic probe resulted in an

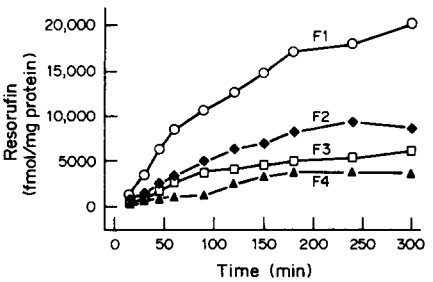

Fig. 1. Time course of P-450$_{scc}$ substrate metabolism in mitochondria prepared from the granulosa layer collected from preovulatory follicles during different stages of maturation. Resorufin fluorescence is measured as the product of P-450$_{scc}$ enzymes at the indicated times after addition of 4 μM of the fluorogenic substrate.

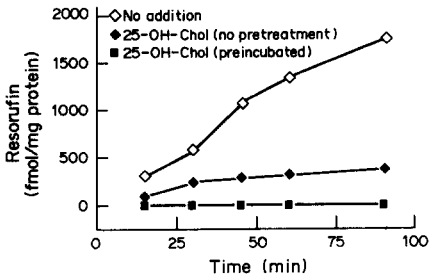

Fig. 2. Effect of 25-OH-Chol on P-450$_{scc}$ activity as measured at the indicated times in mitochondria obtained from the granulosa layer of the largest follicle and incubated with 1 μM of the fluorogenic substrate with or without 25-OH-Chol (1 μM). "No pretreatment" refers to P-450$_{scc}$ probe and 25-OH-Chol added simultaneously. "Preincubated" refers to mitochondria preincubated with 25-OH-Chol for 30 min prior to the addition of P-450$_{scc}$ probe.

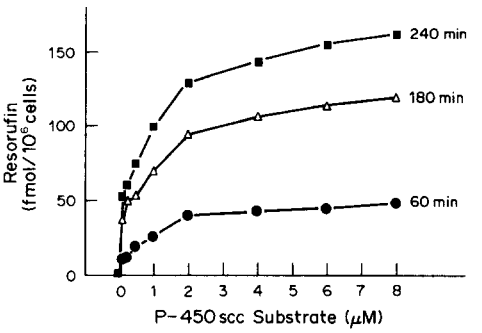

Fig. 4. Time course of P-450$_{scc}$ substrate metabolism in granulosa cells isolated from the largest preovulatory follicle. Resorufin fluorescence was measured as the product of P-450$_{scc}$ enzyme at the indicated times after addition of different concentrations of the fluorogenic substrate.

increase of resorufin fluorescence in a time- and dose-dependent manner (Fig. 4). The amount of resorufin released was influenced by the stage of follicular development such that it was greater in F_1 granulosa cells than in F_2 or F_3 cells (data not shown). The cytochrome P-450$_{scc}$ suicide substrate (20R)20-phenyl-5-pregnene-3β,20-diol (20-PPD) [33] also inhibited the metabolism of the fluorogenic substrate in a concentration related fashion (Fig. 5).

Effect of LH

In order to determine the influence of LH on the metabolism of the P-450$_{scc}$ substrate, granulosa cells were incubated with the fluorogenic substrate in the presence or absence of the gonadotropin. LH (100 ng/ml) increased the cumulative amount of resorufin produced from cells during a 3 h incubation with the P-450$_{scc}$ substrate. When the hormone and substrate were added simultaneously to F_1 and F_3 granulosa cells, LH significantly provoked within 15 min the release of resorufin above control levels in F_1 cells [$P < 0.05$; $n = 3$ experiments; Fig. 6(a)]. The stimulatory effect of LH was significant for 2–3 h. However, the degree to which LH enhanced resorufin production appeared to be related to the duration of the incubation. The gonadotropin's stimulatory effect diminished with time becoming statistically nonsignificant by 4 h, remaining not different from controls thereafter [Fig. 6(a)]. The time-dependent decrease in the relative difference in resorufin production between LH-treated and -untreated cells was apparently due to a decrease in the rate of P-450$_{scc}$ probe metabolism by LH-treated cells. LH (100 ng/ml) also enhanced resorufin fluorescence in F_3 granulosa cells but to a lesser extent than in F_1 cells [$P < 0.05$; Fig. 6(a)]. In F_1 cells LH increased resorufin fluorescence by 31.0 $\pm$ 5.0-fold ($n = 3$) as compared to 5.0 $\pm$ 0.8-fold in F_3 cells over respective unstimulated cells (controls) in a 30 min incubation. However, the extent of stimulation diminished to 2.0 $\pm$ 0.25- and 0.5 $\pm$ 0.02-fold, respectively by 2 and 3 h, in both F_1 and F_3 granulosa cells. The effect of LH

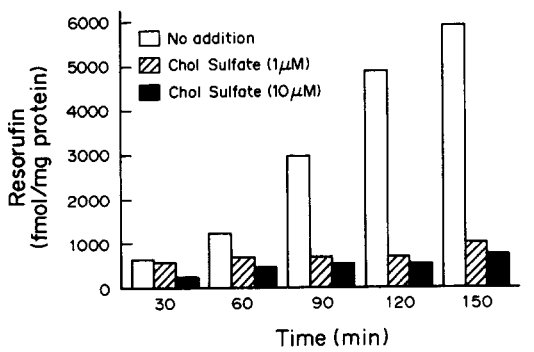

Fig. 3. Dose-related effect of cholesterol sulfate on P-450$_{scc}$ activity in mitochondria measured at the indicated times. Mitochondria isolated from the granulosa layer of the largest follicle (F_1) were preincubated with different concentrations of cholesterol sulfate for 30 min prior to the addition of 2 μM of the fluorogenic substrate.

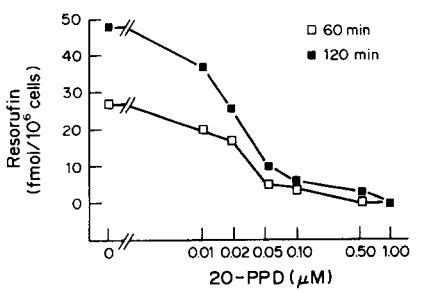

Fig. 5. Effect of (20R)20-phenyl-5-pregnene-3β,20-diol (20-PPD) on P-450$_{scc}$ probe metabolism by granulosa cells of the largest follicle. Cells were preincubated with different concentrations of 20-PPD for 15 min prior to the addition of 3 μM of the fluorogenic substrate.

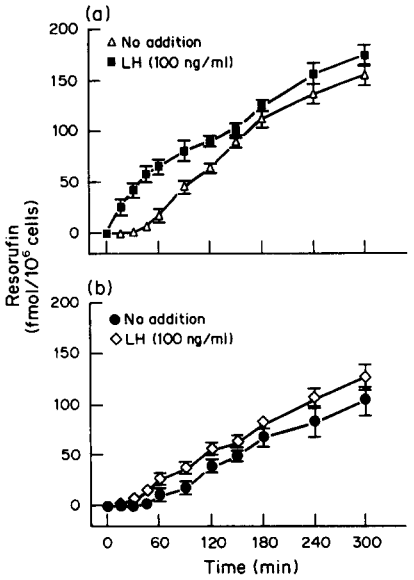

Fig. 6. Effect of LH on P-450$_{scc}$ substrate metabolism as measured at the indicated times in granulosa cells from the first (F_1) and third (F_3) largest follicle incubated with 3 μM of the fluorogenic substrate. LH and P-450$_{scc}$ probe were added simultaneously. Panel a = F_1; Panel b = F_3. Each point is the mean ± SEM of three separate experiments.

was dose-dependent. When different concentrations of LH (added simultaneously with the fluorogenic probe) were incubated with freshly isolated granulosa cells, LH increased the release of resorufin in a dose-related manner (Fig. 7). The degree of stimulation (by each LH dose tested) was influenced by the duration of incubation. The ratio of resorufin released in the presence of LH (I_{LH}) to that in control cells ($I_{no\,LH}$) was calculated (data not shown) for 30, 90 and 180 min incubation periods. The relative LH stimulation was greatest at 30 min (the earliest time point studied) but decreased as the

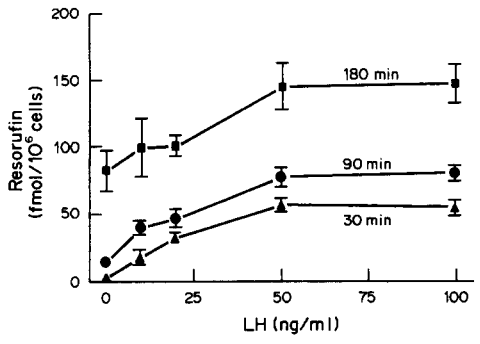

Fig. 7. Dose-response effect of LH on P-450$_{scc}$ substrate metabolism in granulosa cells measured at the indicated times. Granulosa cells from the largest follicle (F_1) were incubated with different concentrations of LH and the fluorogenic substrate (3 μM). LH and P-450$_{scc}$ probe were added simultaneously. Each point is the mean ± SEM of four separate experiments.

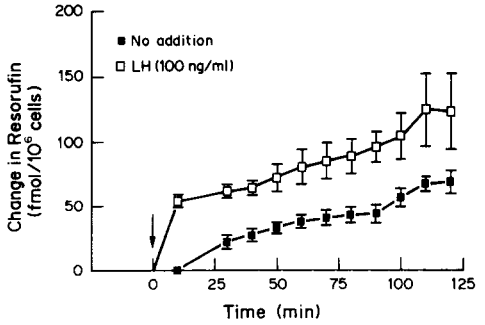

Fig. 8. Effect of preincubation of granulosa cells with the P-450$_{scc}$ substrate on LH action. F_1 granulosa cells were incubated with 3 μM probe for 2 h. LH was then added (arrow) and the incubation continued for denoted times. Results are the mean ± SEM of three separate experiments.

duration of incubation increased (90; 240 min). Concomitantly, the half maximal LH-stimulatory dose (ED_{50}) increased from 6.92 ng/ml at 30 min to 24.5 and 32.4 ng/ml after 90 and 180 min of incubation, respectively. In additional experiments, F_1 granulosa cells were preincubated with P-450$_{scc}$ probe for 2 h prior to addition of LH. Preincubation with the P-450$_{scc}$ substrate was done in order to allow the cells to equilibrate with it. In earlier studies, the rate of fluorogenic substrate uptake (determined by flow cytometry) plateaued at about 30 to 60 min. Figure 8 shows that in cells preloaded with the P-450$_{scc}$ probe LH increased resorufin fluorescence over the control group. By comparison, when the cells were preincubated (2 h) with or without LH before the addition of P-450$_{scc}$ probe, resorufin fluorescence was barely detected in cells that were exposed to LH in the preincubation period (Fig. 9).

Effect of 8-br-cAMP and forskolin

If the facilitatory effect of LH on the P-450$_{scc}$ probe conversion to resorufin is mediated by the

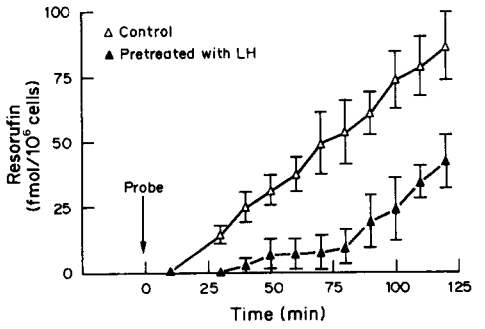

Fig. 9. Effect of preincubation of granulosa cells with LH on P-450$_{scc}$ substrate metabolism. F_1 granulosa cells were incubated with and without LH for 2 h. P-450$_{scc}$ (3 μM) was added and the incubation continued for denoted times. Each point is the mean ± SEM of three separate experiments.

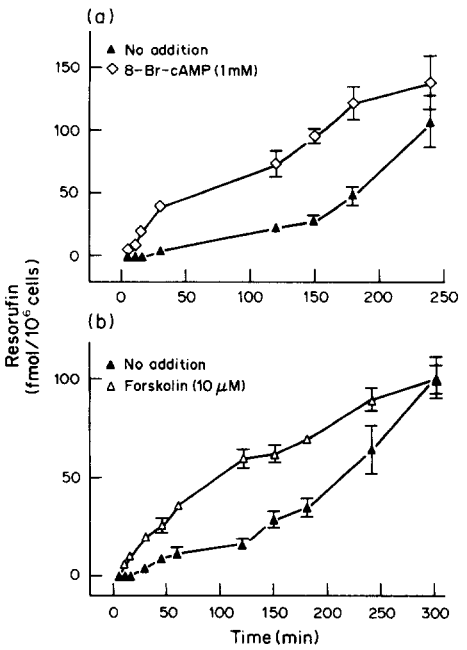

Fig. 10. Time course of 8-br-cAMP and forskolin enhanced P-450$_{scc}$ substrate metabolism in granulosa cells. Total resorufin fluorescence is measured as the product of P-450$_{scc}$ enzyme activity at the indicated times after the simultaneous addition of 3 μM of the fluorogenic substrate 8-br-cAMP (panel a, 1 mM) or forskolin (panel b, 10 μM). Each point is the mean $\pm$ SEM of three separate experiments.

adenylate cyclase second messenger system then cAMP agonists would also be expected to enhance the fluorescence production in cells incubated with the P-450$_{scc}$ probe. As observed with LH both 8-br-cAMP and forskolin treatment increased the cumulative amount of resorufin in cells incubated for 2–3 h with the P-450$_{scc}$ probe. Granulosa cells were incubated with the fluorogenic probe in the presence or absence of 8-br-cAMP. The release of resorufin was significantly elevated above control levels within 10 min [Fig. 10(a); $P < 0.05$; $n = 3$ experiments]. As observed with LH, the degree to which the agonist enhanced the resorufin production appeared to be related to the duration of incubation because the stimulatory effect of the cyclic nucleotide analog diminished by 4 h. Forskolin also stimulated resorufin fluorescence within 15 min of addition to granulosa cells [Fig. 10(b); $P < 0.05$; $n = 3$ experiments]. The temporal pattern of the forskolin effect on the P-450$_{scc}$ substrate metabolism was similar to that observed with LH and 6-br-cAMP.

DISCUSSION

Pituitary gonadotropin hormone stimulation of steroidogenesis in granulosa cells was examined through the application of a recently developed fluorescent substrate for cytochrome P-450$_{scc}$ [28]. The specificity of the P-450$_{scc}$ probe for the cytochrome P-450$_{scc}$ enzyme was evaluated in both mitochondrial preparations and intact cells. The pattern of P-450$_{scc}$ probe metabolism observed for mitochondria obtained from hen ovarian granulosa ($F_1 > F_2 > F_3$) was consistent with earlier results [27]. Furthermore, our data obtained with intact cells is in agreement with the known steroid biosynthetic pattern during follicular maturation [27, 32, 34–36]. LH is the well known biological regulator of steroidogenesis in hen granulosa cells in large preovulatory follicles [32, 34, 36, 37]. In the present study LH enhanced P-450$_{scc}$ substrate metabolism in intact cells and the degree of stimulation increased with increasing stage of follicular maturation, as would be expected from previous studies on the responsiveness of granulosa cells to LH stimulation of steroidogenesis during follicular maturation [27, 32, 34–36]. Forskolin and 8-br-cAMP had effects similar to LH on the P-450$_{scc}$ probe metabolism, suggesting that conversion of the P-450$_{scc}$ to resorufin is influenced by LH and the adenylate cyclase effector system in these cells.

Use of the fluorogenic probe may result in an underestimation of the amount of cholesterol conversion that can be attributed to the effects of the hormone and other agents on cholesterol side-chain cleavage enzyme activity because of the expected effects of the agonists on various steps in the steroid biosynthetic pathway, e.g. mobilization of endogenous P-450$_{scc}$ substrate. In cells treated concurrently with gonadotropins and the P-450$_{scc}$ probe, the effect of LH may have been compromised by the presence of endogenous substrate in the form of cholesterol ester. Facilitatory effects of LH on recruitment or mobilization of intracellular stores of free and esterified cholesterol [14, 38] and on cholesterol esterase activity [39, 40] have been demonstrated in the ovary. In the present study, cells pretreated with LH showed reduced levels of P-450$_{scc}$ probe metabolism. Presumably, prolonged pretreatment of cells with LH optimized conditions for the presence of copious amounts of endogenous cholesterol substrate, which could have effectively competed with exogenous, P-450$_{scc}$ fluorogenic substrate for the P-450$_{scc}$ enzyme. It may be argued that the viability of cells preincubated with LH was decreased leading to a reduction in resorufin

release. The fact that LH-induced progesterone synthesis has been shown to increase through 5 h [32] argues against that possibility. By comparison, the acute, dose-dependent, stimulatory effect of LH was more pronounced in experiments in which the cells were preincubated with the $P\text{-}450_{scc}$ fluorogenic substrate prior to the addition of LH. In those experiments the gonadotropin's effect was observed as early as 10 min after its addition when there was no apparent change in resorufin fluorescence in control cells. Preincubation of the granulosa cells with the $P\text{-}450_{scc}$ probe may have optimized conditions for the presence of exogenous substrate and minimized the availability of endogenous cholesterol substrate. In probe preloaded cells the stimulatory effect of LH on $P\text{-}450_{scc}$ probe metabolism was increased by 25- to 35-fold within 15–30 min compared to 5- to 15-fold in cells treated concurrently with LH and the $P\text{-}450_{scc}$ probe. The validity of the hypothesis that endogenous substrates competed with the $P\text{-}450_{scc}$ probe for the enzyme is supported by the findings in the present study that several other substrates of cytochrome $P\text{-}450_{scc}$ suppressed resorufin fluorescence in cells or mitochondria incubated with the $P\text{-}450_{scc}$ probe. Two well known substrates for cytochrome $P\text{-}450_{scc}$, 25-OH-Chol [23] and cholesterol sulfate [41], suppressed the release of resorufin from the fluorogenic substrate in mitochondrial preparations. Moreover, nanomolar concentrations of the $P\text{-}450_{scc}$ suicide substrate, 20-PPD, attenuated resorufin fluorescence in intact granulosa cells.

There are at least two possible mechanisms that may be invoked to explain enhanced metabolism of the $P\text{-}450_{scc}$ probe by hormone and agonists. A sterol carrier protein (SCP2) has been identified in ovarian cells [42] and it has been shown to be the target of LH-activated second messenger systems [43]. SCP2 transports cholesterol to the inner mitochondrial membrane and could mediate the observed effects of LH on the $P\text{-}450_{scc}$ probe metabolism. This hypothesis is supported by our results (Fig. 3) showing that cholesterol sulfate inhibited the conversion of the $P\text{-}450_{scc}$ probe to resorufin. Cholesterol sulfate is a substrate for $P\text{-}450_{scc}$, and has also been shown to inhibit cholesterol translocation to the $P\text{-}450_{scc}$ enzyme in mitochondria [17]. Therefore, the effects of cholesterol sulfate in the present study may also be explained by an inhibitory effect on translocation of the $P\text{-}450_{scc}$ probe to the enzyme in mitochondria. Our data do not rule out the possibility that LH directly activates the $P\text{-}450_{scc}$ enzyme. However, in studies using 25-OH-chol as a substrate for $P\text{-}450_{scc}$ no effect of gonadotropins or other hormonal agonists on $P\text{-}450_{scc}$ activity was demonstrated [23]. 25-OH-Chol has been used extensively to study the regulation of steroidogenesis in chicken [27] and pig [26] granulosa cells and rat luteal cells [23]. Because 25-OH-chol is highly soluble, its access to the $P\text{-}450_{scc}$ is not limited. The $P\text{-}450_{scc}$ probe has an apparent solubility and hydrophobicity more similar to cholesterol. Therefore, it would be expected not to enter mitochondria freely and to require the assistance of the cell's native translocating protein(s) in order to gain access to the $P\text{-}450_{scc}$ enzyme. In this respect, the $P\text{-}450_{scc}$ fluorogenic probe may provide a means by which $P\text{-}450_{scc}$ substrate translocation may be monitored in real time in single cells. We interpret the findings of the present study to suggest that gonadotropins, through the cAMP-dependent/protein kinase system, may enhance the $P\text{-}450_{scc}$ probe metabolism by influencing the availability of it to $P\text{-}450_{scc}$ enzyme, perhaps by activation of SCP2.

The $P\text{-}450_{scc}$ fluorogenic substrate has advantages over conventional methods for the study of the physiological or pharmacological regulation of the conversion of cholesterol to pregnenolone. The fluorogenic substrate offers the option of: (1) acquiring live time data; (2) using a method that does not involve the use of radioactive compounds; (3) greatly reducing assay time; and (4) analyzing $P\text{-}450_{scc}$ activity in single cells, when combined with flow or image cytometry. For the most part it could be a prime choice of substrate for studies of cytochrome $P\text{-}450_{scc}$ activity in which purified or semi-purified enzyme is used. Because it freely crosses the mitochondrial membrane, 25-OH-chol appears to be an advantageous substrate for studying the regulation of $P\text{-}450_{scc}$ activity directly. However, the fluorogenic $P\text{-}450_{scc}$ probe appears to be a uniquely useful substrate for studying the hormonal regulation of cholesterol redistribution during activation of steroid biosynthesis. The methods and techniques with which evidence has been obtained concerning the acute regulation of steroid biosynthesis by trophic hormones has relied on the use of different kinds of radioactive (carbon-14-labeled or tritiated) compounds in addition to cumbersome chromatographic procedures. Often, more than one steroid product of the $P\text{-}450_{scc}$ reaction

must be measured or further metabolism of pregnenolone must be blocked with pharmacological agents. Using a novel fluorogenic substrate, we have confirmed previous findings with regard to gonadotropin/cAMP regulation (acute) of cholesterol conversion to pregnenolone in a rapid, single endpoint assay.

In summary, LH, 8-br-cAMP, and forskolin each enhanced the metabolism of a new substrate for cytochrome $P\text{-}450_{scc}$ in granulosa cells. The hormonal effects were evident on the cumulative amount of fluoresscence reporter produced, most likely through modification of the redistribution of the fluorogenic substrate to the enzyme. This substrate is a useful tool with which the activity of cholesterol side-chain cleavage enzyme (cytochrome $P\text{-}450_{scc}$) can be assessed in either intact steroidogenic cells or their mitochondrial fractions.

Acknowledgements—We thank Dr S. Raiti, Director of the National Hormone and Pituitary Program, NIDDK, for the gift of ovine LH. We are very grateful to Dr Larry Vickery, University of California, Irvine, for the generous gift of 20-PPD. This work was supported by NIH Grants HD27345 (EKA) and HD22195 (BLM).

REFERENCES

1. Strauss III J. F., Schuler L. A., Rosenblum M. F. and Tanaka T.: Cholesterol metabolism by ovarian tissue. *Adv. Lipid Res.* **18** (1981) 99–157.
2. Gore-Langton R. E. and Armstrong D. T.: Follicular steroidogenesis and its control. In *The Physiology of Reproduction* (Edited by E. Knobil and J. Neill). Raven Press, New York, Vol. 1 (1988) p. 331.
3. Niswender G. D. and Nett T. M.: The corpus luteum and its control. In *The Physiology of Reproduction* (Edited by E. Knobil and J. Neill). Raven Press, New York, Vol. I (1988) p. 489.
4. Hall P. F.: Testicular steroid synthesis: organization and regulation. In *The Physiology of Reproduction* (Edited by E. Knobil and J. Neill). Raven Press, New York, Vol. 1 (1988) p. 975.
5. Goldring N. B., Farkash Y., Goldschmit D. and Orly J.: Immunofluorescent probing of the mitochondrial cholesterol side-chain cleavage cytochrome *P*-450 expressed in differentiating granulosa cells in culture. *Endocrinology* **119** (1986) 2821–2832.
6. Goldring N. B., Durica J. M., Lifka J., Hedin L., Ratoosh S. H., Miller W. L., Orly J. and Richards J. S.: Cholesterol side-chain cleavage *P*450 messenger ribonucleic acid: evidence for hormonal regulation in rat ovarian follicles and constitutive expression in corpora lutea. *Endocrinology* **120** (1987) 1942–1950.
7. Voultilainen R., Tapanainen J., Chung B. C., Matteson K. J. and Miller W. L.: Hormonal regulation of *P*450scc (20,22-desmolase) and *P*450c17 (17a-hydroxylase/17,20-lyase) in cultured human granulosa cells. *J. Clin. Endocr. Metab.* **63** (1986) 202–207.
8. Golos T. G., Miller W. L. and Strauss J. F. III.: Human chorionic gonadotropin and 8-bromo cyclic adenosine monophosphate promote an acute increase in cytochrome *P*450scc and adrenodoxin messenger RNAs in cultured human granulosa cells by a cyclohexamide-insensitive mechanism. *J. Clin. Invest.* **80** (1987) 896–899.
9. Toaff M. E., Strauss J. F. III and Hammond J. M.: Regulation of cytochrome *P*450scc in immature porcine granulosa cells by FSH and estradiol. *Endocrinology* **112** (1983) 1156–1158.
10. Oonk R. B., Parker K. L., Gibson J. L. and Richards J. S.: Rat cholesterol side-chain cleavage cytochrome *P*-450 (*P*-450scc) gene structure and regulation by cAMP *in vitro*. *J. Biol. Chem.* **265** (1990) 22,392–22,401.
11. Mulheron G. W., Wise T. H., Stone R. T. and Ford J. J.: Follicle-stimulating hormone regulation of cytochrome *P*-450 side chain cleavage messenger ribonucleic acid accumulation by porcine granulosa cells isolated from small and medium follicles. *Biol. Reprod.* **43** (1990) 828–834.
12. Spicer L. J., Kao L. C., Strauss J. F. III and Hammond J. M.: 2-Hydroxyestradiol enhanced progesterone production by porcine granulosa cells: dependence on de novo cholesterol synthesis and stimulation of cholesterol side-chain cleavage activity and cytochrome *P*450scc messenger ribonucleic acid levels. *Endocrinology* **127** (1990) 2763–2770.
13. Tilly J. L., Kowalski K. L. and Johnson A. L.: Cytochrome *P*450 side-chain cleavage (*P*450scc) in the hen ovary. II. P450scc messenger RNA, immunoreactive protein and enzyme activity in developing granulosa cells. *Biol. Reprod.* **45** (1991) 967–974.
14. Freeman D. A. and Ascoli M.: Studies on the source of cholesterol used for steroid biosynthesis in cultured Leydig tumor cells. *J. Biol. Chem.* **257** (1982) 14,231–14,238.
15. Pedersen R. C. and Brownie A. C.: Cholesterol side-chain cleavage in the rat adrenal cortex: isolation of a cycloheximide-sensitive activator peptide. *Proc. Natn. Acad. Sci. U.S.A.* **80** (1983) 1882–1886.
16. Toaff M. E., Strauss J. F. III, Flickinger G. L. and Shattil S. J.: Relationship of cholesterol supply to luteal mitochondrial steroid synthesis. *J. Biol. Chem.* **254** (1979) 3977–3982.
17. Lambeth J. D., Xu X. X. and Glover M.: Cholesterol sulfate inhibits adrenal mitochondrial cholesterol side chain cleavage at a site distinct from cytochrome *P*-450scc. *J. Biol. Chem.* **262** (1987) 9181–9188.
18. Mori M. and Marsh J. M.: The site of luteinizing hormone stimulation of steroidogenesis in mitochondria of the rat corpus luteum. *J. Biol. Chem.* **257** (1982) 6178–6183.
19. Nimrod A.: On the synergistic action of androgen and FSH on progestin secretion by cultured granulosa cells. *Molec. Cell. Endocr.* **21** (1981) 51–62.
20. Caron M. G., Goldstein S., Savard K. and Marsh J. M.: Protein kinase stimulation of a reconstituted cholesterol side chain cleavage enzyme system in bovine corpus luteum. *J. Biol. Chem.* **250** (1975) 5137–5143.
21. Veldhuis J. D., Klase P. A., Strauss J. F. III and Hammond J. M.: The role of estradiol as a biological amplifier of the actions of follicle-stimulating hormone: *in vitro* studies in swine granulosa cells. *Endocrinology* **111** (1982) 144–150.
22. Veldhuis J. D., Klase P. A., Strauss J. F. III and Hammond J. M.: Facilitative interactions between estradiol and luteinizing hormone in the regulation of progesterone production by cultured swine granulosa cells: relation to cellular cholesterol metabolism. *Endocrinology* **111** (1982) 441–449.
23. Toaff M. E., Schleyer H. and Strauss J. F. III: Metabolism of 25-hydroxycholesterol by rat luteal mitochondria and dispersed cells. *Endocrinology* **111** (1982) 1785–1790.
24. Dimino M. J., Frances D. E. and Downing J. R.: Steroidogenesis by mitochondria prepared from porcine ovarian tissues. In *Ovarian Follicular Development and*

Function (Edited by A. R. Midgley and W. A. Sadler). Raven Press, New York (1979) pp. 199–201.
25. Dimino M. J., Bieszczad R. R. and Rowe M. J.: Cyclic AMP-dependent protein kinase in mitochondria and cytosol from different-sized follicles and corpora lutea of porcine ovaries. *J. Biol. Chem.* **256** (1981) 10,876–10,882.
26. Veldhuis J. D., Klase P. A., Demers L. M. and Chafouleas J. G.: Mechanisms subserving calcium's modulation of luteinizing hormone action in isolated swine granulosa cells. *Endocrinology* **114** (1984) 441–449.
27. Asem E. K. and Hertelendy F.: Influence of follicular maturation on luteinizing hormone, cyclic 3′,5′-adenosine monophosphate, forskolin and cholesterol stimulated progesterone production in hen granulosa cells. *Biol. Reprod.* **32** (1985) 257–268.
28. Simpson D. J., Unkefer C. J., Whaley T. W. and Marrone B. L.: A mechanism-based fluorogenic probe for the cytochrome *P*-450 cholesterol side chain cleavage enzyme. *J. Org. Chem.* **56** (1991) 5391–5396.
29. Marrone B. L., Simpson D. J., Yoshida T. M., Unkefer C. J., Whaley T. W. and Buican T. N.: Analysis of *P*-450scc activity by fluorescence detection methods. *Endocrinology* **128** (1991) 2654–2656.
30. Gilbert A. B., Evans A. J., Gilbert A. B., Evans A. J., Perry M. M. and Davidson M. H.: A method for separating the granulosa cells, the basal lamina and the theca of the preovulatory ovarian follicle of the domestic fowl (*Gallus domesticus*). *J. Reprod. Fert.* **50** (1977) 179–181.
31. Huang E. S. R., Kao K. J. and Nalbandov A. V.: Synthesis of sex steroids by cellular components of chicken follicles. *Biol. Reprod.* **20** (1979) 454–461.
32. Hammond R. W., Burke W. H. and Hertelendy F.: Influence of follicular maturation on progesterone release in chicken granulosa cells in response to turkey and ovine gonadotropins. *Biol. Reprod.* **24** (1981) 1048–1055.
33. Ascoli M., Freeman D. A., Sheets J. J. and Vickery L. A.: Inhibition of steroidogenesis in cultured Leydig tumor cells by 22-amino-23,24-bisnor-5-cholen-3B-ol and (20R)20-phenyl-5-pregnene-3B,20-diol. *Endocrinology* **113** (1983) 127–132.
34. Bahr J. M., Wang S. C., Huang M. Y. and Calvo F. O.: Steroid concentrations in isolated theca and granulosa layers of preovulatory follicles during the ovulatory cycles of the domestic hen. *Biol. Reprod.* **29** (1983) 326–334.
35. Etches R. J. and Duke C. E.: Progesterone, androstenedione and oestradiol content of the theca and granulosa tissues of the four largest ovarian follicles during the ovulatory cycle of the hen (*Gallus domesticus*). *J. Endocr.* **103** (1984) 71–76.
36. Marrone B. L. and Hertelendy F.: Steroidogenesis by avian ovarian cells: Effects of LH and substrate availability. *Am. J. Physiol.* **244** (1983) E487–E493.
37. Robinson F. E. and Etches R. J.: Ovarian steroidogenesis during follicular maturation in the domestic fowl (*Gallus domesticus*). *Biol. Reprod.* **35** (1986) 1096–1105.
38. Freeman D. A.: Plasma membrane cholesterol: removal and insertion into the membrane and utilization as substrate for steroidogenesis. *Endocrinology* **124** (1989) 2527–2534.
39. Behrman H. R. and Armstrong D. T.: Cholesterol esterase stimulation by luteinizing hormone in luteinized rat ovaries. *Endocrinology* **85** (1969) 474–480.
40. Caffrey J. L., Fletcher P. W., Diekman M. A., O'Callaghan P. L. and Niswender G. D.: The activity of ovine luteal cholesterol esterase during several experimental conditions. *Biol. Reprod.* **21** (1979) 601–608.
41. Tuckey R. C.: Side-chain cleavage of cholesterol sulfate by ovarian mitochondria. *J. Steroid Biochem. Molec. Biol.* **37** (1990) 121–127.
42. Tanaka T., Billheimer J. T. and Strauss J. F. III: Luteinized rat ovaries contain a sterol carrier protein. *Endocrinology* **114** (1984) 553–540.
43. Steinschneider A., McLean M. P., Billheimer J. T., Azhar S. and Gibori G.: Protein kinase C catalyzed phosphorylation of sterol carrier protein 2. *Endocrinology* **125** (1989) 569–571.