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Receptor-Mediated Gonadotropin Action in the Ovary

REGULATORY ROLE OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASE(S) IN INTRACELLULAR ADENOSINE 3′:5′-CYCLIC MONOPHOSPHATE TURNOVER AND GONADOTROPIN-STIMULATED PROGESTERONE PRODUCTION BY RAT OVARIAN CELLS

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The regulatory role of cyclic nucleotide phosphodiesterase(s) and cyclic AMP metabolism in relation to progesterone production by gonadotropins has been studied in isolated rat ovarian cells. Low concentrations of choriogonadotropin (0.4–5 ng/ml) increased steroid production without any detectable increase in cyclic AMP, when experiments were carried out in the absence of phosphodiesterase inhibitors. The concentration of choriogonadotropin (10 ng/ml) that stimulated progesterone synthesis maximally resulted in a minimal increase in cyclic AMP accumulation and choriogonadotropin binding. Choriogonadotropin at a concentration of 10 ng/ml and higher, however, significantly stimulated protein kinase activity and reached a maximum between 250 and 1000 ng of hormone/ml. Higher concentrations (50–2500 ng/ml) of choriogonadotropin caused an increase in endogenous cyclic AMP, and this increase preceded the increase in steroid synthesis. Analysis of dose–response relationships of gonadotropin-stimulated cyclic AMP accumulation, progesterone production and protein kinase activity revealed a correlation between these responses over a wide concentration range when experiments were performed in the presence of 3-isobutyl-1-methylxanthine. The phosphodiesterase inhibitors papaverine, theophylline and 3-isobutyl-1-methylxanthine each stimulated steroid production in a dose-dependent manner. Incubation of ovarian cells with dibutyryl cyclic AMP or 8-bromo cyclic AMP mimicked the steroidogenic action of gonadotropins and this effect was dependent on both incubation time and nucleotide concentration. Maximum stimulation was obtained with 2 mM-dibutyryl cyclic AMP and 8-bromo cyclic AMP, and this increase was close to that produced by a maximally stimulating dose of choriogonadotropin. Other 8-substituted derivatives such as 8-hydroxy cyclic AMP and 8-isopropylthio cyclic AMP, which were less susceptible to phosphodiesterase action, also effectively stimulated steroidogenesis. The uptake and metabolism of cyclic [^{3}H]AMP in ovarian cells was also studied in relation to steroidogenesis. When ovarian cells were incubated for 2 h in the presence of increasing concentrations of cyclic [^{3}H]AMP, the radioactivity associated with the cells increased almost linearly up to 250 μM-cyclic [^{3}H]AMP concentration in the incubation medium. The ^{3}H label in the cellular extract was recovered mainly in the forms ATP, ADP, AMP, adenosine and inosine, with cyclic AMP accounting for less than 1% of the total tissue radioactivity. Incubation of cyclic AMP *in vitro* with ovarian cells resulted in a rapid breakdown of the nucleotide in the medium. The degradation products in the medium have been identified as AMP, adenosine and inosine. The rapid degradation of cyclic AMP by phosphodiesterase(s) makes it difficult to correlate changes in cyclic AMP concentrations with steroidogenesis. These observations thus provide an explanation for the previously observed lack of cyclic AMP accumulation under conditions in which low doses of choriogonadotropin stimulated steroidogenesis without any detectable changes in cyclic AMP accumulation.

The initial step in the action of gonadotropins in the ovary is the interaction of hormone with specific binding sites on the cell membrane (Gospodarowicz, 1973; Lee & Ryan, 1973; Menon & Kiburz, 1974; Haouer & Saxena, 1974; Rao, 1974; Bellisario & Bahl, 1975; Azhar & Menon, 1976; Azhar *et al.*, 1976) followed by the stimulation of cyclic AMP production with subsequent stimulation of protein kinase and steroidogenesis (Marsh *et al.*, 1966; Dorrington & Kilpatrick, 1967; Hermier *et al.*, 1971;

Stansfield *et al.*, 1972; Kammerman *et al.*, 1972; Menon, 1973; Koch *et al.*, 1974; Menon & Kiburz, 1974; Marsh, 1975, 1976; Kawano *et al.*, 1975; Mills, 1975; Clark & Menon, 1976; Clark *et al.*, 1976; Channing & Tsafriri, 1977; Zeleznik *et al.*, 1977; Conti *et al.*, 1977; Azhar & Menon, 1978; Menon & Azhar, 1978). The available evidence suggests that cyclic AMP may be an intracellular mediator of gonadotropins in ovarian steroidogenesis (Marsh *et al.*, 1966; Dorrington & Kilpatrick, 1967; Hermier *et al.*, 1971; Stansfield *et al.*, 1972; Mills, 1975; Channing & Tsafriri, 1977). Previous studies from this laboratory have shown that although gonadotropin stimulation of progesterone synthesis in ovarian cells was accompanied by an increase in cyclic AMP formation, low concentrations of lutropin and choriogonadotropin were found to stimulate steroidogenesis *in vitro* without any significant detectable increase in cyclic AMP concentration (Clark & Menon, 1976). These observations have revealed a discrepancy in the relationship between cyclic AMP accumulation and progesterone production in response to low concentrations of tropic hormone, thereby questioning the intermediary role of cyclic AMP in steroidogenesis in response to low doses of the hormone. Similarly, in the testicular system it has been demonstrated that there is a discrepancy between the amounts of gonadotropins required to saturate the binding sites and to maximally stimulate cyclic AMP formation and steroidogenesis (Catt & Dufau, 1973; Moyle & Ramachandran, 1973; Rommerts *et al.*, 1973; Mendelson *et al.*, 1975). A similar dissociation between cyclic AMP accumulation and steroid synthesis in response to low doses of tropic hormone has also been described in isolated adrenal cells (Beale & Sayers, 1972; Hudson & McMartin, 1975). However, a positive correlation between activation of cyclic AMP-dependent protein kinase and steroidogenesis by gonadotropins has been demonstrated in bovine corpus-luteum slices (Ling & Marsh, 1977) and Leydig cells (Cooke *et al.*, 1976). By assaying free and occupied cyclic AMP-binding sites of the regulatory subunit of protein kinase in basal and hormone-stimulated Leydig cells Dufau *et al.* (1977) have demonstrated progressive changes in cyclic AMP production and cyclic AMP-receptor activity during steroidogenesis by low concentrations of gonadotropin.

These observations have led us to investigate the quantitative relationship between cyclic AMP accumulation and progesterone production in response to low doses of gonadotropins and the probable role of cyclic nucleotide phosphodiesterases in these processes. In the present study we have examined (*a*) dose- and time-dependent accumulation of cyclic AMP, activation of cyclic AMP-dependent protein kinase and progesterone production in response to choriogonadotropin and phosphodiesterase inhibitor by the cells, (*b*) the ability of various cyclic AMP phosphodiesterase inhibitors to enhance the steroidogenesis and to potentiate gonadotropin action, (*c*) effectiveness of various 8-substituted cyclic AMP derivatives in stimulating progesterone production in comparison with other cyclic nucleotides, and (*d*) some characteristics of cyclic AMP metabolism in relation to hormone-stimulated steroidogenesis. The findings from the present studies provide evidence to suggest that: (1) low doses of choriogonadotropin stimulate steroidogenesis through changes in cyclic AMP concentrations; and (2) the relatively fast metabolism of cyclic AMP within cells makes the demonstration of changes in cyclic AMP concentrations in response to low doses of choriogonadotropin technically difficult.

Experimental

Materials

Sprague–Dawley rats that were 26 days old were purchased from Spartan Farms, Hazlett, MI, U.S.A. [8-^{3}H]Adenosine 3′:5′-cyclic monophosphate (sp. radioactivity 27Ci/mmol) and cyclic [U-^{14}C]AMP (sp. radioactivity 287mCi/mmol) were purchased from Amersham Corp., Arlington Heights, IL, U.S.A. [*methoxy*-^{3}H]Inulin (sp. radioactivity 5Ci/mmol) was obtained from New England Nuclear Corp., Boston, MA, U.S.A. Cyclic AMP, dibutyryl cyclic AMP, cyclic IMP, cyclic UMP, cyclic GMP, cyclic CMP, cyclic dAMP, 8-amino cyclic AMP, 8-hydroxy cyclic AMP, cyclic XMP, ATP, ADP, 5′-AMP, adenosine, adenine, inosine and mixed histone (type IIA) were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. 8-Methylthio cyclic AMP, 8-benzylthio cyclic AMP, 8-isopropylthio cyclic AMP, 8-bromo cyclic AMP, 8-bromo cyclic GMP and carrier-free [^{32}P]P_i were purchased from ICN Pharmaceuticals, Irvine, CA, U.S.A. Bovine serum albumin (fraction V) was supplied by Reheis Chemical Co., Phoenix, AZ, U.S.A. Collagenase (type CLS) and deoxyribonuclease I were the products of Worthington Biochemicals Corp., Freehold, NJ, U.S.A. 3-Isobutyl-1-methylxanthine was supplied by Aldrich Chemical Co., Milwaukee, WI, U.S.A. Eagle's Minimum Essential Medium with Earle's salts (Medium 109) was purchased from Grand Island Biological Co., Grand Island, NY, U.S.A. Purified human choriogonadotropin (CR-119; 11500 units/mg) was generously provided by Dr. R. Canfield, Columbia University, NY, U.S.A., and Population Research Branch, National Institute of Child Health and Human Development. ^{125}I-labelled choriogonado-

tropin (sp. radioactivity 50 μCi/μg) was prepared as described by Dufau *et al.* (1972). [γ-^{32}P]ATP was synthesized by the method of Glynn & Chappell (1964). Dowex AG 1-X2 (200–400 mesh) was purchased from Bio-Rad Laboratories, Richmond, CA, U.S.A. All other reagents were obtained from regular commercial sources and were of highest purity.

Methods

Preparation of dispersed ovarian cells. Interstitial cells were prepared by collagenase dispersion from the ovaries of 25-day-old rats as described previously (Kawano *et al.*, 1975; Clark & Menon, 1976). The cells were washed twice and resuspended in Medium 109 containing 1 mg of bovine serum albumin/ml (50 mg original weight of tissue per ml). Cells were counted in a haemocytometer and viability was determined by dye exclusion after staining in 0.02% Nigrosin. DNA was determined by the method of Burton (1956), and all results are expressed per μg of DNA.

Incubation conditions and measurement of progesterone, cyclic AMP accumulation and protein kinase activation. Determination of progesterone accumulation. Approx. $(2.5–4)\times10^6$ cells were incubated at 37°C for 2h (unless otherwise stated) under CO_2/O_2 (1:19, v/v) in a volume of 400 μl of Medium 109 containing 0.1% bovine serum albumin in the presence or absence of choriogonadotropin or other test substances. After incubation, the reaction was stopped by placing the sample tubes in a boiling-water bath for 3 min, and 0.6 ml of water and 10 μl (approx. 10000 c.p.m.) of [1,2-^{3}H]progesterone (to monitor recovery) was added and the samples left in the cold overnight. After 12h, samples were extracted with light petroleum and assayed for progesterone by radioimmunoassay as described previously (Kawano *et al.*, 1975; Clark & Menon, 1976).

Cyclic AMP measurements. Cells were incubated as described above for progesterone production, with the exception that 3-isobutyl-1-methylxanthine (0.5 mM) was also present (unless otherwise noted). After incubation, usually for 2h, the tubes were boiled for 5 min, transferred to ice and 0.75 ml of 10% trichloroacetic acid (w/v), 0.15 ml of 1 M-HCl, 180 μl of water and 20 μl of cyclic [^{3}H]AMP (approx. 2000 c.p.m.) were added. The samples were stored in the cold overnight. The precipitated proteins were then removed by centrifugation at 5000*g* for 10 min at 4°C. The supernatant fractions were processed and assayed for cyclic AMP as described by Gilman (1970). Results are expressed as pmol of cyclic AMP formed per μg of DNA.

Assay of protein kinase activity. Cell suspensions (1.2×10^7 cells) in 400 μl of Medium 109 containing 0.1% bovine serum albumin were incubated with or without test substances at 37°C in O_2/CO_2 (19:1, v/v) for 120 min. After incubation cells were centrifuged and homogenized in 200 μl of 10 mM-Tris/HCl buffer, pH 7.2, containing 1 mM-EDTA and 0.5 mM-3-isobutyl-1-methylxanthine. After centrifugation at 20000*g* for 20 min in the cold (4°C) the clear supernatant was used to assay protein kinase activity by a slight modification of the method of Reimann *et al.* (1971). The incubation mixture contained, in a final volume of 75 μl, 12 mM-potassium phosphate, pH 6.8, 20 mM-NaF, 4 mM-$MgCl_2$, 0.22 mM-[γ-^{32}P]ATP (90000–100000 c.p.m./nmol), 10 mg of mixed histone II/ml, enzyme preparation, and where required, 2 μM-cyclic AMP. The reaction was stopped after 5 min of incubation at 30°C by spotting 50 μl of these mixtures on filter-paper discs and immersing in ice-cold 10% trichloroacetic acid as described by Corbin & Reimann (1974), with the modification (Azhar & Menon, 1978) that discs were washed 4 times with 10% trichloroacetic acid with at least one overnight wash. They were then washed for 30 min in ethanol and 30 min in diethyl ether before drying and counting in a Beckman LS230 liquid-scintillation spectrophotometer in vials containing 10 ml of a solution of 4g of Omnifluor/litre of toluene. Protein kinase activity was expressed as the activity ratio, i.e. the ratio of activity in the absence of cyclic AMP to that in the presence of 2 μM-cyclic AMP (Corbin *et al.*, 1973).

Uptake and metabolism of cyclic [^{3}H]AMP. Uptake experiments were initiated by the addition of a mixture of the labelled (5 μCi/tube) and unlabelled cyclic AMP to obtain the defined final concentrations as given in respective Tables and Figures. Unless otherwise indicated, the experiments were carried out at 37°C in Medium 109 containing 3% (w/v) albumin in the presence of CO_2/O_2 (1:19, v/v). The final incubation volume was 400 μl and the cell concentration ranged between 4×10^6 and 6×10^6 cells/ml. Incubations were terminated (usually after 2h) by adding 2 ml of ice-cold Medium 109 to each tube. Appropriate controls were run simultaneously, which were identical with experimental sets except that the incubation was terminated immediately after the addition of cyclic [^{3}H]AMP. Cells were immediately centrifuged (2000 rev./min). The supernatant was discarded, and the cells were washed with the same volume of the ice-cold medium. The washing procedure did not last more than 4 to 5 min and the efflux of intracellular radioactivity occurring during this prodecure was negligible (2–3% of the total uptake). The residual extracellular radioactivity that was still associated with the cells after the washing procedure did not exceed 3–5% of the total uptake. This value was similar to that found when extracellular marker [^{3}H]inulin was used

(Boumendel-Podevin & Podevin, 1977). The results were corrected on this basis to account for extracellular trapping of cyclic [^{3}H]AMP. The cell pellet after the final wash was resuspended in 1.5ml of trichloroacetic acid (5%, w/v) containing cyclic [^{14}C]AMP (6000c.p.m. to monitor the recovery of cyclic [^{3}H]AMP) and a portion of the resulting suspension was counted for radioactivity to determine total uptake. The remainder of the suspension was centrifuged to remove the precipitated material. The radioactivity in a portion of the resulting supernatant was also measured to determine soluble radioactivity and the remaining supernatant was analysed to determine the intracellular fate of cyclic AMP.

The supernatant was extracted 5 times with water-saturated diethyl ether (2vol.), freeze-dried and analysed by descending paper chromatography on Whatman 3MM paper (4cm×46cm) with ethanol/1M-ammonium acetate, pH7.4 (7:3, v/v) as solvent system. The chromatograms were developed for 21h (Kawano *et al.*, 1975). Strips 1cm long were eluted with 1.5ml of water and radioactivity associated with a volume of 0.5ml was measured in 10ml of Herberg's fluid (Rapkin, 1964). In some cases freeze-dried material was also analysed by chromatography on Dowex-1 and alumina columns (Saloman *et al.*, 1974; Ortmann & Perkins, 1977). In all the cases standards were run alongside the unknowns.

Assay of phosphodiesterase. The radioisotope method of Thompson & Appleman (1971), as modified by Boudreau & Drummond (1975), was used to determine cyclic AMP phosphodiesterase activity (Azhar & Menon, 1977). The reaction mixture contained 10mM-Tris/HCl (pH7.5), 1mM-$MgCl_2$, 3.75mM-2-mercaptoethanol, 1μM-cyclic [^{3}H]AMP (containing 1.2×10^5c.p.m.) and 20μl of enzyme extract in a final volume of 0.2ml. After incubation at 30°C for 10min the tubes were frozen in a solid CO_2/methanol mixture, then boiled for 3min in a boiling-water bath. The tubes were cooled to room temperature, 50μl of a solution of snake venom (*Ophiophagus hanna*) (1mg/ml) was added to each tube, and the tubes were incubated at 30°C for 10min. The incubation was terminated by the addition of 1ml of a slurry of Dowex anion-exchange resin (AG 1-X2, 200–400 mesh) in 3mM-acetic acid (1 part resin, 2 parts acetic acid). After centrifugation, 0.5ml of clear supernatant was counted for radioactivity after the addition of 10ml of Herberg's fluid (Rapkin, 1964) by using a Beckman LS230 scintillation counter.

Phosphodiesterase (cyclic AMP) activity of the media incubated with ovarian cells (10μM- to 250μM-cyclic AMP as substrate) for 2h was assayed as follows. The reaction mixture contained 10mM-Tris/HCl (pH7.5), 1mM-$MgCl_2$, 3.75mM-2-mercaptoethanol, 200μg of snake venom (*Ophiophagus hanna*) and 0.1ml of incubation medium in a final volume of 0.2ml. The tubes were incubated at 30°C for 1h, and the incubation was terminated by the addition of 1ml of a slurry of Dowex anion-exchange resin. Other details were as described above.

Protein and DNA measurements. Protein concentration was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. DNA was assayed by the procedure of Burton (1956).

Results

Effect of cyclic nucleotide phosphodiesterase inhibitor on cyclic AMP accumulation, protein kinase activation and progesterone production in response to choriogonadotropin

In the absence of the added 3-isobutyl-1-methylxanthine, choriogonadotropin up to 5ng/ml did not affect the basal cyclic AMP production, whereas 50 and 100ng of hormone/ml increased cyclic AMP accumulation about 5- and 6-fold respectively (Fig. 1*a*). In contrast, in the presence of phosphodiesterase inhibitor, an increase in cyclic AMP production was detectable with choriogonadotropin concentrations as low as 0.65ng/ml, and increased further with increasing concentrations of choriogonadotropin up to 50ng/ml, reaching a maximum at about 100ng/ml. The effect of different concentrations of choriogonadotropin on protein kinase activity is shown in Fig. 1(*b*). Choriogonadotropin (10ng/ml), which maximally stimulated steroidogenesis (Fig. 1*c*), also stimulated protein kinase activity significantly, both in the presence and absence of phosphodiesterase inhibitor. However, protein kinase activity continued to increase with increasing concentrations of hormone and reached a maximum between 250 to 1000ng/ml (results not shown).

Unlike cyclic AMP accumulation, progesterone production was detectable with very low concentrations of choriogonadotropin even in the absence of phosphodiesterase inhibitor (Fig. 1*c*). Progesterone production showed a significant increase with 0.5ng of choriogonadotropin/ml and increased linearly with increasing concentrations of hormone up to 10ng/ml and then reached a plateau. Addition of 3-isobutyl-1-methylxanthine (0.5mM) in the incubation medium itself stimulated progesterone production. Further addition of inhibitor along with increasing concentrations of gonadotropin shifted the dose–response curve, and maximum steroid production was observed with a choriogonadotropin concentration of 2ng/ml. Surprisingly, 3-isobutyl-1-methylxanthine (0.5mM) in the presence of higher concentrations of choriogonadotropin, i.e. 10–100ng/ml, decreased the progesterone response (Fig. 1*c*); the reason for this effect is presently unknown.

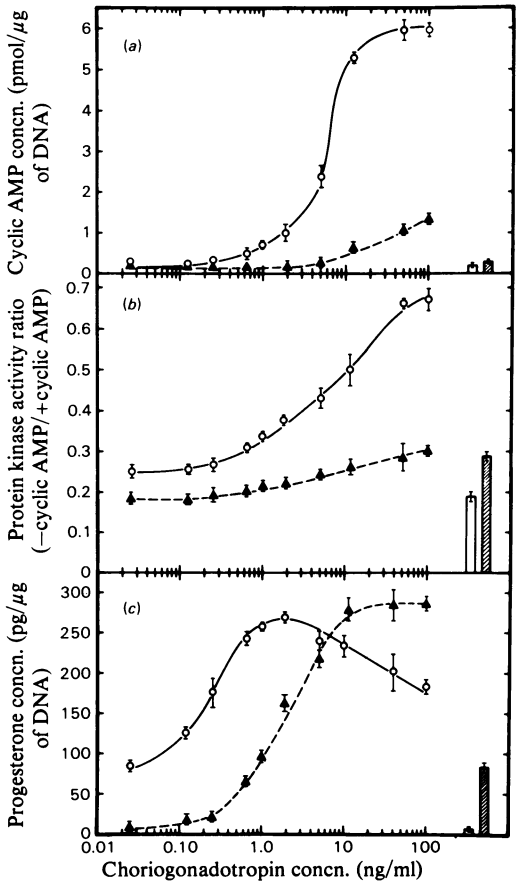

Fig. 1. *Effect of increasing concentrations of choriogonadotropin and phosphodiesterase inhibitor on cyclic AMP accumulation, protein kinase activity and progesterone production in rat ovarian cells*

Ovarian cells (4×10^6 for cyclic AMP and progesterone production, 1.2×10^7 for protein kinase activation) were incubated with increasing concentrations of choriogonadotropin. Where required 0.5mM-3-isobutyl-1-methylxanthine was also added in a final volume of 0.4ml of Medium 109 containing 0.1% bovine serum albumin. Incubations were carried at 37°C for 2h in the presence of O_2/CO_2 (19:1, v/v). After incubation, respective samples were processed for cyclic AMP (*a*), protein kinase activity (*b*) and progesterone concentration (*c*) as described in the Experimental section. The results are means for triplicate samples on duplicate determinations. ▲, Choriogonadotropin alone; ○, choriogonadotropin in the presence of 0.5mM-3-isobutyl-1-methylxanthine; open bars, without any test substance; hatched bar, with 0.5mM-3-isobutyl-1-methylxanthine. Vertical bars through symbols indicate ± S.E.M.

Time- and dose-dependent accumulation of cyclic AMP and progesterone production in response to choriogonadotropin and cyclic nucleotide phosphodiesterase inhibitors

Incubation of ovarian cells for 2min to 2h with 0.4 or 2.5ng of choriogonadotropin/ml in the absence of 3-isobutyl-1-methylxanthine did not elicit an increase in cyclic AMP accumulation over control values. However, with 250ng to 2.5μg of choriogonadotropin/ml without added phosphodiesterase inhibitor, ovarian cells showed increased cyclic AMP concentrations even after 2min of incubation and reached a plateau after 45–60min of incubation. Addition of 3-isobutyl-1-methylxanthine (0.5mM) potentiated the gonadotropin-stimulated cyclic AMP synthesis. Even with concentrations as low as 0.4ng of choriogonadotropin/ml, small but significant stimulation of cyclic AMP was observed when cells were incubated in the presence of phosphodiesterase inhibitor. Incubation of cells with 50ng/ml, 260ng/ml or 2.5μg/ml along with 3-isobutyl-1-methylxanthine (0.5mM) caused an immediate increase in cyclic AMP concentration, which continued to increase with increasing incubation time (Fig. 2).

With maximally stimulating doses of choriogonadotropin, i.e. 50ng/ml, 250ng/ml or 2.5μg/ml, an increase in progesterone production was evident after 20–30min, whereas with 0.4ng/ml a significant increase in progesterone was not observed until after 90min. Addition of 3-isobutyl-1-methylxanthine (0.5mM) itself stimulated steroidogenesis in a time-dependent manner. Similarly, 3-isobutyl-1-methylxanthine (0.5mM) greatly potentiated the action of low doses of choriogonadotropins (0.4 and 2.5ng/ml) on steroidogenesis (Fig. 2). However, with high doses, i.e. 50ng/ml, 250ng/ml or 2.5μg/ml of hormone, addition of 3-isobutyl-1-methylxanthine (0.5mM) inhibited the maximum response at all time points compared with that observed in the presence of hormone alone (Fig. 2). The inhibitory action of 3-isobutyl-1-methylxanthine was dependent on the concentrations of both inhibitor and hormone. With a low concentration of 3-isobutyl-1-methylxanthine (0.5mM) no inhibition in progesterone production was observed, whereas concentrations of 0.25–1mM inhibited the response elicited by higher doses (50ng/ml, 250ng/ml or 2.5μg/ml) of hormone (results not shown). In the absence of either hormone or 3-isobutyl-1-methylxanthine no increase in progesterone production was detectable even after 2h of incubation.

Effect of various concentrations of cyclic nucleotide phosphodiesterase inhibitors on progesterone production and phosphodiesterase activity

Four different compounds known to be inhibitors of cyclic nucleotide phosphodiesterases (Appleman

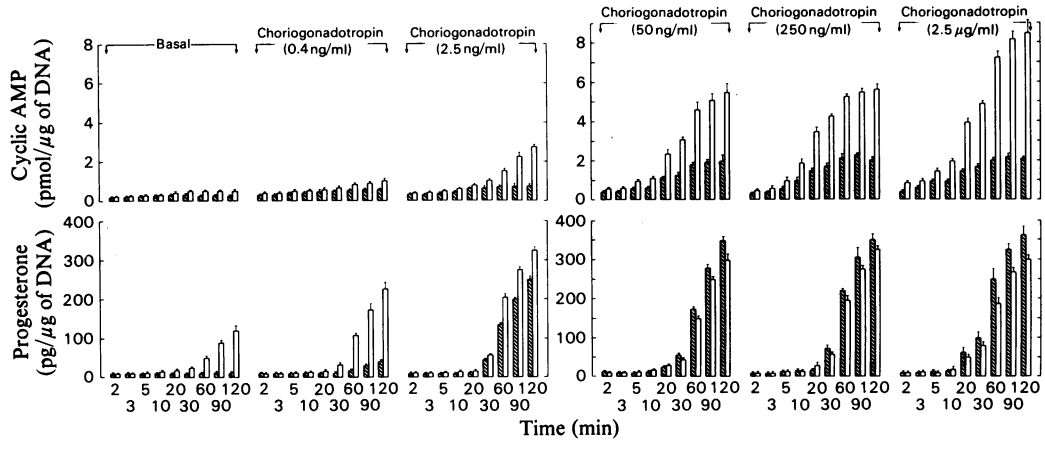

Fig. 2. *Effect of incubation time on the kinetics of cyclic AMP accumulation and progesterone production in rat ovarian cells in response to choriogonadotropin and 3-isobutyl-1-methylxanthine*

Ovarian cells (2.5×10^6) were incubated with different concentrations of choriogonadotropin in the presence or absence of 3-isobutyl-1-methylxanthine (0.5 mM) for indicated time periods in a final volume of 0.4 ml of Medium 109 containing 0.1% bovine serum albumin. Other details were the same as described in the legend to Fig. 1 and also in the Experimental section. Hatched bar, without phosphodiesterase inhibitor; open bar, with 3-isobutyl-1-methylxanthine (0.5 mM). Results are means with S.E.M. indicated by vertical bars.

et al., 1973; Peytreman *et al.*, 1973) were tested for their effects on progesterone accumulation in ovarian cells. Papaverine was active at the lower concentrations, whereas 3-isobutyl-1-methylxanthine was almost equally effective at higher concentrations (Table 1). Theophylline and caffeine also stimulated steroidogenesis at higher concentrations. All these drugs also inhibited both soluble and particulate-associated cyclic AMP phosphodiesterases in a dose-related manner. For the soluble enzyme, 3-isobutyl-1-methylxanthine was most effective, followed by papaverine, and then theophylline (Table 2). In contrast, papaverine was most effective against the particulate-associated phosphodiesterase, followed by 3-isobutyl-1-methylxanthine, and then theophylline. Although the relative magnitudes of the effects on phosphodiesterase and steroid production varied, all the compounds resembled each other in producing both effects in ovarian cells (Tables 1 and 2).

Effect of 8-substituted derivatives of cyclic AMP and other cyclic nucleotides on progesterone production by rat ovarian cells

The addition of dibutyryl cyclic AMP or 8-bromo cyclic AMP to the incubation medium resulted in an increase in progesterone production in a time- and dose-dependent manner. Maximum stimulation was obtained with 1–2 mM concentrations of cyclic nucleotides at 2 h of incubation. This increase was close to that produced by a maximally stimulatory dose of choriogonadotropin (10 ng/ml). Further, when a maximum stimulatory dose (2 mM) of either dibutyryl cyclic AMP or 8-bromo cyclic AMP was added along with a maximum effective dose of choriogonadotropin (10 ng/ml), no additive effect was observed, suggesting both agents act through a similar mechanism (results not shown). In contrast, addition of exogenous cyclic AMP in a concentration range of 10 μM to 1 mM did not affect steroidogenesis, whereas concentrations of 2 mM or higher slightly enhanced progesterone production. Addition of 3-isobutyl-1-methylxanthine (0.25 mM) potentiated the action of cyclic nucleotides when lower concentrations (50 μM or less) were tested. However, as in the case of choriogonadotropin, higher concentrations of inhibitor decreased the progesterone production stimulated by the maximum stimulatory dose of cyclic nucleotides (results not shown).

Effects of some other 8-substituted derivatives of cyclic AMP on steroid production are shown in Table 3. When lower concentrations (0.25 mM) of these nucleotides were tested, 8-hydroxy cyclic AMP was most effective followed by 8-bromo cyclic AMP and 8-isopropylthio cyclic AMP. At 2.5 mM concentration, both 8-hydroxy cyclic AMP and 8-bromo cyclic AMP were equally effective, followed by 8-methylthio cyclic AMP and 8-isopropylthio cyclic AMP. Methylamino cyclic AMP and 8-amino cyclic AMP were least effective. Other cyclic nucleotides, such as 8-bromo cyclic GMP, cyclic XMP,

cyclic IMP, cyclic dAMP, cyclic dTMP, cyclic GMP, cyclic CMP, and 5′-AMP up to 5mM concentrations were without any effect.

Table 1. *Effect of cyclic nucleotide phosphodiesterase inhibitors on progesterone synthesis by rat ovarian cells*
Ovarian cells (2.5×10^6) were incubated with the indicated concentrations of phosphodiesterase inhibitors or choriogonadotropin as described in the legend to Fig. 1. Other details were as stated in the Experimental section. Results are means for triplicate samples on duplicate determinations ± S.E.M.

Addition	Concentration (mM)	Progesterone concn. (pg/μg of DNA)
No addition	0	4.5±0.2
3-Isobutyl-1-methylxanthine	0.05	21±2
	0.10	38±3
	0.25	64±4
	0.50	93±3
	1.0	116±7
	1.5	106±8
Papaverine	0.05	23±3
	0.10	53±2
	0.25	88±4
	0.50	115±9
	0.75	159±5
	1.00	188±11
Theophylline	0.10	6.7±0.8
	0.50	11.6±1.1
	1.00	14.2±1.6
	2.5	33±2.1
	5.0	46±1.0
	10	56±0.9
Caffeine	0.1	5.2±0.6
	0.5	5.3±0.4
	1.0	5.0±0.8
	2.5	9.3±0.70
	5.0	14.5±0.8
	10	22.3±1.3
Choriogonadotropin	10ng/ml	395±15

Uptake and metabolism of exogenous cyclic [^{3}H]AMP

From the above results it is clear that although various 8-substituted cyclic derivatives were effective in stimulating steroidogenesis, cyclic AMP itself could not enhance progesterone production to any great extent. These results could be attributed to the following possible causes. (*a*) The penetration of the exogenous nucleotides into the ovarian cells *in vitro* may be inefficient, and/or (*b*) the added cyclic nucleotide may be destroyed by cyclic AMP phosphodiesterase already present in the ovarian cells. We have checked both these possibilities by following the uptake and metabolism of cyclic AMP. Ovarian cells incubated with 0.1mM-cyclic [^{3}H]AMP showed that the cellular radioactivity was increasing in an essentially linear fashion over a 120min period. The results presented in Fig. 3 show concentration-dependent uptake of cyclic [^{3}H]AMP by ovarian cells. As evident from these results, significant amounts of radioactivity were associated with acid-precipitable fraction, suggesting the possibility that considerable degradation of cyclic [^{3}H]AMP had occurred either intracellularly or extracellularly. We then examined the nature of the radioactivity present in the medium and cells after incubation for 120min with 0.1mM-cyclic [^{3}H]AMP. Again, extensive degradation of cyclic [^{3}H]AMP was observed in the medium, and the major radioactive component co-migrated with adenosine, adenine and inosine, and to a lesser extent with 5′-AMP. In contrast, the radioactivity from the cellular extract was recovered mainly in the form of ATP, ADP, AMP and adenosine, whereas less than 1% of the total radioactivity was recovered as cyclic AMP (Table 4). To determine whether or not the degradation of cyclic AMP to 5′-AMP and adenosine occurred extracellularly, unlabelled 5′-AMP, adenosine and adenine were added to the incubation medium at a concentration of 1mM, along with 0.1mM-cyclic [^{3}H]AMP. Unlabelled adenosine effectively inhibited the uptake

Table 2. *Effect of different phosphodiesterase inhibitors on soluble and particulate-associated cyclic nucleotide phosphodiesterase activities from rat ovarian interstitial cells*
Ovarian cells (5×10^7) were homogenized in sucrose buffer (0.25M-sucrose in 5mM-Tris/HCl, pH7.5) and the homogenate was centrifuged at 400*g* for 10min. The resulting supernatant was further centrifuged at 105000*g* for 90min and the supernatant was saved. The sedimented material was rinsed twice with sucrose buffer and finally resuspended in a volume equivalent to the original homogenate. Both these fractions were then tested for phosphodiesterase activity in the presence of different concentrations (0.1–500μM) of inhibitors. Other details were the same as described in the Experimental section. Results are means for triplicate determinations.

Inhibitor	Cyclic AMP phosphodiesterase ...	ID_{50} (μM^{-1})*	
		Soluble	Particulate
Theophylline		240	270
3-Isobutyl-1-methylxanthine		4.5	9.2
Papaverine		6.4	0.80

* Concentration required for 50% inhibition of cyclic AMP phosphodiesterase activity.

Table 3. *Effect of 8-substituted derivatives of cyclic AMP on progesterone production in rat ovarian cells*
Incubation conditions were the same as described in the legend to Fig. 1, except that the concentrations of various 8-substituted cyclic AMP derivatives or choriogonadotropin were included and 3-isobutyl-1-methylxanthine was omitted from all incubations. Incubation time was 2h. Results are means ± S.E.M.

Addition	[Cyclic nucleotide] (mM)	Progesterone concn. (pg/μg of DNA) ... 0	0.25	2.5
None		7.8±0.8		
8-Methylamino cyclic AMP			10±1.5	125±5
8-Amino cyclic AMP			11.2±0.9	141±10
8-Methylthio cyclic AMP			57±7	406±10
8-Benzylthio cyclic AMP			17.5±3.3	252±10
8-Isopropylthio cyclic AMP			131±6.4	375±12
8-Bromo cyclic AMP			188±10	477±13
8-Hydroxy cyclic AMP			336±25	480±21
8-Azido cyclic AMP			39±10	322±19
8-Dimethylamino cyclic AMP			19±6	290±11
Choriogonadotropin (10ng/ml)		478±34		

of cyclic [^{3}H]AMP, whereas 5'-AMP inhibited to a lesser extent and adenine was ineffective (results not shown). Similarly, addition of papaverine (0.1mM) or 3-isobutyl-1-methylxanthine (0.5mM) inhibited cyclic [^{3}H]AMP uptake (approx. 70%) with a parallel increase in radioactivity associated with cyclic AMP in the medium.

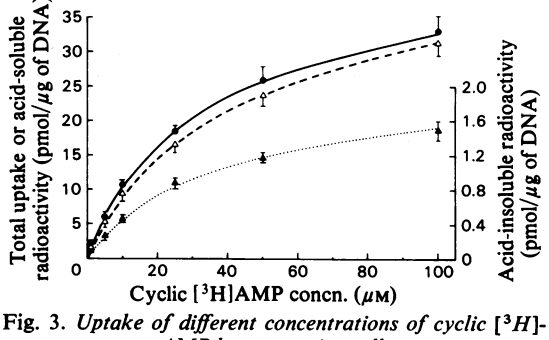

Fig. 3. *Uptake of different concentrations of cyclic [^{3}H]-AMP by rat ovarian cells*
Uptake of cyclic [^{3}H]AMP by ovarian cells was measured as described in the Experimental section, except that the incubation time was 2h. Results are means for triplicate samples with S.E.M. indicated by vertical bars through the symbols. ●, Total uptake; △, acid-soluble radioactivity; ▲, acid-insoluble radioactivity.

These results demonstrate that the majority of cyclic AMP is first hydrolysed by membrane-associated phosphodiesterase to 5'-AMP and then to adenosine by 5'-nucleotidase and the resulting nucleoside is taken up by the cells. Further, whatever intact cyclic AMP is taken up by the cells is also hydrolysed instantaneously. To preclude the possibility that cyclic degradation in the medium resulted from metabolism by enzymes leaking from cells, the cells were preincubated in the absence of cyclic AMP and then washed and transferred to another tube for incubation with cyclic AMP. In another set of experiments the ovarian cells were incubated in the absence of cyclic AMP, the cells were removed and the incubation medium was tested for its ability to metabolize cyclic [^{3}H]AMP. Under these conditions no degradation of cyclic [^{3}H]AMP was observed.

Discussion

Previous studies from this laboratory have shown that at physiological concentrations of gonado-

Table 4. *Recovery of cyclic AMP in the extract prepared from the ovarian cells incubated with cyclic [^{3}H]AMP*
Incubation conditions and other details were identical with those described in the Experimental section. Results are means ± S.E.M.

Cyclic AMP concn. in incubation medium (μM)	Cyclic [^{3}H]AMP* (pmol/μg of DNA) Total uptake	Recovery by paper chromatography	Recovery by column chromatography
100	33.1±0.2	0.21±0.01	0.16±0.04
250	49.1±1.0	0.39±0.03	0.27±0.02

* The intracellular concentrations of cyclic AMP were calculated by assuming the specific activity of intracellular cyclic [^{3}H]AMP was the same as for cyclic [^{3}H]AMP in the incubation medium.

tropin, the stimulation of progesterone production could not be correlated well with the accompanying changes in cyclic AMP concentrations in ovarian cells (Clark & Menon, 1976). The present study is an extension of these previous observations, to evaluate the role of cyclic AMP. We have utilized kinetic studies on cyclic AMP formation and steroidogenesis in response to phosphodiesterase inhibitors and gonadotropin, as well as following the qualitative and quantitative changes in cyclic AMP uptake and degradation and the function of cyclic nucleotide phosphodiesterase in these processes.

The kinetic studies of progesterone production, protein kinase activation and cyclic AMP accumulation in response to choriogonadotropin revealed many marked differences. When the experiments were performed in the absence of phosphodiesterase inhibitor, steroid production was partially dissociated from cyclic AMP accumulation. These observations are in agreement with previous studies reported for Leydig cells, where testosterone production was shown to be stimulated by doses of choriogonadotropin that did not result in detectable increases in cyclic AMP (Catt & Dufau, 1973; Moyle & Ramachandran, 1973; Rommerts *et al.*, 1973; Mendelson *et al.*, 1975; Cooke *et al.*, 1976). However, in the present study a correlation was observed between progesterone production and increase in protein kinase activity ratio, although the latter required higher concentrations for maximum stimulation. Similarly, Cooke *et al.* (1976), by following protein kinase activity, and Dufau *et al.* (1977), by assaying occupied and unoccupied cyclic AMP-binding sites in relation to gonadotropin-stimulated testosterone production in Leydig cells, have also made similar observations. Ling & Marsh (1977), with bovine corpus-luteum slices, have reported that lutropin (10ng/ml), which stimulated progesterone production maximally, also resulted in small but significant stimulation of protein kinase activity. Since activation of protein kinase presumably reflects changes in cyclic AMP concentrations, the failure in the present study to detect changes in cyclic AMP in response to low doses of hormone reflects the possibility that the small increase in cyclic AMP is too difficult to detect by the current methodology or that cyclic AMP is rapidly hydrolysed by very active cyclic nucleotide phosphodiesterase(s). The latter possibility seems to be most likely and further strengthened by the observation that when experiments were performed in the presence of 3-isobutyl-1-methylxanthine a better correlation between cyclic AMP, protein kinase and progesterone responses was observed. As evident from Figs. 1 and 2, low concentrations of choriogonadotropin (0.4ng/ml), which in the absence of phosphodiesterase inhibitor showed a slight increase in steroidogenesis, produced almost the maximum steroidogenic response when tested in the presence of 3-isobutyl-1-methylxanthine. Similarly, we could not achieve the maximum cyclic AMP response in the absence of added phosphodiesterase inhibitors even by employing supermaximal concentrations of hormones. Addition of 3-isobutyl-1-methylxanthine greatly potentiated the action of gonadotropin. These observations clearly indicate that a cyclic nucleotide phosphodiesterase(s) is very active in rat ovarian cells, hydrolysing the small amounts of cyclic AMP produced in response to low doses of tropic hormones. The presence of a high activity of cyclic AMP phosphodiesterase in the ovarian cells was also reflected by an increase in progesterone production when inhibitors of phosphodiesterase, such as papaverine, or 3-isobutyl-1-methylxanthine, were added.

Additional support for the above conclusions was obtained from the observation that various 8-substituted cyclic AMP derivatives enhanced steroidogenesis. The effects of choriogonadotropin on the stimulation of steroidogenesis in ovarian cells can also be duplicated by 8-bromo cyclic AMP and other 8-substituted derivatives of cyclic AMP, but to a lesser extent or not at all by cyclic AMP. Further, various 8-substituted derivatives of cyclic AMP, for example 8-hydroxy cyclic AMP, 8-bromo cyclic AMP and 8-isopropylthio cyclic AMP, which are hydrolysed at rates of less than 0.1% that of cyclic AMP (Muneyama *et al.*, 1971; Miller *et al.*, 1973, 1975; Khwaja *et al.*, 1975), showed considerable steroidogenic activity even at lower concentrations. Our results also indicate that no cyclic [^{3}H]AMP was detected in ovarian cells after incubation with labelled nucleotides for 2h. Since extracellular cyclic nucleotide phosphodiesterase activity was detected in the ovarian cells, some of the extracellular cyclic AMP was converted into 5′-AMP, adenosine and inosine before entry into the cells. This conclusion is further strengthened by the observation that AMP, adenosine and inosine elicited inhibition of ^{3}H uptake from the medium, whereas phosphodiesterase inhibitors papaverine and 3-isobutyl-1-methylxanthine decreased the amount of metabolic products of cyclic [^{3}H]AMP in the incubation medium. The extracellular metabolism of cyclic AMP described in the present paper may explain our observation that cyclic AMP added *in vitro* to incubation medium had a very limited effect on steroidogenesis in the ovarian cells. Further, the rapid breakdown of cyclic AMP externally and within the ovarian cells may explain the observation that no increment in cyclic AMP was detected when the cells were stimulated with low or physiological concentrations of gonadotropin.

The inhibitory effect of higher doses of 3-isobutyl-1-methylxanthine on steroidogenesis, especially at higher concentrations of choriogonadotropin or

cyclic nucleotides, could be due to secondary effects in addition to its well known action on cyclic AMP phosphodiesterases (Appleman *et al.*, 1973). Methylxanthines have been shown to inhibit steroidogenesis in the adrenal (Halkerston *et al.*, 1966; Kitabchi *et al.*, 1971) and testicular systems (Mendelson *et al.*, 1975; Cooke *et al.*, 1976; Williams *et al.*, 1976). Several possibilities may be considered for this inhibitory effect. Since in rat ovarian cells progesterone synthesis is dependent on macromolecular synthesis (Kawano *et al.*, 1975) it is possible that methylxanthines inhibit progesterone synthesis by inhibiting protein synthesis, especially when cyclic nucleotide phosphodiesterase inhibitors have been shown to inhibit protein synthesis in many systems (Halkerston *et al.*, 1966; Leier & Jungmann, 1971; Stellwagen, 1974). The possibility that the high concentration of cyclic AMP generated in the presence of 3-isobutyl-1-methylxanthine could lead to the desensitization of cellular responses should also be considered. In addition the inhibition of uptake of precursors (Faust *et al.*, 1977) required for steroidogenesis by methylxanthines cannot be ruled out at the present time.

The experiments described in the present paper provide further evidence in favour of the intermediary role of cyclic AMP in steroidogenesis in response to low doses of hormone. We have presented evidence for the rapid degradation of cyclic AMP and the appearance of degradation products such as ATP, ADP, 5'-AMP and adenosine in the cellular extracts and incubation medium. These observations as well as those on cyclic [^{3}H]AMP-uptake studies, the kinetics of cyclic AMP formation in response to choriogonadotropin and the possible intracellular compartmentation of cyclic AMP reported previously (Menon & Azhar, 1978) point out the complicating problems associated with the demonstration of the intermediary role of cyclic AMP in steroidogenesis in the ovary.

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References

Appleman, M. M., Thompson, W. J. & Russell, T. R. (1973) *Adv. Cyclic Nucleotide Res.* **3**, 65–98

Azhar, S. & Menon, K. M. J. (1976) *J. Biol. Chem.* **251**, 7398–7404

Azhar, S. & Menon, K. M. J. (1977) *Eur. J. Biochem.* **73**, 73–82

Azhar, S. & Menon, K. M. J. (1978) *Biochem. Biophys. Res. Commun.* **81**, 205–211

Azhar, S., Hajra, A. K. & Menon, K. M. J. (1976) *J. Biol. Chem.* **251**, 7405–7412

Beale, R. & Sayers, G. (1972) *Arch. Biochem. Biophys.* **148**, 70–76

Bellisario, R. & Bahl, O. P. (1975) *J. Biol. Chem.* **251**, 3837–3844

Boudreau, R. J. & Drummond, G. I. (1975) *Anal. Biochem.* **63**, 388–399

Boumendel-Podevin, E. F. & Podevin, R. A. (1977) *J. Biol. Chem.* **252**, 6675–6681

Burton, K. (1956) *Biochem. J.* **62**, 315–323

Catt, K. J. & Dufau, M. L. (1973) *Nature (London) New Biol.* **244**, 219–221

Channing, C. P. & Tsafriri, A. (1977) *Metabolism* **26**, 413–468

Clark, M. R. & Menon, K. M. J. (1976) *Biochim. Biophys. Acta* **444**, 23–32

Clark, M. R., Azhar, S. & Menon, K. M. J. (1976) *Biochem. J.* **158**, 175–182

Conti, M., Harwood, J. P., Dufau, M. L. & Catt, K. J. (1977) *J. Biol. Chem.* **252**, 8869–8874

Cooke, B. A., Lindh, M. L. & Janszen, F. H. A. (1976) *Biochem. J.* **160**, 439–446

Corbin, J. D. & Reimann, E. M. (1974) *Methods Enzymol.* **38**, 287–290

Corbin, J. D., Soderling, T. R. & Park, C. R. (1973) *J. Biol. Chem.* **248**, 1813–1821

Dorrington, J. H. & Kilpatrick, R. (1967) *Biochem. J.* **104**, 725–730

Dufau, M. L., Tsuruhara, T. & Catt, K. J. (1972) *Biochim. Biophys. Acta* **278**, 281–289

Dufau, M. L., Tsuruhara, T., Horner, K. A., Podesta, E. & Catt, K. J. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 3419–3423

Faust, J. R., Goldstein, J. L. & Brown, M. S. (1977) *J. Biol. Chem.* **252**, 4861–4871

Gilman, A. G. (1970) *Proc. Natl. Acad. Sci. U.S.A.* **67**, 305–312

Glynn, I. M. & Chappell, J. B. (1964) *Biochem. J.* **90**, 147–149

Gospodarowicz, D. (1973) *J. Biol. Chem.* **248**, 5042–5049

Halkerston, I. D. K., Feinstein, M. & Hechter, O. (1966) *Proc. Soc. Exp. Biol. Med.* **122**, 896–900

Haour, F. & Saxena, B. B. (1974) *J. Biol. Chem.* **249**, 2195–2205

Hermier, C., Combarnous, Y. & Jutisz, M. (1971) *Biochim. Biophys. Acta* **244**, 625–633

Hudson, A. M. & McMartin, C. (1975) *Biochem. J.* **148**, 539–544

Kammerman, S., Canfield, R. E., Kolena, J. & Channing, C. P. (1972) *Endocrinology* **91**, 65–74

Kawano, A., Gunaga, K. P. & Menon, K. M. J. (1975) *Biochim. Biophys. Acta* **385**, 88–100

Khwaja, T. A., Boswell, K. H., Robins, R. K. & Miller, J. P. (1975) *Biochemistry* **14**, 4238–4244

Kitabchi, A. E., Wilson, D. B. & Sharma, R. K. (1971) *Biochem. Biophys. Res. Commun.* **44**, 898–904

Koch, Y., Zor, U., Chobsieng, P., Lamprecht, S. A., Pomerantz, S. & Lindner, H. R. (1974) *J. Endocrinol.* **61**, 179–191

Lee, C. Y. & Ryan, R. J. (1973) *Biochemistry* **12**, 4609–4615

Leier, D. J. & Jungmann, R. A. (1971) *Biochim. Biophys. Acta* **239**, 320–328

Ling, W. Y. & Marsh, J. M. (1977) *Endocrinology* **100**, 1571–1578

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Marsh, J. M. (1975) *Adv. Cyclic Nucleotide Res.* **6**, 136–199

Marsh, J. M. (1976) *Biol. Reprod.* **14**, 30–53

Marsh, J. M., Butcher, R. W. & Sutherland, E. W. (1966) *J. Biol. Chem.* **241**, 5436–5440

Mendelson, C., Dufau, M. L. & Catt, K. J. (1975) *J. Biol. Chem.* **250**, 8818–8823

Menon, K. M. J. (1973) *J. Biol. Chem.* **248**, 494–501

Menon, K. M. J. & Azhar, S. (1978) *Biochem. J.* **172**, 433–442

Menon, K. M. J. & Kiburz, J. (1974) *Biochem. Biophys. Res. Commun.* **56**, 363–371

Miller, J. P., Boswell, K. H., Muneyama, K., Simon, L. N., Robins, R. K. & Shuman, D. A. (1973) *Biochemistry* **12**, 5310–5319

Miller, J. P., Beck, A. H., Simon, L. N. & Meyer, R. B. (1975) *J. Biol. Chem.* **250**, 426–431

Mills, T. M. (1975) *Endocrinology* **96**, 440–445

Moyle, W. R. & Ramachandran, J. (1973) *Endocrinology* **93**, 127–134

Muneyama, K., Bauer, R. J., Shuman, D. A., Robins, R. K. & Simon, L. M. (1971) *Biochemistry* **10**, 2390–2396

Ortmann, R. & Perkins, J. P. (1977) *J. Biol. Chem.* **252**, 6018–6025

Peytreman, A., Nicholson, W. E., Liddle, G. W., Hardman, J. G. & Sutherland, E. W. (1973) *Endocrinology* **92**, 2864–2872

Rao, C. V. (1974) *J. Biol. Chem.* **249**, 2864–2872

Rapkin, E. (1964) *Int. J. Appl. Radiat. Isot.* **15**, 69–74

Reimann, E. M., Walsh, D. A. & Krebs, E. G. (1971) *J. Biol. Chem.* **246**, 1986–1995

Rommerts, F. F. G., Cooke, B. A., van der Kemp, J. W. C. M. & van der Molen, H. J. (1973) *FEBS Lett.* **33**, 114–118

Saloman, Y., Londs, C. & Rodbell, M. (1974) *Anal. Biochem.* **58**, 541–548

Stansfield, D. A., Franks, D. J., Wilkinson, G. H. & Horne, J. R. (1972) *J. Steroid Biochem.* **3**, 643–653

Stellwagen, R. H. (1974) *Biochim. Biophys. Acta* **338**, 428–439

Thompson, W. J. & Appleman, M. M. (1971) *Biochemistry* **10**, 311–316

Williams, C. D., Horner, A. D. & Catt, K. J. (1976) *Endocrine Res. Commun.* **3**, 343–353

Zeleznik, A. J., Keyes, P. L., Menon, K. M. J., Midgley, A. R. & Reichert, L. E. (1977) *Am. J. Physiol.* **233**, E229–E239