Selective Activation of Rabbit Ovarian Protein Kinase Isozymes in Rabbit Ovarian Follicles and Corpora Lutea*

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The magnitude of activation of the type I and type II forms of cAMP-dependent protein kinase was investigated in estrous follicles and corpora lutea (CL) obtained from ovaries of control rabbits and rabbits injected acutely with human chorionic gonadotropin (hCG). To this end, a chromatographic technique which permitted quantitative evaluation of the in vivo activation state of the two forms of cAMP-dependent protein kinase was developed and verified. Results revealed that in follicles obtained from ovaries of untreated estrous rabbits, 15% of the soluble cAMP-dependent protein kinase, all of which exists as the type II isozyme, is activated. Intravenous administration of a single bolus of hCG promoted a concentration-dependent activation (in 10 min) of this protein kinase isozyme. In CL obtained from ovaries of control, 4-day pseudopregnant rabbits, 32% of the total soluble cAMP-dependent protein kinase exists as the type I form and 68% exists as the type II form. Both types of protein kinase are approximately 10% dissociated in CL from ovaries of untreated rabbits. Upon intravenous administration of hCG, only the type I form of cAMP-dependent protein kinase is further activated (in 10 min). Dissociation of this protein kinase is dependent upon the time and concentration of hCG. Preferential activation of the type I form of cAMP-dependent protein kinase in CL is also demonstrable in in vitro studies using exogenous cAMP. These data suggest that the physiological state of the two forms of cAMP-dependent protein kinase while in estrous rabbit follicles is the type II isozyme of cAMP-dependent protein kinase while in CL of 4-day pseudopregnant rabbits, it is the type I enzyme form.

Many of the effects induced in ovarian tissues by LH7 are thought to be mediated by cAMP (1-8). Indeed, ovarian follicles and CL exhibit an adenyl cyclase system which is highly responsive to LH stimulation (7, 9-14) and accumulate cAMP upon LH stimulation (15-25). As cAMP has been shown to affect cellular functions only through cAMP-dependent protein kinases, it is presumed that the gonadotropin-induced events in ovarian tissues mediated by cAMP occur via cAMP-dependent protein kinases. We have previously characterized, by a number of parameters, the types of cAMP-dependent protein kinase present in the cytosolic fractions of various rabbit ovarian tissues and have found that estrous rabbit follicles contain exclusively the type II form of cAMP-dependent protein kinase while CL of hCG-induced pseudopregnancy and pregnancy contain both type I and II forms of cAMP-dependent protein kinase. Indeed, hCG administration promotes a partial activation of total soluble protein kinases in CL, based upon an increase in the protein kinase activity ratio (measured in the absence versus the presence of exogenous cAMP in the assay), it was not possible to evaluate whether both or only one of the protein kinase enzymes was being activated.

The purpose of the current experiments was to determine which enzyme form of soluble cAMP-dependent protein kinase is activated in ovarian follicles and CL by the in vivo administration of hCG. Results reveal that acute hCG administration promotes a concentration-dependent activation of the soluble type II enzyme of cAMP-dependent protein kinase in estrous rabbit follicles and of the type I isoenzyme in CL of 4-day pseudopregnant rabbits. The type II form of cAMP-dependent protein kinase in CL is activated only in response to in vitro incubation with high levels of exogenous cAMP.

EXPERIMENTAL PROCEDURES

Animals—The care and maintenance of rabbits has been previously described (28). Follicles (2 mm in diameter) were obtained from estrous rabbits which were neither pseudopregnant nor pregnant. CL were obtained from the ovaries of 4-day pseudopregnant rabbits. The day after the induction of ovulation by exogenous hCG (100 IU, iv) was counted as day 1 of pseudopregnancy. All injections were iv in 0.4-0.5 ml of saline into a peripheral ear vein.

Tissue Preparation and DEAE-cellulose Chromatography—Following animal killing by cervical dislocation, ovaries were immediately removed and placed in iced 10 mM potassium phosphate buffer, pH 7.0, and follicles or CL were immediately dissected free of adhering tissue. Dissected tissues were homogenized in 0.4 ml of ice-cold 10 mM potassium phosphate buffer, pH 6.5, containing 0.5 mM EDTA, 0.5 mM MIX, and 0.1% Triton X-100. The homogenate was centrifuged at 10,000 × g for approximately 15 s, and 0.2 ml of the supernatant was applied and immediately loaded onto a column (1 × 1 cm) of DEAE-cellulose (Whatman; stored in 2 M NaCl at 4 °C) equilibrated within 24 h of use with 10 mM potassium phosphate buffer containing 0.5 mM EDTA and 0.5 mM MIX, pH 6.5. Tissue preparations and column loading were performed within 6–7 min of animal killing in order to reduce reassociation of any dissociated type II cAMP-de-
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The column was washed with equilibration buffer until the eluate was free of material that adsorbed at 280 nm. Protein kinases were eluted with a linear gradient between solutions of 10-400 mM potassium phosphate, pH 6.5, containing 0.5 mM EDTA and 0.5 mM MIX. Mixtures were collected with a Gilson microfractionator (Model FC-060 M) at a flow rate of 0.225 ml/min. Portions of 10-100 p l aliquots were assayed for CAMP-binding activities. Animal killing, column chromatography, and protein kinase and CAMP-binding assays were performed on the same day. Recovery of type I and type II protein kinases from DEAE-cellulose was consistently 80-90%. Extraction of the charcoal did not alter the recovery of the protein kinases with chromatography.

All materials and methods for assay of protein kinase and CAMP-binding activities, for protein determinations, and for preparation of the heat-stable protein kinase inhibitor were as described previously (28).

Conductivity determinations were made on even-numbered fractions eluted from all columns using a Myron L DS meter (Model EP) (Myron L Co., Encinitas, CA).

Final concentrations of all solutions are indicated in the text.

Photography, Gel Electrophoresis, and Autoradiography—[32P]-Azidoadenosine 3′:5′-monophosphate ([32P]-AMP, 90 Ci/mM) was obtained from ICN Chemical and Radiosotope Division, Irvine, CA or from Schwarz/Mann, Spring Valley, NY. Photofluorographic labeling of 40-ml aliquots of the column fractions was done in a final volume of 50 ml containing 1 mM MgCl2, 0.1 mM ATP, 5-7 × 10-7 M [32P]-AMP, in the absence and presence of 0.1 mM cAMP. The reaction mixture was preincubated in darkness for 60 min at room temperature and then exposed to UV minerlght (Ultraviolet Products, Inc., San Gabriel, CA, Model R-52) for 10 min at a distance of 10 cm. The preincubation was originally performed at 4 °C; however, at room temperature, labeling of the regulatory subunit of the type II form of CAMP-dependent protein kinase was improved without an increase of lower molecular weight bands. Reactions were stopped by the addition of 20 ml of stop solution containing 30% glycerol, 3% SDS, 150 mM Tris-HCl, pH 8.7, 0.04% bromophenol blue, and 10% β-mercaptoethanol, and tubes were placed in a boiling water bath for 3 min. Proteins in the labeled preparations were separated by SDS-polyacrylamide gel electrophoresis as described by Rudolph and Krueger (32) using an SE 500 series slab gel electrophoresis unit (Hoeffer Scientific Instruments, San Francisco, CA). After electrophoresis, gels were stained, destained, dried, and placed with Kodak X-Omat XRP-5 x-ray film for 12 h to 7 days. Molecular weights were estimated on each gel from the migration rates of chymotrypsinogen, bovine serum albumin, ovalbumin, catalase, and myoglobin.

RESULTS

Rationale for and Verification of Homogenization and Chromatography Conditions—In order to demonstrate the activational status of each of the two forms of CAMP-dependent protein kinase in CL, specific procedures were required. Firstly, tissue homogenization had to be performed in a low ionic buffer since the type I form of protein kinase in CL and other tissues rapidly dissociated in high ionic strength buffer (27, 29). However, use of a low ionic strength buffer required that tissue manipulation time be strictly limited since the type II form of protein kinase rapidly reassociated under these conditions (27, 29-31). Secondly, chromatography procedures were needed which not only would separate the two protein kinase holoenzymes but would also separate the dissociated regulatory and catalytic subunits of protein kinases proportionate to the extent of activation of each of the forms of CAMP-dependent protein kinase.

Separation of the soluble luteal protein kinase activity into the type I and type II holoenzyme forms of CAMP-dependent protein kinase was achieved using DEAE-cellulose, eluting the protein kinases with a linear gradient of potassium phosphate, pH 6.5 (not shown, Ref. 28). Apparent holoenzyme concentration of both forms of protein kinase was demonstrated by the co-elution of catalytic protein kinase activity (measured in the presence of exogenous cAMP and [3H]AMP-binding activity. However, this chromatographic procedure did not result in the separate elution of dissociated regulatory and catalytic subunits of the protein kinase from the apparent holoenzymes upon in vitro dissociation of the kinases with cAMP (not shown). (Protein kinases in the luteal supernatant were dissociated by incubating the supernatant with 500 μM CAMP for 30 min at 4 °C.)

A number of additions were made to the basic potassium phosphate buffer (pH 6.5) in an attempt to achieve chromatographic separation of the dissociated regulatory and catalytic subunits upon in vitro activation from the holoenzymes in control tissues. The inclusion of 0.5 mM EDTA and 0.5 mM MIX in the potassium phosphate homogenization and column elution buffers resulted in differential binding of the apparent holoenzymes and dissociated catalytic and regulatory subunits in CL (to be described later in the text).

Differential binding to DEAE-cellulose of the apparent protein kinase holoenzymes and the dissociated catalytic and regulatory subunits was verified with the following protein kinase "standards": 1) a crude rat heart supernatant as the source of type I holoenzyme of CAMP-dependent protein kinase (30) and 2) partially purified protein kinase from beef heart (Sigma Chemical Co., St. Louis, MO) as the source of the type II CAMP-dependent protein kinase (30) (Fig. 1). 1) The soluble type I form of protein kinase in control rat heart eluted as an apparent holoenzyme, with catalytic protein kinase and [3H]AMP-binding activities exactly co-eluting as a single peak (Fig. 1A). Upon activation of this type I form of protein kinase by preincubation of rat heart supernatant with 0.5 μM CAMP (30 min, 4 °C) and subsequent elution from DEAE-cellulose, [3H]AMP-binding activity now eluted separately from catalytic protein kinase activity (Fig. 1B). Conductivity measurements of the column eluates revealed that the catalytic protein kinase activity of the activated enzyme (Fig. 1B) eluted in the same position as the intact holoenzyme of control rat hearts (Fig. 1A) while the [3H]AMP-binding activity of the active enzyme was used (Fig. 1A) to elute at a concentration of 0.5 μM CAMP. 2) Control beef heart protein kinase eluted as a single peak of catalytic protein kinase and [3H]AMP-binding activities (Fig. 1C). Partial activation of this type II protein kinase, by preincubation of the enzyme with 5 μM CAMP (30 min, 4 °C), and subsequent elution from DEAE-cellulose resulted in the separate elution of the dissociated protein kinase catalytic activity from the [3H]AMP-binding activities and the nonactivated portion of the type II holoenzyme (Fig. 1D). Conductivity measurements revealed that the dissociated form of catalytic protein kinase activity derived from beef heart type II protein kinase (Fig. 1D) now eluted coincidentally with both the type I protein kinase holoenzyme (Fig. 1A) and with the protein kinase catalytic activity derived from the type I protein kinase holoenzyme (Fig. 1B), while [3H]AMP-binding activity from the activated beef heart protein kinase eluted at its original salt concentration (Fig. 1, compare C and D). Catalytic protein kinase (Sigma Chemical Co., St. Louis, MO) eluted as a single peak of activity coincidently with the type I protein kinase holoenzyme (not shown). No catalytic or [3H]AMP-binding activities eluted in the column flow-through and wash volumes when fresh enzyme preparations were used.

X-100, especially from the mitochondrial and microsomal fractions, as well as the truly soluble forms of protein kinases.
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Identification of the cAMP-binding activities shown in Fig. 1 as the protein kinase regulatory subunits R-I and R-II, respectively, was confirmed by the electrophoretic separation of the proteins binding 8-N3-[32P]cAMP (Fig. 2). Thus, when R-I is dissociated from the catalytic kinase subunit, it binds more tightly to a DEAE-cellulose column than when it is associated with the protein kinase catalytic subunit. The binding of R-II to DEAE-cellulose, however, is not affected by the catalytic protein kinase subunit.

Fig. 1. Differential binding properties on DEAE-cellulose of protein kinase catalytic and cAMP-binding activities derived from rat and beef hearts. The rat heart preparation (A and B) was a 10,000 × g (15 s) supernatant fraction prepared from fresh rat heart homogenized in the presence of 10 mM potassium phosphate, 0.5 mM EDTA, 0.5 mM MIX, and 0.1% Triton X-100, pH 6.5, as described under "Experimental Procedures." A, control rat heart preparation applied to the column within 7 min of animal killing. B, rat heart supernatant was incubated at 4 °C for 30 min with 0.5 μM cAMP and then applied to a separate column. The beef heart preparation (C and D) was obtained from Sigma Chemical Co., St. Louis, MO. C, lyophilized beef heart protein kinase was reconstituted in 10 mM potassium phosphate buffer, 0.5 mM MIX, and 0.5 mM EDTA, pH 6.5, and immediately applied to a column. D, beef heart enzyme was incubated at 4 °C for 30 min with 5 μM cAMP and then applied to a column. Enzyme activities were eluted with a linear potassium phosphate gradient containing 0.5 mM MIX and 0.5 mM EDTA, pH 6.5, as indicated in D. Aliquots of odd-numbered fractions were assayed for catalytic protein kinase activity in the presence of 0.45 μM cAMP (○) and for cAMP-binding activity (◇) as described under "Experimental Procedures." Representative elution profiles from 4-7 experiments are shown. For A and B, 3.0 and 2.8 mg of protein were applied to each column; for C and D, approximately 200 μg of protein were applied to each column.

Fig. 2. Autoradiographs showing 8-N3-[32P]cAMP incorporation into cAMP-binding proteins obtained upon DEAE-cellulose chromatography of rat heart and beef heart. Tissue manipulations and chromatographic procedures are described in the legend to Fig. 1. Aliquots (40 μl) of column fractions were incubated with 8-N3-[32P]cAMP in the absence (−) or presence (+) of 0.1 mM cAMP, irradiated, and subjected to SDS-polyacrylamide gel electrophoresis, as described under "Experimental Procedures." Molecular weights (MW × 10^3) were determined by the relative migration rates of protein standards, as described under "Experimental Procedures." A, DEAE-cellulose fractions obtained from control rat heart tissue. Columns 1 and 2, fraction 11; columns 3 and 4, fraction 23. B, DEAE-cellulose fractions obtained from rat heart supernatant incubated with 0.5 μM cAMP. Columns 1 and 2, fraction 15; columns 3 and 4, fraction 21. C, DEAE-cellulose fractions obtained from control beef heart extract. Columns 1 and 2, fraction 15; columns 3 and 4, fraction 29. D, DEAE-cellulose fractions obtained from beef heart extract incubated with 5 μM cAMP. Columns 1 and 2, fraction 15; columns 3 and 4, fraction 29.

Evidence that the catalytic protein kinase activity (derived from incubation of either beef heart or rat heart supernatants with cAMP) which eluted with less than 0.05 M salt (see Fig. 1, B and D) consisted of free catalytic subunits of cAMP-dependent protein kinases was based upon the absence of coincident cAMP-binding activity (Fig. 1, B and D; Fig. 2, B and D) as well as upon the sensitivity of this protein kinase activity to inhibition by the heat-stable protein kinase inhibitor (>90% inhibition by saturating concentrations of the inhibitor).

These chromatographic conditions thus permitted the separate elution of the two holoenzymes of cAMP-dependent protein kinase, each detected as co-eluting catalytic protein kinase and [3H]cAMP-binding activities, as well as the elution of the dissociated catalytic and regulatory subunits, detected as catalytic protein kinase activity in the absence of [3H]cAMP-binding activity and as [3H]cAMP-binding activity in the absence of catalytic protein kinase activity, respectively. Equivalent elution profiles were obtained for both type I and II protein kinase holoenzymes and dissociated subunits when either 0.7 or 1.0 mM EDTA or 0.5 mM EGTA was substituted.
for 0.5 mM EDTA in the homogenization and column elution buffers (data not shown).

**Activation of Soluble cAMP-dependent Protein Kinase in Rabbit CL**—Previous experiments, in which CL were homogenized in a medium containing 0 or 0.5 M NaCl and subsequent protein kinase activity ratios were determined within 30 min of animal killing) in 10,000 × g (30 s) supernatant fractions, revealed that the total soluble protein kinase in CL from ovaries of untreated pseudopregnant rabbits was already 60% activated and that the iv injection of 100 IU of hCG resulted in a maximal 80-86% activation of the soluble luteal protein kinases and, in fact, provide no information about the state of activation of the individual luteal CAMP-dependent protein kinases. The purpose of the current experiments was to determine the degree of activation of each of the two soluble cAMP-dependent kinases in CL of control and of hCG-treated rabbits using the nondissociating homogenization and chromatography conditions (described in the previous section).

The location of the type I and II forms of protein kinases upon DEAE-cellulose chromatography of a luteal supernatant was verified by assays for catalytic protein kinase activity and by evaluating the electrophoretic mobility of proteins specifically labeled with 8-N3-[32P]cAMP. Protein kinase activity derived from CL of 4-day pseudopregnant rabbits eluted as two distinct peaks of protein kinase activity (Fig. 3A and B). Studies with the photoaffinity probe showed that cAMP-binding activity associated with peak 1 migrated as R-I and that associated with peak 2 migrated as R-II (Fig. 4A). When the luteal kinase isozymes were dissociated in vitro, by incubating the luteal supernatant with 500 μm CAMP (30 min, 4°C), and subjected to DEAE-cellulose chromatography, the binding patterns of the dissociated catalytic, R-I, and R-II subunits were equivalent to those of the dissociated rat heart and beef heart standards, respectively (Fig. 3C and Fig. 4B).

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**Fig. 3.** DEAE-cellulose chromatography of the soluble protein kinase activity in CL dissected from ovaries of 4-day pseudopregnant rabbits. Dissected CL were homogenized and supernatants were obtained from homogenates as described in the legend to Fig. 1. In all panels, CL were obtained from untreated pseudopregnant rabbits. A, luteal supernatant was applied to a column within 7 min of animal killing. B, luteal supernatant was incubated at 4°C for 2 h (2 h control) and then applied to a separate column. C, luteal supernatant was incubated at 4°C for 30 min with 500 μm CAMP and then applied to a separate column. Column chromatography and assays for catalytic protein kinase activity (●) and cAMP-binding activity (○) are described in the legend to Fig. 1 and under “Experimental Procedures.” Representative elution profiles from 3 experiments are shown. CL derived from 1 or 2 rabbits were used for each experiment. For A, B, and C, 3.6, 1.5, and 2.0 mg of protein were applied to the column, respectively.

**Fig. 4.** Autoradiographs showing 8-N3-[32P]cAMP incorporation into cAMP-binding proteins obtained from DEAE-cellulose chromatography of rabbit CL. Tissue manipulations and chromatographic procedures are described in the legend of Fig. 3. Aliquots (40 μl) of column fractions were incubated with 8-N3-[32P]cAMP in the absence (−) or presence (+) of 0.1 mm CAMP, irradiated, and subjected to SDS-polyacrylamide gel electrophoresis, as described under “Experimental Procedures.” A, DEAE-cellulose fractions obtained from CL of 4-day pseudopregnant rabbits. Columns 1 and 2, fraction 17; columns 3 and 4, fraction 33. B, DEAE-cellulose fractions obtained from a CL supernatant which was incubated (4°C, 30 min) with 500 μm CAMP. Columns 1 and 2, fraction 13; columns 3 and 4, fraction 23, columns 5 and 6, fraction 37.
Column fractions containing \(^3\text{H}\)cAMP-binding activities corresponding to dissociated R-I and R-II were consistently devoid of associated protein kinase catalytic activity. Thus, dissociated catalytic subunits from both isoenzyme forms eluted first, followed by R-I and then by R-II.

The distribution of the type I and II forms of cAMP-dependent protein kinase in CL of 4-day pseudopregnant rabbits was evaluated by DEAE-cellulose chromatography of luteal supernatants which had been preincubated (4°C) for 1 h (Fig. 3B). The purpose of the 2-h incubation was to permit reassociation of any of the type II cAMP-dependent protein kinase which may have been dissociated in vivo. Based upon the area distributions (proportional weights of peak paper areas for each column profile) of \(^3\text{H}\)cAMP-binding and catalytic protein kinase activities in 3 experiments, 32% of the total soluble cAMP-stimulated protein kinase existed as the type I holoenzyme form and 68% existed as the type II holoenzyme form of cAMP-dependent protein kinase in CL of 4-day pseudopregnant rabbits. For each experiment, the proportional distribution of the catalytic protein kinase and \(^3\text{H}\)cAMP-binding activity peaks was determined from the proportional weights of the peak paper areas. The degree of activation of the type I and II forms of cAMP-dependent protein kinase was then determined by the deviation of these proportional distributions of catalytic protein kinase and cAMP-binding activity peaks for each experiment from those of the 2-h control luteal supernatant values. Thus, activation of the type I isozyme was based upon the proportional increase in cAMP-binding activity at elution locations other than the type I holoenzyme location and activation of the type II isozyme was based upon the proportional increases in catalytic protein kinase activity at the elution location of the type I holoenzyme.

The degree of activation of type I and type II protein kinases was determined in supernatants of CL of 4-day pseudopregnant rabbits which were not acutely treated, in supernatants of CL of 4-day pseudopregnant rabbits which were acutely injected with various concentrations of hCG, and in luteal supernatants incubated with cAMP. In CL subjected to tonic gonadotropin secretion (i.e., CL from untreated, pseudopregnant rabbits), both the type I and type II forms of cAMP-dependent protein kinase were activated approximately 10% (Fig. 5). When a single iv bolus of hCG was injected into 4-day pseudopregnant rabbits, only the type I form of cAMP-dependent protein kinase was activated (Fig. 5). (Rabbits were killed 10 min after hCG injection and tissue extract was applied to the column within 7 min of animal killing.) Additionally, the type I form of cAMP-dependent protein kinase was activated in a concentration-dependent manner, with increasing concentrations of hCG promoting increased activation of the type I protein kinase (Fig. 5). The time course of protein kinase activation, in response to 25 IU of hCG/kg of body weight, revealed that maximal activation of the type I form of protein kinase occurred within 5 min of the iv hCG injection and that this enzyme remained dissociated for at least 30 min (Fig. 6). Control saline injections followed by animal killing at 2, 5, or 30 min did not promote significant activation of the type I form of protein kinase (Fig. 6). These results from the saline-injected rabbits demonstrate that the activation of the type I cAMP-dependent protein kinase in response to in vivo administration of hCG is not a nonspecific, stress-induced event. The type II isozyme appeared to be 20% activated at 5 and 10 min, and again at 30 min post-hCG administration (Fig. 6). However, control saline injections also promoted an equivalent degree of type II protein kinase activation at these times. These results indicate that the observed 20% activation of the type II protein kinase isozyme is a nonspecific effect hCG.

In contrast to the results obtained upon in vivo administration of hCG, in vitro incubation of luteal supernatants with

**Fig. 5.** Concentration-dependent activation of the soluble protein kinases (PK) in CL of 4-day pseudopregnant rabbits in response to the in vivo administration of hCG. CL were dissected from ovaries of untreated rabbits or rabbits injected iv with various doses of hCG, as indicated on the abscissa, and killed 10 min postinjection. Dissected CL were homogenized and a supernatant was obtained from homogenates as described in the legend to Fig. 1. All data shown in the figure were derived from DEAE-cellulose column profiles in which supernatant fractions were applied to the column within 7 min of animal killing. The degree of activation of the type I (○) and type II (◆) forms of cAMP-dependent protein kinase is indicated on the ordinate and was determined by the deviation of the proportional distribution of catalytic protein kinase and cAMP-binding activity peaks from those of the 2-h control luteal supernatant values. Values on the graph represent either single determinations or means ± s.e. of 4, 3, 5, 3, 2, and 3 separate determinations for 0, 1, 2.5, 5, 10, 18, and 25 IU of hCG/kg of body weight, respectively. Statistical treatment of the data is included to measure technical precision.

**Fig. 6.** Time course of the activation of type I and type II cAMP-dependent protein kinases (PK) in CL obtained from ovaries of 1) untreated pseudopregnant rabbits, 2) rabbits injected iv with saline, or 3) rabbits injected iv with 25 IU of hCG/kg of body weight. CL were dissected and homogenized, and a supernatant was obtained from homogenates as described in the legend to Fig. 1. All data shown on the figure were derived from DEAE-cellulose column profiles in which supernatant fractions were applied to columns within 7 min of animal killing. Control animals received saline injections. Determination of the degree of activation by hCG of the type I (○) and type II (◆) and by saline of the type I (△) and type II (○) forms of cAMP-dependent protein kinase is described in the legend to Fig. 5. Values of CL kinase activation following hCG injection represent either single determinations or means ± s.e. of 4, 2, 3, and 6 separate determinations for 0, 5, 10, and 30 min after hCG injection. Values of CL kinase activation following saline injections represent either a single determination at 2 min or 2 determinations each at 5 min and 30 min.
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500 μM cAMP caused nearly total dissociation of both luteal cAMP-dependent protein kinases (Fig. 3C). Incubation with 5 μM caused 100% activation of the type I protein kinase and only a partial activation (approximately 50%) of the type II protein kinase (not shown).

To prove that the quantitative differential activation of luteal protein kinases occurred in vivo in response to hCG-induced elevations of cAMP and not in vitro during extraction and/or chromatography procedures, the following 3 types of experiments were performed. 1) In the first, charcoal (1% in 0.1% bovine serum albumin) was added to the cell extract to remove unbound cAMP, thereby preventing protein kinase activation during the preparation of the luteal cell extract, the following experiment was performed. DEAE-cellulose chromatography. The resulting elution profile of protein kinase and cAMP-binding activities revealed that the forward dissociation reaction of the type I isozyme was completely inhibited during the preparation of the luteal cell extract, the following experiment was performed (34). Crude type I isozyme (rat heart supernatant) was added with rabbit CL to the homogenization media.

To support further that the forward dissociation reaction of the type I isozyme occurred in vitro, the chromatographic elution profile of the type I isozyme was completely inhibited during the preparation of the luteal cell extract, the following experiment was performed (34). Crude type I isozyme (rat heart supernatant) was added with rabbit CL to the homogenization media. Extracts were performed in the absence and presence of charcoal and protein kinase assays were performed both on the heterogenous kinase extracts and on the exogenous kinase. The data presented in Table I compare the protein kinase activity ratios of the mixture of rat heart and luteal protein kinases versus those of the rat heart kinases. CL were obtained from control, 4-day pseudopregnant rabbits or 4-day pseudopregnant rabbits 10 min after the iv injection of 100 IU of hCG. The data show that the protein kinase activity ratio of the heterogenous type I enzyme was not increased by the extract of control or hCG-stimulated luteal tissue. 3) Lastly, experiments were conducted to test that the chromatography procedure did not promote dissociation of the type I or type II protein kinase isozymes. To this end, untreated crude type I (rat heart supernatant) or beef heart type II (Sigma Chemical Co., St. Louis, MO) protein kinases were mixed with CL from 4-day pseudopregnant rabbits (injected with 100 IU of hCG, 10 min), extracts were made, and DEAE-cellulose chromatography was performed as previously described. As shown in Fig. 7, both isotypes remained at least 90% reassociated. Although the protein kinase activity of the endogenous luteal kinase appears to be negligible compared with exogenous enzyme, one would predict that the theoretical cAMP present in the hCG-treated luteal extracts (approximately 10-12 nmol (35)) is not limiting.

Taken together, these experiments prove that the differential activation of the type I isoform occurred in vivo. Activation of Soluble cAMP-dependent Protein Kinase in Estrous Rabbit Follicles—Estrous rabbit follicles exhibit ex-

**TABLE I**

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Fig. 7. Effect of luteal extracts from hCG-treated rabbits on the chromatographic elution profile of exogenous type I (A) or type II (B) protein kinase isozyme. Crude type I isozyme (rat heart, see Fig. 1) or type II isozyme (see Fig. 1) were mixed with CL from 4-day pseudopregnant rabbits which were injected with 100 IU of hCG (10 min). Extracts of the kinase mixtures were made and chromatography was performed as described in the legend to Fig. 1. For A, 17.5 mg of protein was loaded, 10 mg of which was derived from the rabbit CL. For B, 110 mg of protein was loaded, 8.2 mg of which was derived from the rabbit CL.
clusively the type II form of cAMP-dependent protein kinase upon DEAE-cellulose chromatography of the soluble fraction (26). Previous results have shown that the iv injection of 25 IU of hCG/kg of body weight (an ovulatory dose) to estrous rabbits results in 90% activation of the type II form of cAMP-dependent protein kinase in follicles (27). In these experiments, follicles were homogenized in a medium containing 0.5 M NaCl to prevent protein kinase subunit reassociation (27, 29), and subsequent protein kinase activity ratios were determined in 10,000 X g (10 min) supernatant fractions (within 60 min of animal killing). Using these conditions, we observed no significant activation of protein kinase over control levels (of which the protein kinase activity ratio was 0.57 ± 0.04, n = 5) in response to 1.0, 2.5, 5.0, or 10.0 IU of hCG/kg of body weight. In addition to our inability to demonstrate a dose-dependent activation of the soluble protein kinase in follicles, the results of these experiments were clouded by the dissociating effects of 0.5 M NaCl on the type II form of cAMP-dependent protein kinase (27). The present experiments were designed to determine the degree of activation of the type II form of cAMP-dependent protein kinase in follicles from ovaries of untreated estrous rabbits and to determine whether the iv administration of hCG prompted a concentration-dependent activation of this protein kinase.

Results revealed that the soluble type II form of cAMP-dependent protein kinase in follicles obtained from untreated estrous rabbits was activated 15%. (Fig. 8), based upon the elution of 15% of the total catalytic protein kinase activity with a salt concentration of less than 0.1 M (not shown). The injection of various concentrations of hCG, iv, to estrous rabbits followed by death after 10 min resulted in the concentration-dependent activation of the follicular type II form of cAMP-dependent protein kinase (Fig. 8).

A comparison of the concentration dependence of the degree of hCG-induced activation within 10 min of the soluble cAMP-dependent protein kinases in follicles of estrous rabbits to that in CL of 4-day pseudopregnant rabbits revealed that the magnitude of activation of the type II cAMP-dependent protein kinase in follicles was equivalent to (or not different from) that of the type I form of cAMP-dependent protein kinase in CL (Fig. 9).

**DISCUSSION**

Of the many functions triggered by LH (hCG) in mature, preovulatory ovarian follicles, the following events are believed to be mediated by cAMP: steroidogenesis, oocyte maturation, luteinization, and ovulation (1-8). Since hormone-induced variations in intracellular cAMP levels are believed to be translated by cAMP-dependent protein kinases, then one can presume that all of these LH (hCG)-induced, cAMP-triggered events in follicles are mediated through cAMP-dependent protein kinases. Rabbit ovarian follicles exhibit only the type II form of cAMP-dependent protein kinase in the soluble fraction (27). Activation of the protein kinase in follicles is dependent upon the concentration of hCG presented to the follicle. Presumably, then, the steroidogenic, luteinizing, ovulatory, and oocyte maturational events triggered in preovulatory follicles by LH are all initiated intracellularly by cAMP and the message is transferred to regulatory proteins by the type II isozyme of cAMP-dependent protein kinase. However, analysis of these LH-triggered functions in follicles reveals that they seem to occur independently of each other (5, 8, 36, 37). Furthermore, initiation of each event requires a different minimal threshold concentration of LH (36), although all of these functions can be simultaneously triggered by the single preovulatory surge of LH. It is difficult to explain how a single enzyme can independently regulate these functions. Possibly, the magnitude of activation of the soluble type II protein kinase in follicles dictates the specific event to be initiated by designating a specific substrate(s) whose activity is modified through a phosphorylation reaction catalyzed by the catalytic subunit of the cAMP-dependent protein kinase. It is also possible in follicles, as has been suggested in cardiac tissue by Hayes et al. (38), that selective functions are translated intracellularly by activation of compartmentalized pro-
tein kinases, which in turn regulate the activity of substrates within the same or in a different cellular compartment.

In CL, the major physiological effect triggered by LH (hCG) is luteolysis; the magnitude of which is dependent upon the concentration of LH. A second event induced experimentally in rabbit CL by large concentrations of LH (hCG) is luteolysis. A large body of evidence indicates that the luteal steroidogenic response to LH is mediated by cAMP (5, 39) via cAMP-dependent protein kinases (40-42). Both type I and type II forms of cAMP-dependent protein kinase are present in the soluble fraction of rabbit CL (26, 27) and are activated approximately 10% in CL of 4-day pseudopregnant rabbits. In vitro both forms of cAMP-dependent protein kinase are also activated by exogenous cAMP; however, in vitro, exogenous hCG promotes specific activation of only the type I enzyme. These results suggest that it is the type I form of cAMP-dependent protein kinase in CL of 4-day pseudopregnant rabbits which is the more important mediator of acute hCG-induced responses. Since CL differentiate from follicles, these results are rather surprising since it is the type II form of protein kinase in follicles which seems to be the mediator of the cAMP-regulated responses. Even more surprising is the observation that the magnitude of activation of the type I protein kinase in CL is not different from that of the type II protein kinase in follicles in response to equal concentrations of hCG. Although intracellular cAMP levels were not measured in follicles and CL, adenyl cyclase data reveal that CL of 4-day pseudopregnant rabbits have the capacity to synthesize in vitro markedly greater levels of cAMP compared to follicles in response to a saturating concentration of LH (35). Thus, it is likely that CL synthesize greater levels of cAMP as compared to follicles in response to the iv injection of 25 IU of hCG/kg of body weight. Since only the type I form of cAMP-dependent protein kinase in CL is activated in response to this concentration of hCG, possibly with luteinization the type II enzyme becomes localized in a less "accessible compartment" within the luteal cells. It is not known if all luteal cells contain both isozymes of cAMP-dependent protein kinase. Selective activation of the type I isozyme in vitro may also reflect the presence of the type I isozyme in cells with an LH (hCG)-responsive adenyl cyclase and the presence of type II isozyme in other cells, possibly ones without an LH response cyclase system.

Furthermore, the type I form of cAMP-dependent protein kinase has also been reported in lymphocytes in response to concanavalin A (43). These authors correlated concanavalin A-stimulated activation of the type I form of protein kinase, determined under nondissociating homogenization and chromatography conditions, with increased ornithine decarboxylase activity. Interestingly, the combination of dibutyryl cAMP and concanavalin A caused nearly total activation of both type I and II forms of cAMP-dependent protein kinase but inhibited the induction of ornithine decarboxylase. Thus, in lymphocytes and CL, each of which contains both type I and type II forms of cAMP-dependent protein kinase, only the type I form of protein kinase is acutely activated under seemingly physiological conditions and the type II form of protein kinase is markedly activated (under the conditions studied) only in response to pharmacologically high levels of cAMP. Studies in other tissues containing both forms of cAMP-dependent protein kinase have indicated that both forms of protein kinase are activated in response to hormonal stimulation although the quantitative degree of selective protein kinase activation at different hormone concentrations was not evaluated (30, 31, 33, 44).

In all tissues thus far examined, only two forms of cAMP-dependent protein kinase have been shown to exist. There is widespread tissue, and in some cases, species specificity in the molecular proportions of the two enzymes. Moreover, the molecular proportions of the two enzymes differ in a single tissue during growth and differentiation (26, 31, 45-48). Although widespread differences in the distribution of the two forms of cAMP-dependent protein kinase are suggestive of distinct physiological functions for the two enzymes, the observation that different concentrations of LH (hCG) can trigger totally different physiological events in a single tissue (ovarian follicles) which contains only a single soluble form of cAMP-dependent protein kinase suggests that each form of cAMP-dependent protein kinase is capable of mediating a number of distinct biological functions. The molecular basis for the differential functions of either form of cAMP-dependent protein kinase is not clear, since the catalytic subunits of both enzymes appear to be indistinguishable (49-51). However, as shown in CL as well as in other tissues, both forms of cAMP-dependent protein kinase are not always activated under physiological conditions. The selective activation of one form of the enzyme certainly suggests a regulatory role for this enzyme which is distinct from the other form of cAMP-dependent protein kinase.

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Selective Activation of Soluble cAMP-dependent Protein Kinase Isozymes