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ENKEPHALIN-MEDIATED INHIBITION OF FORSKOLIN-STIMULATED RABBIT LUTEAL ADENYLYL CYCLASE ACTIVITY

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Summary: Forskolin at 25-100 μM elicited 10- to 15-fold stimulation of rabbit luteal adenylyl cyclase activity in the absence of guanine nucleotides. Addition of saturating concentrations of GTP or guanyl-5'-yl imidodiphosphate [GMP-P(NH)P] inhibited forskolin stimulation by 15-25% and 35-45%, respectively, in Na^+-free media. The further addition of 8 μM [D-Ala^2, Met^5] enkephalin amide (Da-ENK) caused an additional 16-24% inhibition of activity in the presence of GTP plus forskolin, but did not alter enzymatic activity in the presence of forskolin alone or forskolin plus GMP-P(NH)P. Inhibition by guanine nucleotide alone or Da-ENK plus GTP was only observed in the presence of forskolin. Maximal inhibition by Da-ENK was observed at 25 μM forskolin. Da-ENK reduced the IC_{50} for GTP by 2.3-fold but did not alter the IC_{50} for GMP-P(NH)P. Addition of Na^+ above 3 mM attenuated the inhibitory responses to GTP and GTP plus Da-ENK, but not to GMP-P(NH)P or GMP-P(NH)P plus Da-ENK. Above 100 mM, Na^+ inhibited enzymatic activity in the presence of forskolin, forskolin plus GTP and forskolin plus GMP-P(NH)P in the absence and presence of Da-ENK. These findings suggest that the rabbit corpus luteum contains an inhibitory receptor for opiate peptides that couples to adenylyl cyclase.

The rabbit corpus luteum contains an adenylyl cyclase system that is stimulated by luteinizing hormone, catecholamines, and prostaglandins (1-4). Regulation of hormone binding to its receptor and activation of adenylyl cyclase by Mg^{++} and guanine nucleotides (3,4) is consistent with the notion that the luteal cyclase system consists of a catalytic moiety and a stimulatory guanine nucleotide- and Mg^{++}-binding regulatory component (for review on the component nature of adenylyl cyclase see ref. 5). In addition, we have recently described a Mg^{++}- and guanine nucleotide-dependent inhibition of the forskolin activated luteal enzyme (6,7) indicating the presence of an inhibitory guanine nucleotide- and Mg^{++}-binding inhibitory regulatory component and suggesting the presence of an receptor coupled to adenylyl cyclase in the rabbit corpus luteum. Studies by Shu-Dong et al. (8) demonstrated the presence of opiate peptides in the rat corpus luteum. Since

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opiates have been shown to inhibit adenylyl cyclase activity in other systems (5,9) we investigated the actions of the enkephalin analog Da-ENK[1] on forskolin activated rabbit luteal adenylyl cyclase in order to identify a potential inhibitory receptor.

MATERIALS AND METHODS

Materials: Inorganic ^{32}P was purchased from International Chemical and Nuclear Corp. (Irvine, Calif.). [^{3}H]cAMP (10-20 Ci/mmol) was from Schwarz/Mann (Orangeburg, N.Y.). ATP (Catalog No. A-2383), GTP, GMP-P(NH)P, EDTA, Tris, diTris creatine phosphate, creatine phosphokinase, hCG, and Da-ENK were from Sigma Chemical Co. (St. Louis, MO). Forskolin was from Calbiochem (La Jolla, Calif.). The components of the nucleoside triphosphate-regenerating system used in the adenylyl cyclase assay were subjected to purification steps to decrease contamination with "guanine nucleotide-like" compounds as previously described (10). All other chemicals and reagents were of the highest commercially available purity and were used without further purification. [α-^{32}P]ATP (> 50 Ci/mmol) was synthesized according to the procedure of Walseth and Johnson (11).

Animals and Membranes: New Zealand White rabbits (3.0-4.5 kg) were used throughout. Pseudopregnancy was induced by injection of 100 IU hCG in saline, iv. The rabbits were killed by cervical dislocation on day 7 of psuedopregnancy (the day of hCG injection was day 0). The ovaries were removed and placed in ice-cold Krebs-Ringer bicarbonate, pH 7.4, until dissection of corpora lutea. The dissected corpora lutea were homogenized in ice-cold 27% wt/wt sucrose in 10 mM Tris-HCl, 1 mM EDTA, pH 7.5 and membrane particles prepared as previously described (1). Protein was determined by the method of Lowry et al. (12) using bovine serum albumin (fraction V) as standard.

Adenylyl Cyclase Assays: Adenylyl cyclase activity was determined at 32.5°C for 10 min in 50 µl of medium containing 0.1 mM ATP (with 3-5 x 10^6 CPM of [α-^{32}P]ATP), 3.0 mM $MgCl_2$, 1.0 mM EDTA, 1.0 mM cAMP (with approximately 10,000 cpm of [^{3}H]cAMP), 20 mM diTris creatine phosphate, 0.2 mg/ml creatine kinase, 0.02 mg/ml myokinase and 25 mM Tris-HCl, pH 7.5. Forskolin was generally used at a concentration of 25 µM in the assay and was added as a stock solution of 25 mM dissolved in 100% ethanol. All assays contained 1% ethanol in order to keep the forskolin in solution. Assays were stopped by the addition of 100 µl of "stopping solution" consisting of 10 mM cAMP, 40 mM ATP, and 1% sodium dodecylsulfate. The [^{32}P]cAMP formed and the [^{3}H]cAMP added to monitor recovery were isolated according to Salomon et al. (13) using Dowex and alumina chromatography as modified by Bockaert et al. (14). All experiments were repeated a minimum of three times with similar results and representative experiments are presented.

RESULTS

Forskolin was a potent activator of rabbit luteal adenylyl cyclase activity. At a concentration of 100 µM, forskolin produced a 10- to 15-fold stimulation of cyclase activity, in Na^+-free media, in the absence of added

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[1] ABBREVIATIONS: Da-ENK, [D-Ala^2, Met^5]enkephalin amide; GMP-P(NH)P, guanyl-5'-yl imidodiphosphate; hCG, human chorionic gonadotropin; Tris-RS, nucleoside triphosphate regenerating system made with diTris creatine phosphate; CL, corpus lutuem.

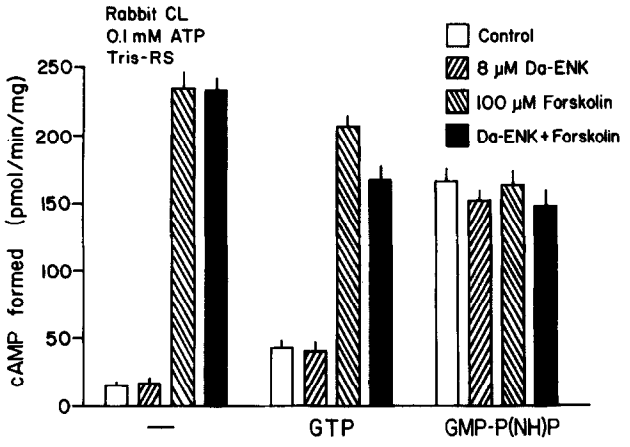

Figure 1: Effects of Da-ENK, forskolin, GTP and GMP-P(NH)P on rabbit luteal adenylyl cyclase activity. Adenylyl cyclase activities were determined as described in Materials and Methods. When present, the Da-ENK concentration was 8 µM, the forskolin concentration was 100 µM, and the GTP and GMP-P(NH)P concentrations were both 25 µM. The membrane protein content was 15 µg/50-µl assay. Values represent the mean ± SD of triplicate determinations.

guanine nucleotide or Da-ENK (Fig. 1). Addition of 8 µM Da-ENK did not alter the ability of 100 µM forskolin to stimulate cyclase activity nor did Da-ENK alter basal activity. GTP at a concentration of 25 µM elicited a 2-to 3-fold stimulation of cyclase activity that was not altered by Da-ENK. Enzymatic activity in the presence of GTP plus forskolin was 15-25% less than that observed in the presence of forskolin alone and the further addition of Da-ENK produced an additional 16-24% decrease in adenylyl cyclase activity. In the presence of 25 µM GMP-P(NH)P cyclase activity was 8- to 10-fold higher than basal activity and the addition of Da-ENK did not significantly alter the ability of GMP-P(NH)P to stimulate the enzyme. Adenylyl cyclase activity assayed in the presence of GMP-P(NH)P plus forskolin was reduced by 35-45% compared to forskolin alone and the addition of Da-ENK did not produce any further decrease in enzymatic activity.

Inhibition of adenylyl cyclase activity by guanine nucleotide or Da-ENK plus GTP was only observed in the presence of forskolin. The maximal difference in enzymatic activity between the GTP and GTP plus Da-ENK treated enzyme was observed at a forskolin concentration of 25 µM (Fig. 2). Neither GTP nor Da-ENK, alone or in combination, significantly altered the K_{act} for forskolin which was 5 µM.

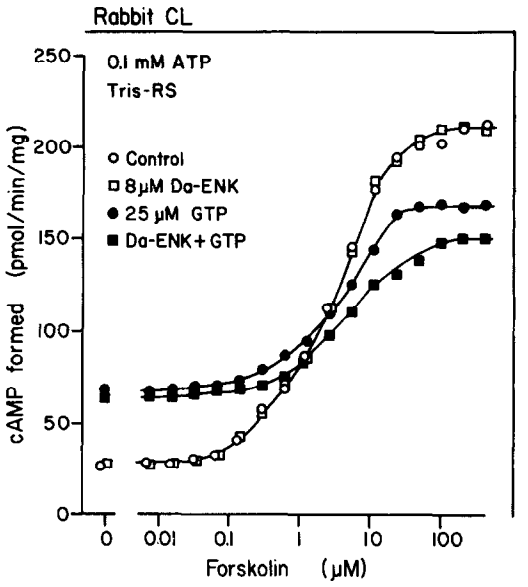

Figure 2: Effects of Da-ENK and GTP on concentration-effect curves for forskolin. Assays were determined as described in Materials and Methods. When present, the Da-ENK concentration was 8 μM and the GTP concentration was 25 μM. The membrane protein content was 4.5 μg 50-μl assay. Each point represents the mean of duplicate determinations.

In addition to enhancing GTP-mediated inhibition of forskolin activated adenylyl cyclase, Da-ENK altered the sensitivity of the enzyme to inhibition by GTP (Fig. 3). The IC_{50} values for GTP to inhibit the forskolin stimulated enzyme were 2.3 ± 0.2-fold (mean ± SD, n = 4) lower in the presence of Da-ENK than in its absence. In contrast, the inability of Da-ENK to enhance GMP-P(NH)P-mediated inhibition was accompanied by no change in the IC_{50} values for GMP-P(NH)P in the presence and absence of Da-ENK (Fig. 3).

In addition to GTP, Blume et al. (15) have demonstrated a requirement for Na^+ in order to obtain Da-ENK mediated inhibition of adenylyl cyclase in neuroblastoma-glioma hybrids. Therefore, we examined the effects of Na^+ on inhibition of forskolin activated luteal adenylyl cyclase by guanine nucleotides in the presence and absence of Da-ENK. Below 100 mM, Na^+ had little or no effect on adenylyl cyclase activity on the forskolin or forskolin plus GMP-P(NH)P treated enzyme when assays were performed in the presence or absence of Da-ENK (Fig. 4). On the other hand, Na^+ concentrations between 3 and 100 mM attenuated the inhibitory action of GTP on the forskolin activated

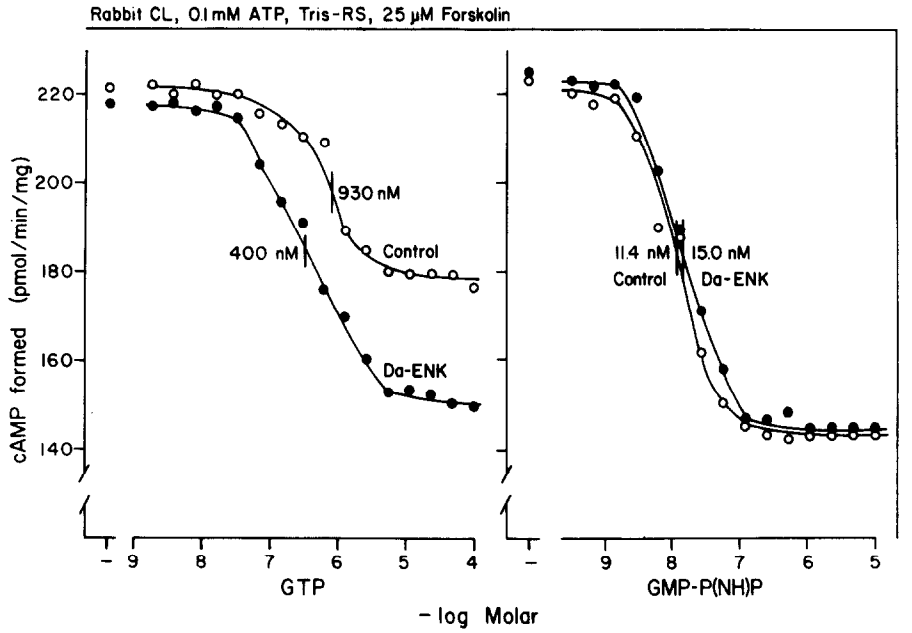

Figure 3: Effects of Da-ENK on concentration-effect curves for GTP and GMP-P(NH)P in the presence of 25 µM forskolin. Adenylyl cyclase activities were determined as described in Materials and Methods. When present, the Da-ENK concentration was 8 µM. The membrane protein content was 5.5 µg/50-µl assay. Each point represents the mean of duplicate determinations. The vertical lines and values next to the curves represent IC_{50} values.

enzyme in a concentration dependent manner both in the presence and absence of Da-ENK. Above 100 mM, Na^+ produced concentration dependent inhibition of adenylyl cyclase activity under all assay conditions tested.

DISCUSSION

In the present study we have shown that the enkephalin analog Da-ENK inhibits forskolin activated rabbit luteal adenylyl cyclase. As shown for hormone-mediated inhibition of adenylyl cyclase in other systems (5,9,16), Da-ENK induced inhibition of the rabbit luteal enzyme is a GTP-dependent process. Da-ENK reduces the GTP concentration required to inhibit cyclase activity. Da-ENK induced inhibition could not be demonstrated in the presence of GMP-P(NH)P. A similar phenomenon has been reported for epinephrine-mediated inhibition of platelet adenylyl cyclase (17). As demonstrated for adipocyte cyclase (18,19), Na^+ reversed the GTP-dependent inhibition of luteal adenylyl cyclase. However, unlike most other systems (for review see 16), Na^+ was neither required for hormone-mediated inhibition

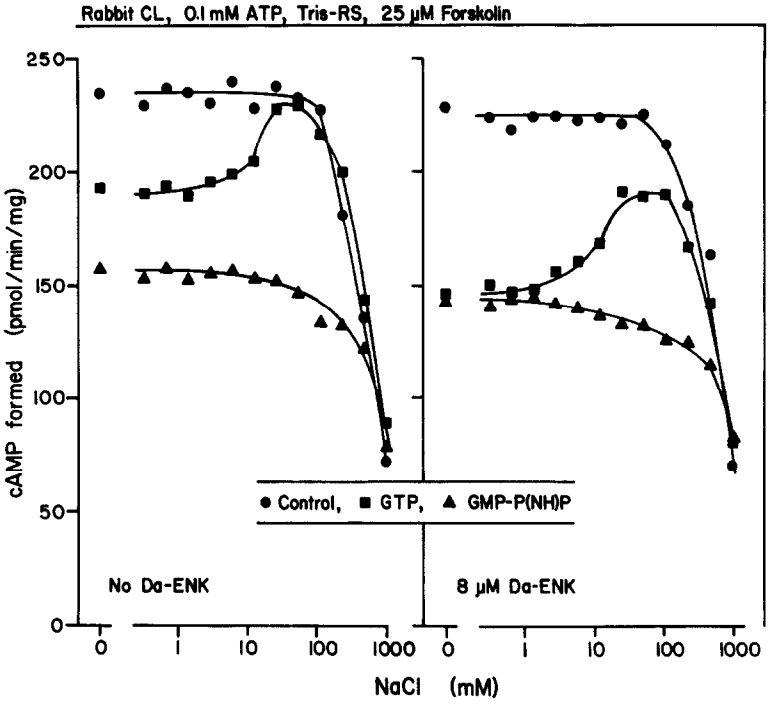

Figure 4: Effects of Da-ENK, GTP and GMP-P(NH)P on concentration-effect curves for NaCl in the presence of 25 µM forskolin. Adenylyl cyclase activities were determined as described in Materials and Methods. When present, the Da-ENK concentration was 8 µM, and the concentrations of both GTP and GMP-P(NH)P were 25 µM. The membrane protein content was 4.0 µg/50-µl assay. Each point represents the mean of duplicate determinations.

of luteal cyclase nor did Na^+ potentiate the inhibitory action of Da-ENK. Increasing Na^+ attenuated the inhibitory action of both GTP and GTP plus Da-ENK such that at physiological concentrations of Na^+, GTP was no longer inhibitory while GTP plus Da-ENK was inhibitory. Although the extent of luteal adenylyl cyclase inhibition by Da-ENK (16-24%) was small compared to hormone-induced inhibition of adenylyl cyclase in most other systems (40-80%; 16) it was comparable to somatostatin-mediated inhibition of forskolin-activated S49 cyc$^-$ lymphoma cell adenylyl cyclase (20,21).

The ability of Da-ENK to inhibit forskolin activated luteal adenylyl cyclase in a GTP-dependent manner supports the suggestion that the rabbit corpus luteum contains an inhibitory as well as a stimulatory guanine nucleotide- and Mg^{++}-binding regulatory component (6,7). Further, the findings of the present study suggests a potential function for the opiate

peptides which have been localized in the corpus luteum (8). These opiate peptides could bind to opiate receptors and negatively regulate adenylyl cyclase activity, resulting in the inhibition of cAMP production and reduced progesterone output from the corpus luteum under normal physiological conditions. Further, the existence of such an inhibitory process may provide a mechanism by which rabbit luteal adenylyl cyclase persistently activated by luteinizing hormone (22) may be turned-off.

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