The ability of gonadotropin releasing hormone (GnRH) to elevate cellular levels of mRNA for β-subunit of luteinizing hormone (LH) has been examined in monolayer cultures from rat pituitary. Low concentrations of GnRH (100 pM) induced a 68-fold increase in LH-β mRNA, while higher concentrations of GnRH were less effective. The low concentrations of GnRH (100 pM) did not result in altered GnRH receptor levels (92 ± 12% compared to controls) after 24 h treatment but did increase protein kinase C activity to 249 ± 16%. The protein kinase C activator, phorbol 12-myristate 13-acetate, at concentrations (2-20 nM) which did not deplete protein kinase C, stimulated LH-β mRNA levels 2-5-fold after 24 h. Higher concentrations of phorbol 12-myristate 13-acetate, which depleted protein kinase C activity, substantially reduced the ability of 100 pM GnRH to stimulate increases in LH-β mRNA levels. As previously observed, protein kinase C-depleted cells exhibited normal LH release in response to GnRH stimulation. These studies demonstrate that low concentrations of GnRH may have an important role in regulation of gonadotropin biosynthesis. Furthermore, the results suggest that activation of protein kinase C is sufficient to stimulate increases in LH-β mRNA levels and that protein kinase C is necessary for normal GnRH stimulation of LH-β mRNA levels. Accordingly, we postulate that protein kinase C may mediate the action of GnRH on LH-β mRNA levels.

Gonadotropin releasing hormone (GnRH) is a hypothalamic decapetide which regulates release of the gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone, from the anterior pituitary. LH is a heterodimer containing noncovalently linked α- and β-subunits. The α-subunit is common to several glycoprotein hormones while the β-subunit is unique and confers the biological specificity of the dimer (1). The results of several studies suggest that the concentration of β-subunit mRNA is rate-limiting in the synthesis of glycoprotein hormones (2-7). Recently, it has become clear that GnRH is able to stimulate increases in LH-β mRNA concentrations and regulate LH biosynthesis as well as LH release (2-8).

The mechanism by which GnRH mediates increased LH-β mRNA levels has not been identified. However, the intracellular mediators of GnRH effects on LH release have been the subject of intensive investigation. Multiple lines of evidence suggest that an increase in intracellular calcium ion concentrations is a sufficient and necessary signal for stimulation of LH release and up-regulation of GnRH receptor (9). As is the case with many hormonal systems which mobilize calcium in response to receptor occupancy, it is now well established that agonist occupancy of the GnRH receptor causes rapid hydrolysis of polyphosphoinositides, leading to the accumulation of inositol phosphates and 1,2-diacylglycerols (10-13). These events appear to be regulated by a G-protein (14). The demonstrations that synthetic diacylglycerols (15) and phorbol esters (16, 17) stimulate the release of LH suggested a possible role for protein kinase C in mediating gonadotropin release in response to GnRH. However, recent studies in our laboratory have shown that the percentage of cellular LH released in response to GnRH is not affected by the depletion of protein kinase C from pituitary cells in vitro whether measured by ['H]phorbol dibutyrate binding, by enzymatic or immunological activity, or by the ability of cells to respond to phorbol esters or diacylglycerols (18, 19). It has also been shown that homologous down-regulation of cell surface receptor number is unaltered in protein kinase C-depleted cells (20). These observations suggest that it is unlikely that GnRH regulation of LH release or GnRH receptor loss are mediated by protein kinase C. A recent study (21) implicated protein kinase C in up-regulation of agonist occupied GnRH receptors.

In this study, we have investigated the effects of GnRH on LH-β mRNA levels in pituitary cultures and have found that a low concentration of GnRH profoundly stimulates LH-β mRNA levels. We have used this system to examine the role of protein kinase C in mediating GnRH effects on LH-β mRNA levels. These studies demonstrated that GnRH-stimulated increases in LH-β mRNA concentrations are blocked by prior treatment of cells with high doses of PMA (which results in protein kinase C loss). This phenomenon is not related to changes in GnRH receptor numbers or changes in GnRH-stimulated LH release. These results suggest that protein kinase C activation may mediate GnRH-induced changes in rat LH-β mRNA.
EXPERIMENTAL PROCEDURES

Preparation and Challenge of Pituitary Cell Cultures—Female weanling Spague-Dawley (Harlan, Indianapolis, IN) rats were used as a source of pituitary tissue in these experiments and were killed by cervical dislocation. Primary pituitary cells were prepared by enzymatic dispersion as described (22) and suspended (cells from 0.75 pituitaries/ml) in medium 199 (M199, Gibco) containing 0.3% bovine serum albumin (BSA, Fraction V, Sigma), 2.5% fetal bovine serum, 10% horse serum (M. A. Bioproducts, Walkersville, MD), 20 μg/ml gentamicin sulfate (Sigma), and 10 nM HEPES pH 7.4 (plating medium). Two- or 4-ml aliquots of this cell suspension were placed in 22- or 35-mm wells of 12- or 6-well cluster culture plates (Costar, Cambridge, MA), respectively, and were maintained in a 7% carbon dioxide-saturated atmosphere at 37 °C. After 2 days, the plating medium was decanted and the cells were washed twice with M199 (37 °C, 2 ml/well) containing 0.3% BSA and 10 mM HEPES, pH 7.4 (M199/BSA) to remove serum and nonadherent cells. The cells were then incubated for the indicated time periods with either 2 or 4 ml of M199/BSA containing the indicated concentrations of hormone. Media were collected at the end of treatment periods for determination of LH release by radioimmunoassay.

Binding Experiments—Dispersed pituitary cells were prepared, suspended in plating medium (cells from 6.7 pituitaries in 1 ml), and maintained in culture as described above. GnRH receptor binding was assayed as described previously (20) using buserelin ([p-Ser(Bu)3,Pro-NH2]GnRH (Roechst-Roussel Pharmaceuticals), Sommerville, NJ), a metabolically stable GnRH agonist analog (22). The cells were then washed with 2 ml of M199/BSA at 37 °C and incubated at 40 min at this temperature and then removed to 23 °C and the temperature was equilibrated for 20 min. The M199/BSA was then decanted and replaced with 500 μl of M199/BSA containing 200-2000 pM [125I]iodo-Tyr6-buserelin (0.5-1.0 Ci/mg as- 

Radioimmunoassay—Luteinizing hormone was measured by radioimmunoassay using antisera (C-102) prepared and characterized in our laboratory (23). Highly purified rat LH (LH I-6, National Institute for Arthritis, Diabetes and Digestive and Kidney Diseases (NIADDK)) was iodinated by a modification (23) of the method of Hunter and Greenwood (24) and a second preparation of rat LH (RP-2, NIADDK) was used as the reference protein. Immobilized protein A was used to separate free and bound hormone (25).

Analysis of LH-β mRNA—RNA was prepared by solubilization of cultured pituitary cells in guanidine HCl and sedimentation through CsCl using a modification (26) of previously described procedures (27). RNA samples (0.2 to 4 μg of total RNA) were transferred to nitrocellulose by filtration using a multiphasic manifold (Bethesda Research Laboratories) and immobilized by incubation at 80 °C under reduced pressure for 2 h (28). Filters containing the immobilized RNA were hybridized to a cloned LH-β cDNA (29) using previously described conditions (28). The LH-β cDNA was radiolabeled to a specific activity of 0.5-1.0 × 10^6 cpm/μg of DNA by the random primer method (20). Relative LH-β mRNA levels were determined by densitometry of multiple dilutions of RNA samples.

Protein Kinase C Assay—In order to assess the effects of protein kinase C depletion, cells were prepared and plated as described above and challenged on the second day of culture for 24 h with 1 μM PMA or vehicle alone (0.5% MeSO in M199/BSA containing 10% horse serum and 2.5% fetal calf serum). The cells were then washed and incubated an additional 24 h in plating medium with or without GnIH (100 pM). The effectiveness of PMA pretreatment on depletion of protein kinase C was assessed at the end of each incubation by measuring Triton X-100-extractable protein kinase C activity. Cells were homogenized in 5 ml of 25 mM Tris-HCl, pH 7.5, containing 0.25 M sucrose, 2.5 mM MgCl2, 2.5 mM EGTA, 50 mM 2-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride with 0.3% Triton X-100. The suspension of cells was shaken gently for 30 min at 4 °C and applied to a DEAR-Bio-Gel A column (Bio-Rad) which had been equilibrated with buffer. The columns were then washed with 10 ml of buffer, followed by 2 ml of buffer containing 20 mM NaCl. Protein kinase C was eluted with 5 ml of buffer containing 100 mM NaCl and concentrated to 1 ml in a Centricell Ultrafilter (Polysciences). We have previously found that 95% of the protein kinase C activity elutes in the 20-100 mM fraction (31). Kinase activity of this fraction was assayed by determination of the rate of transfer of 32P from [γ-32P]ATP to histone as previously described (32). The standard assay solution contained in 250 μl: 5 nmol of Tris-HCl, pH 7.5, 1.25 μmol

FIG. 1. Time course of GnRH-mediated changes in LH release, rat LH-β mRNA, and GnRH receptor numbers. Cells were prepared and maintained in culture as described under "Experimental Procedures," were treated with GnRH (100 pM, □) or M199/BSA (no sera) alone (●), and incubated for the indicated time. At the end of each treatment period, medium was collected for LH determination by radioimmunnoassay; release is expressed as percent of control (A) where control refers to the amount of LH released at 0 time (media added to cells and immediately removed). GnRH receptor numbers (Bmax) were determined by saturation analysis using [125I]iodo-Tyr6-buserelin (Bmax) as described under "Experimental Procedures" and compared to control values (B). To evaluate changes in rat LH-β mRNA, RNA was extracted, purified, and then assayed by cDNA hybridization and autoradiography as described under "Experimental Procedures" (C). Values shown are the mean ± S.E. of data obtained from three separate experiments (p < 0.01).
Stimulation of LH-β mRNA

**Fig. 2.** Dose response of GnRH-mediated changes in LH release, GnRH receptor number, rat LH-β mRNA, and cellular protein kinase C after a 24-h exposure.

Cells were prepared and maintained in culture as described under "Experimental Procedures" and then incubated for 24 h in a range of concentrations (10⁻¹¹-10⁻⁷ M) of GnRH. At the end of the treatment period, medium was collected for LH determination by radioimmunoassay. LH release (A) is expressed as percent of control (medium alone) (n = 4). GnRH receptor numbers (B) were determined using [¹²⁵I]-labeled GnRH as described under "Experimental Procedures" and compared to control values (n = 3). Relative changes in rat LH-β mRNA in response to GnRH (C) were evaluated by extraction and purification of RNA followed by LH-β cDNA hybridization and autoradiography, as described under "Experimental Procedures" (n = 4). Cellular protein kinase C (D) was determined by Triton X-100 extraction, DEAE-cellulose chromatography, and transfer of ³²PO₄ to histone as described under "Experimental Procedures" (n = 3). Values shown are the mean ± S.E. (* P < 0.05). *PKC, protein kinase C.*
of magnesium nitrate, 50 µg of histone (Sigma Type III-S), 2.5 nmol [γ-32P]ATP, and 50 µl of the concentrated column eluate. The solution was supplemented either with ECTA (1 mM); CaCl2 (1 mM); or CaCl2 and lipids (10 µg of phosphatidylserine and 1 µg of 1,2-diolein, Sigma) as indicated. The assay reaction was started by the addition of enzyme and was continued 5 min at 30°C. The reaction was stopped by precipitation of protein on 3 M filter paper (Whatman) in 10% trichloroacetic acid. After washing, 32P incorporation into precipitated protein was determined by liquid scintillation spectrometry. Kinase activity was assessed as pmol of 32P incorporation/µg of protein from the 20-100 mM NaCl column eluate and expressed as a percentage of control. The Ca2+- and lipid-dependent kinase minus the Ca2+-dependent kinase activity of this fraction was used as a measure of cellular protein kinase C (21). Protein was assayed according to the Bradford method (23) using BSA as a standard.

Materials—Natural sequence GnRH was obtained from the National Pituitary Agency (Baltimore, MD). [γ-32P]ATP (1000-1500 dpm/pmole) was synthesized according to the method of Glynn and Chappel (34). PMA (Sigma) was prepared as a 0.1 mM stock solution in MeSO. Concentration of MeSO did not exceed 0.5% (v/v) in the bioassay medium, and this vehicle had no measurable effect on LH release, mRNA levels, or kinase activity.

RESULTS

The time courses of LH release, GnRH receptor numbers, and LH-β mRNA expression in response to 100 pm GnRH are shown in Fig. 1. Cumulative LH release (Fig. 1A) was significantly different from control values after 24 h and continued to increase throughout the observed treatment period. GnRH receptor levels (Fig. 1B) rapidly dropped and then recovered at 6 h and were comparable to control levels for the remaining 24 h of the treatment period. A statistically significant increase in rat LH-β mRNA in response to GnRH was first detected after 6 h, reached maximum levels at 24 h (Fig. 1C), and decreased thereafter. Since the measured response of rat LH-β mRNA was maximal 24 h after the addition of GnRH, treatment periods of 24 h were used in subsequent experiments.

GnRH-stimulated cumulative LH release (Fig. 2A) showed a dose dependence with half-maximal stimulation occurring at approximately 500 pm GnRH, after 24 h of stimulation. By contrast, GnRH receptor numbers (Fig. 2B) showed a dose-dependent decline. Half-maximal decrease occurred at approximately 500 pm GnRH with 24 h of continuous exposure to GnRH. The GnRH dose dependence of rat LH-β mRNA was biphasic (Fig. 2C) with 24 h of stimulation. All doses of GnRH examined increased the relative concentration of LH-β mRNA with a maximal response occurring at 100 pm GnRH (780% of control, p < 0.01). In addition, we examined total cellular protein kinase C activity under the same incubation condition (Fig. 2D). Again, a biphasic response was observed with maximal increase in protein kinase C activity measured at 100 pm GnRH (249% of control, p < 0.01 compared to control). These findings indicate that continuous stimulation with GnRH, at doses which do not reduce GnRH receptor levels, produce increases in both total protein kinase C activity and LH-β mRNA.

In order to evaluate the role of protein kinase C activation in the regulation of LH-β mRNA, we determined the effects of PMA pretreatment of GnRH-stimulated LH release and LH-β mRNA levels. Cells were pretreated for 24 h with PMA (1 µM) or vehicle alone (0.5% MeSO) in M199/BSA, supplemented with 10% horse serum and 2.5% fetal calf serum, as shown in Fig. 3A. This produced a significant reduction in total cellular protein kinase C activity (95 ± 4.85% of control, p < 0.01) after 24 h, which was maintained throughout the second treatment period (48 h). It was also observed that GnRH stimulated an increase in protein kinase C activity in both the PMA-treated and -untreated control cells. PMA pretreatment did not reduce the responsiveness of gonadotropes to LH-releasing effects of GnRH (Fig. 3B). We also evaluated the effects of PMA pretreatment on GnRH receptor numbers (Fig. 3C). No significant change in βmax was observed using this protocol. We then evaluated the effects of protein kinase C depletion on GnRH-stimulated LH-β mRNA levels (Fig. 3D). In the pretreatment period a small, but not statistically significant, increase in LH-β mRNA was observed with PMA. Treatment with GnRH (100 pm) for an additional 24 h stimulated a marked increase in LH-β mRNA in the cells pretreated with vehicle alone (687% of control, p < 0.01). Depletion of protein kinase C by pretreatment with PMA abolished the subsequent GnRH-stimulated increase in LH-β mRNA.

DISCUSSION

Investigations using GnRH-deficient animals and humans have shown that GnRH is essential for maintenance of LH synthesis in vivo (35-37), but until recently little has been known about the regulatory effects of GnRH on LH subunit mRNAs. The cloning of cDNAs coding for the β-subunit of rat LH (29, 38) has allowed investigators to examine the regulation of LH-β mRNA levels. Indeed, recent studies have shown that GnRH can stimulate increases in LH-β mRNA levels when delivered in a pulsatile manner (2, 8) at a fre-
Stimulation of LH-β mRNA

**FIG. 3.** The role of protein kinase C in regulation of GnRH mediated LH release, cellular protein kinase C, GnRH receptor number, and rat LH-β mRNA.

Cells were prepared and maintained in culture as described under "Experimental Procedures" and then incubated for 24 h with 1000 nM PMA (●) or vehicle alone (0.5% MeSO (□)) in M199/BSA containing 10% horse serum and 2.5% fetal bovine serum. LH release, cellular protein kinase C, GnRH receptor number, and LH-β mRNA were measured at the end of the pretreatment period (PreRx) or after an additional 24 h of incubation with medium alone (M199/BSA) or 100 pM GnRH (GnRH). Cellular protein kinase C (A) was determined by Triton X-100 extraction, DEAE-cellulose chromatography, and transfer of 32P to histone as described under "Experimental Procedures." LH released (B) was assayed by radioimmunoassay and expressed as a percentage of the total cellular LH as determined by the measured released LH divided by the Triton X-100 extractable plus the released LH. GnRH receptor number (Bmax, C) was determined using [3H]-buserelin saturation analysis as described under "Experimental Procedures." Rat LH-β mRNA (D) was measured by extraction and purification of RNA followed by LH-β cDNA hybridization and autoradiography. Relative changes in LH-β mRNA were calculated by densitometric analysis (area under the curve/μg of RNA) as described under "Experimental Procedures." Values shown are the mean ± S.E. of data obtained from three separate experiments (p < 0.01). PKC, protein kinase C.
Stimulation of LH-β mRNA

Consistent with previous findings from this laboratory, cells treated with high concentrations of PMA retained the ability to increase LH release in response to GnRH and showed no marked change in GnRH receptor numbers (18, 19). These findings suggest that protein kinase C activation is required for GnRH regulation of LH-β gene expression, although it is not required for GnRH stimulation of LH release. Thus, GnRH receptor occupancy likely leads to increases in LH release and LH-β mRNA levels through response pathways including both common and separate elements. Activation of polyphosphoinositide hydrolysis is apparently a key event in the regulation of LH-β mRNA, with the subsequent accumulation of diacylglycerols and activation of protein kinase C required for regulation of LH gene expression.

The data presented in this report are consistent with the view that protein kinase C mediates GnRH effects on LH-β gene expression. Subsequent steps involved in protein kinase C effects on LH-β gene expression may involve the phosphorylation of chromosomal proteins associated with the LH-β gene. Recent studies have demonstrated that the transcription factor, AP-2, mediates protein kinase C effects on the transcription of specific genes (40). The GnRH-regulated cell cultures described in this report should provide a useful system for further analysis of the mechanisms involved in GnRH- and protein kinase C-mediated stimulation of LH-β gene expression.

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REFERENCES

1. Pierce, J. G., and Parsons, T. F. (1981) Annu. Rev. Biochem. 50, 485-495
2. Papavasiliou, S. S., Zmili, S., Khoury, S., Landefeld, T. D., Chin., W. W., and Marshall, J. C. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 4026-4029
3. Gharib, J., Bowers, S. M., Need, L. R., and Chin, W. W. (1986) J. Clin. Invest. 77, 582-589
4. Chin, W. W., Shupnik, M. A., Ross, D. S., Habener, J. F., and Ridgway, E. C. (1985) Endocrinology 116, 873-878
5. Grotjan, H. E., Berkowitz, A. S., and Keel, B. A. (1985) Mol. Cell. Endocrinol. 41, 205-209
6. Peters, B. P., Krzesicki, R. F., Hartle, R. J., Penini, F., and Rudder, R. W. (1984) J. Biol. Chem. 129, 5123-5130
7. Gurr, J. A., and Kourides, I. A. (1984) Endocrinology 115, 830-832
8. Leung, K., Kaynard, A. H., Negrini, B. P., Kim, K. E., Maurer, R. A., and Landefeld, T. D. (1987) Mol. Cell. Endocrinol. 41, 420-429
9. Conn, P. M., Huckle, W. R., Andrews, W. V., and McArdle, C. A. (1987) Recent Prog. Horm. Res. 43, 29-68
10. Snyder, G. D., and Blesadale, J. E. (1982) Mol. Cell. Endocrinol. 28, 65-69
11. Andrews, W. V., and Conn, P. M. (1986) Endocrinology 118, 1148-1158
12. Schrey, M. P. (1985) Biochem. J. 226, 563-569
13. Huckle, W. R., and Conn, P. M. (1987) Endocrinology 120, 160-169
14. Andrews, W. V., Staley, D. D., Huckle, W. R., and Conn, P. M. (1986) Endocrinology 119, 2537-2546
15. Conn, P. M., Ganong, B. R., Ebeling, J., Staley, D., Neilde, J. E., and Bell, R. M. (1985) Biochem. Biophys. Res. Commun. 126, 552-559
16. Smith, M. A., and Vale, V. W. (1988) Endocrinology 107, 1425-1431
17. Smith, W. A., and Conn, M. (1984) Endocrinology 114, 553-559
18. McArdle, C. A., Huckle, W. R., and Conn, P. M. (1987) J. Biol. Chem. 262, 5029-5035
19. McArdle, C. A., and Conn, P. M. (1988) Methods Enzymol. 168, in press
20. McArdle, C. A., Gorospes, W. C., Huckle, W. R., and Conn, P. M. (1987) Mol. Endocrinol. 1, 420-429

frequency similar to that observed for pulsatile GnRH secretion in vivo. Thus, previous studies have emphasized the need for pulsatile GnRH treatment at particular frequencies in order to stimulate increases in LH-β mRNA levels.

The present findings demonstrate that nonpulsatile treatment with low concentrations of GnRH in static cultures can stimulate increases in LH-β mRNA levels and suggest a possible physiological role for low levels of GnRH in maintenance of LH biosynthesis. The results also suggest an essential role of protein kinase C in permitting GnRH stimulation of LH-β mRNA level. It is conceivable that both basal and pulsatile GnRH secretion in vivo are important components responsible for regulation of LH biosynthesis. Besides the possible physiological importance of these findings, the ability of GnRH to stimulate LH-β mRNA levels in static cultures provides a simplified system for further studies of the mechanism of GnRH action on LH gene expression.

We have used the static culture system to investigate the possible role of protein kinase C in mediating GnRH effects on LH-β mRNA. Several lines of evidence suggest that protein kinase C might have a role to mediate GnRH effects on LH-β mRNA levels. GnRH stimulates both diacylglycerol production (11) and protein kinase C redistribution in vitro (39) and in vivo (31). Furthermore, phorbol esters and diacylglycerols (which activate protein kinase C) stimulate LH release (15, 16). However, recent studies suggest that protein kinase C activity is not required for GnRH stimulation of LH release. GnRH-stimulated LH release can be uncoupled from inositol phospholipid turnover (13) as well as protein kinase C redistribution (31). In addition, the demonstration that, in protein kinase C-depleted cells, both GnRH-stimulated LH release and GnRH receptor down-regulation are unaffected (18, 19) indicates that protein kinase C does not play an obligatory role in the acute response of gonadotropes to GnRH with regard to these cellular processes.

In contrast, the present findings demonstrate that stimulation of protein kinase C activity is a sufficient and necessary signal for regulation of LH-β mRNA levels by GnRH. Treatment of pituitary cell cultures with relatively low concentrations of PMA leads to increases in LH-β mRNA levels, suggesting that activation of protein kinase C can regulate LH mRNA subunit gene expression. PMA at a concentration of 2 nM was found to increase LH-β mRNA to a level similar to that observed with the most effective dose of GnRH. While low concentrations of PMA stimulate LH-β mRNA gene expression, high concentrations of PMA, which deplete protein kinase C total activity, the effects of GnRH are also diminished. Although GnRH stimulates relatively small increases in protein kinase C total activity, the effects of GnRH are also known to involve redistribution of protein kinase C (31, 39) which likely amplifies the response. This may also account for the slight differences in the concentration dependence of GnRH effects on LH-β mRNA and protein kinase C activity. It should also be noted that although the protein kinase C depletions are present in these experiments may have unexpected effects on other signalling pathways.
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21. Huckle, W. R., McArdle, C. A., and Conn, P. M. (1988) J. Biol. Chem. 263, 3296–3302
22. Marian, J., and Conn, P. M. (1979) Mol. Pharmacol. 16, 196–201
23. Smith, W. A., Cooper, R. L., and Conn, P. M. (1982) Endocrinology 111, 1843–1848
24. Hunter, W. M., and Greenwood, F. C. (1962) Nature 194, 495–496
25. Gupta, R., and Morton, D. L. (1979) Clin. Chem. 25, 752–756
26. Maurer, R. A. (1981) Nature 294, 94–97
27. Glisin, V., Crkvenjakov, R., and Byus, C. (1974) Biochemistry 13, 2633–2637
28. Thomas, P. S. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 5201–5205
29. Tepper, M. A., and Roberts, J. L. (1984) Endocrinology 115, 385–391
30. Feinberg, A. P., and Vogelstein, B. (1983) Anal. Biochem. 132, 6–13
31. McArdle, C. A., and Conn, P. M. (1986) Mol. Pharmacol. 29, 570–576
32. Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U., and Nishizuka, Y. (1982) J. Biol. Chem. 257, 7847–7851
33. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
34. Glynn, M., and Chappell, J. B. (1964) Biochem. J. 90, 147–149
35. Charlton, H. M., Halpin, D. M. G., Iddon, C., Rosie, R., Levy, G., McDowell, I. F. W., Megson, A., Morris, J. F., Bramwell, A., Speight, A., Ward, B. J., Broadhead, J., Davey-Smith, G., and Fink, G. (1983) Endocrinology 113, 555–544
36. Valk, T. W., Corley, K. P., Kelch, R. P., and Marshall, J. C. (1980) J. Clin. Endocrinol. Metab. 51, 730–738
37. Liu, T., Jackson, G. L., and Gorski, J. (1976) Endocrinology 98, 151–163
38. Chin, W. W., Godine, J. E., Klein, D. R., Chang, A. S., Tan, L. K., and Habener, J. F. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 4649–4653
39. Hirota, K., Hirota, T., Aguilera, G., and Catt, K. J. (1987) J. Biol. Chem. 262, 3245–3246
40. Angel, P., Imagawa, M., Chiu, R., Stein, B., Imbra, R. J., Rahm- dorf, H. J., Jonat, C., Herrlich, P., and Karin, M. (1987) Cell 49, 729–739