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Specific regulation by steroid hormones of the amount of type I cyclic AMP-dependent protein kinase holoenzyme

(castration/hypophysectomy/adrenalectomy/prostate/testosterone maintenance)

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ABSTRACT **The total amounts of type I and type II cytoplasmic cyclic AMP-dependent protein kinase activities were measured in various tissues of intact rats and rats subjected to castration, hypophysectomy, or adrenalectomy. After castration, the total amount of type I activity decreased rapidly in classically steroid-responsive tissues such as the ventral prostate and levator ani muscle and less rapidly in the liver. After hypophysectomy and adrenalectomy, type I activity in the liver decreased to the same extent as after castration. Type I activity could be maintained in the ventral prostate and levator ani muscle at control levels by the daily injection of dihydrotestosterone. Furthermore, after post-castration regression of the prostate for 3 days, three daily subcutaneous injections of dihydrotestosterone resulted in a complete restoration of type I activity to the intact level. The amount of type II activity was not altered by any of the experimental ablations. This study provides evidence linking steroid action to the ability of steroid-responsive tissues to maintain a substantial activity of type I cyclic AMP-dependent protein kinase.**

Since the proposal of the second-messenger theory of hormone action (1), a rapidly expanding body of literature has confirmed the involvement of cyclic AMP in the regulation of anabolic events stimulated by trophic hormones. Indeed, cyclic AMP-mediation of hypertrophy always seems to be expressed through a temporally linked series of events (2). Instrumental in the elucidation of this series of events has been the extensive substantiation of an earlier hypothesis of Kuo and Greengard (3) that all the effects of cyclic AMP are mediated through cyclic AMP-dependent protein kinases. Because certain tissues have sufficient cyclic AMP levels to activate these enzymes, at least partially, it now seems that the measurement of further activation, rather than measurement of detectable elevation of cyclic AMP, is the parameter of choice for study of the cellular events produced by trophic agents.

The involvement of cyclic nucleotides in the action of steroid hormones has been less clear, with some controversy evident in the literature. Singhal *et al.* (4) have accumulated a considerable body of evidence that exogenous cyclic AMP can exert estromimetic or andromimetic effects *in vivo*, that the absolute levels of cyclic AMP and its rate of biosynthesis are dependent on the gonadal status of the animal, and that methylxanthines, potent inhibitors of cyclic AMP phosphodiesterase, can potentiate steroidal doses. They also have shown that adenylate cyclase activity, cyclic AMP levels, and polyamine levels decrease in the ventral prostate of the rat after anti-androgen administration (5).

Other events involved in a trophic response also are altered by androgens. Wilson and Ahmed (6) have demonstrated that the androgenic status of the animal can have profound effects on the phosphorylation of nuclear proteins by [^{32}P]ATP in the rat ventral prostate. Liao and Fang (7) have shown that nucleolar RNA polymerase is selectively enhanced by androgens.

Increasing evidence suggests that the total amount of type I and type II cyclic AMP-dependent protein kinase activity present in a tissue must be considered as well as the activation of varying pool sizes of protein kinases in response to specific stimuli. The relative amounts of type I and type II protein kinase vary among tissues; a striking example is the almost total lack of type I in bovine heart contrasted to type I predominating in rat heart (8, 9).

Recent evidence suggests different biological roles for type I and type II cyclic AMP-dependent protein kinases. In rat testes, Lee *et al.* (10) were able to show an alteration in the amount of type I and type II as a function of ontogeny. Whereas testes from fetal and 2-day-old rats exhibit only type I protein kinase, type II protein kinase levels increase gradually during the first postnatal weeks to reach maximal levels in testes of 25-day-old rats. Developmental alterations of type II protein kinase take place at a time coincident with the acquisition of the capacity for complete spermatogenesis and steroidogenesis. These studies suggest that type II protein kinase may be involved in the process of differentiation which is known to be sensitive to cyclic AMP levels and cyclic AMP-dependent protein kinase activation patterns (11, 12). Gharrett *et al.* (13) have reported the appearance of type I protein kinase in 3T3 cells after viral transformation.

Recent evidence from several sources suggests the selective actions of the two types of protein kinase in mediating the effects of cyclic AMP. Costa *et al.* (14) obtained evidence for cell cycle-specific expression of type I and type II protein kinases during the Chinese hamster ovary cell cycle: the activity and amount of type I cyclic AMP-dependent protein kinase are elevated during mitosis and the amount is relatively low during early G_1 and S phases; type II protein kinase activity and amount are low during mitosis, markedly increase at the G_1/S border, and decline rapidly during middle to late S phase. The use of puromycin, administered early in G_1, suggests that the increase in type II kinase at the G_1/S border was due to *de novo* synthesis of the kinase. This cell cycle-specific expression of type I and type II protein kinases suggests an orderly sequence of phosphorylation during the cell cycle, presumably catalyzed by a specific type of protein kinase. In isoproterenol stimulation of cardiac hypertrophy in the rat, type I protein kinase was increased in specific activity 2-fold after daily injection of the drug for 10 days (15). Recent reports indicate that the stimulation of mitogenesis in human lymphocytes by plant lectins such as phytohemagglutinin and concanavalin A is related to early activation of type I protein kinase and is inhibited by the simultaneous early activation of both type I and type II protein kinase that occurs in response to nonspecific stimuli such as

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analogues of cyclic AMP, prostaglandins, and high concentrations of concanavalin A (16). The above studies emphasize the importance of the types of protein kinase present, their relative amounts, and their selective activation. These parameters affect the ability of cyclic AMP to act as a second messenger (for review, see ref. 17).

Therefore, we examined the pool sizes of the cytoplasmic cyclic AMP-dependent protein kinase isozymes (type I and type II) in the tissues of intact rats and rats subjected to endocrine ablation (hypophysectomy, gonadectomy, or adrenalectomy). We present evidence that the total amount of type I cyclic AMP-dependent protein kinase activity decreases rapidly after endocrine ablation in classically steroid-responsive tissues (the ventral prostate and levator ani) and less rapidly in the liver. Type I protein kinase activity in the liver is decreased after hypophysectomy and adrenalectomy to the same extent as after castration. After castration, daily injections of dihydrotestosterone maintain control levels of type I cyclic AMP-dependent protein kinase in the ventral prostate and levator ani. After regression of the prostate for 3 days post-castration, three daily subcutaneous injections of 5α-dihydrotestosterone (500 μg/100 g of body weight) result in a complete return of type I protein kinase to intact levels. Our observations indicate that type II cyclic AMP-dependent protein kinase activity is not regulated by either steroid hormones or polypeptide hormones.

MATERIALS AND METHODS

Materials. [γ-^{32}P]ATP (5 Ci/mmol) and Omnifluor were purchased from New England Nuclear, Boston, MA. Unlabeled ATP was obtained from Grand Island Biological Co., Grand Island, NY. Trihydroaminomethane (Trizma), 5α-dihydrotestosterone (5α-androstan-17β-ol-3-one), 1,2-propanediol (propylene glycol), and phenylmethylsulfonyl fluoride were purchased from Sigma Chemical Co., St. Louis, MO. 3-Isobutyl-1-methylxanthine was obtained from Calbiochem, San Diego, CA, and DEAE-cellulose (Cellex-D, 0.51 meq/g) from Bio-Rad Laboratories, Richmond, CA. Tissues analyzed were taken from male Sprague–Dawley rats (200–220 g) housed in hanging metal cages and maintained on Purina rat chow *ad libitum* on a 12-hr photoperiod.

Animal Procedures. Animals to be castrated and their weight-matched controls were obtained from Hilltop, Chatsworth, CA. Bilateral orchiectomy was performed via the scrotal route under light ether anesthesia, and animals were killed by cervical dislocation up to 7 days postoperatively. Bilaterally adrenalectomized animals, hypophysectomized animals, and age-matched controls were purchased from Zivic-Miller, Allison Park, PA. Adrenalectomized animals were maintained on drinking water containing 0.9% saline. These animals were routinely killed 8 days postoperatively as were the hypophysectomized animals.

5α-Dihydrotestosterone was dissolved in warmed propylene glycol (500 μg/200 μl) and injected subcutaneously at a dose level of 500 μg/100 g of body weight; control animals received the injection vehicle alone. Injections were performed at 24-hr intervals. Animal procedures were generally carried out at the same time each day to avoid diurnal variations in experimental parameters. Tissues for analysis were removed rapidly and frozen on dry ice. Analysis was performed on pooled tissues from three or four animals.

DEAE-Cellulose Chromatography of the Protein Kinase Activities. Tissues were homogenized at 4° in 10 volumes of buffer (5 mM Tris, pH 7.5/4 mM EDTA/10 mM NaF) with a Polytron (Brinkman Instruments, Westbury, NY). The homogenates were centrifuged at 20,000 × *g* for 15 min, and 2.5 ml of the resulting supernatant was applied to a DEAE-cellulose column (0.7 × 10 cm) previously equilibrated with the above buffer. Protein concentration was assessed by the method of Bradford (18) to ensure that corresponding amounts of protein were present in each supernatant. Columns were washed with 15 ml of buffer, and the kinases were eluted with a linear salt gradient (0–0.35 M NaCl), total volume 30 ml. Fractions (1 ml) were collected and assayed for protein kinase activity. Type I and type II cyclic AMP-dependent protein kinases purified approximately 20-fold from rat heart and beef heart, respectively (8, 19), were used to determine the conditions required to separate and elute the kinases from DEAE-cellulose columns. To assess the recovery rate, prostates from intact rats and from rats castrated 3 days previously were assayed for activity of cyclic AMP-dependent protein kinase in the supernatant solution. After DEAE-cellulose chromatography, 85–90% of the supernatant kinase activity was recovered consistently. The pellets were extracted with 300 mM NaCl, dialyzed to remove salt, and rechromatographed on DEAE-cellulose. No more than 10% of the total amount of type II protein kinase was recovered in the salt-extracted pellet and less than 5% of type I. With samples from either castrated or intact rats, the amount of cyclic AMP-dependent protein kinase type I or type II recovered after salt extraction of the pellet was similar.

Protein Kinase Assay. Protein kinase activity was determined as described by Corbin *et al.* (20) with minor modifications. A 50-μl aliquot of each fraction was assayed in a total volume of 75 μl containing 20 mM sodium phosphate (pH 6.8), 0.5 mM 3-isobutyl-1-methylxanthine, 20 mM NaF, 25 mM $Mg(OAc)_2$, 200 μg of mixed calf thymus histone, 10 μM cyclic AMP, and 0.5–1.0 μCi of [γ-^{32}P]ATP plus sufficient unlabeled ATP to increase the total ATP concentration to 0.1 mM. The reaction was started by addition of 25 μl of the assay mixture containing the ATP and allowed to proceed for 10 min at 30°. A 50-μl aliquot was then removed and spotted on Whatman 3 MM paper filters. The filters were washed in 10% trichloroacetic acid for 20 min followed by two 10-min washes in 5% trichloroacetic acid and a final wash for 5 min in 95% ethanol. The filters were air dried and assayed for radioactivity in 5 ml of toluene-Omnifluor scintillation fluid. The reaction was linear for 20 min in a volume up to 75 μl under the conditions described above. When added to the protein kinase assay, cyclic AMP (10 μM) stimulated the kinase activity eluting as type I isozyme greater than 10-fold and type II kinase more than 5-fold for all rat tissues described.

RESULTS

Alterations in the Amount of Cyclic AMP-Dependent Protein Kinases in Ventral Prostate and Levator Ani in Response to Castration. The total amounts of type I and type II cyclic AMP-dependent protein kinase activity in the ventral prostate and levator ani were measured each day after castration. Both tissues, which are known to be steroid-responsive, had a marked decrease in type I kinase activity in response to castration (Fig. 1). In the ventral prostate, type I activity decreased to 70% of the intact controls within 1 day and to 56% and 57% of control on days 2 and 3, respectively. The amount of type II isozyme did not change in the prostate at any time after castration. In the levator ani, there was a substantial reduction in type I protein kinase similar to that observed in the prostate. Within 3 days after castration, type I had decreased to 48% of that in the intact controls. There was also some reduction in type II kinase, to 79% of that in the intact controls within 3 days. The marked reduction of type I isozyme in both the ventral prostate and levator ani occurred within 24 hr after castration.

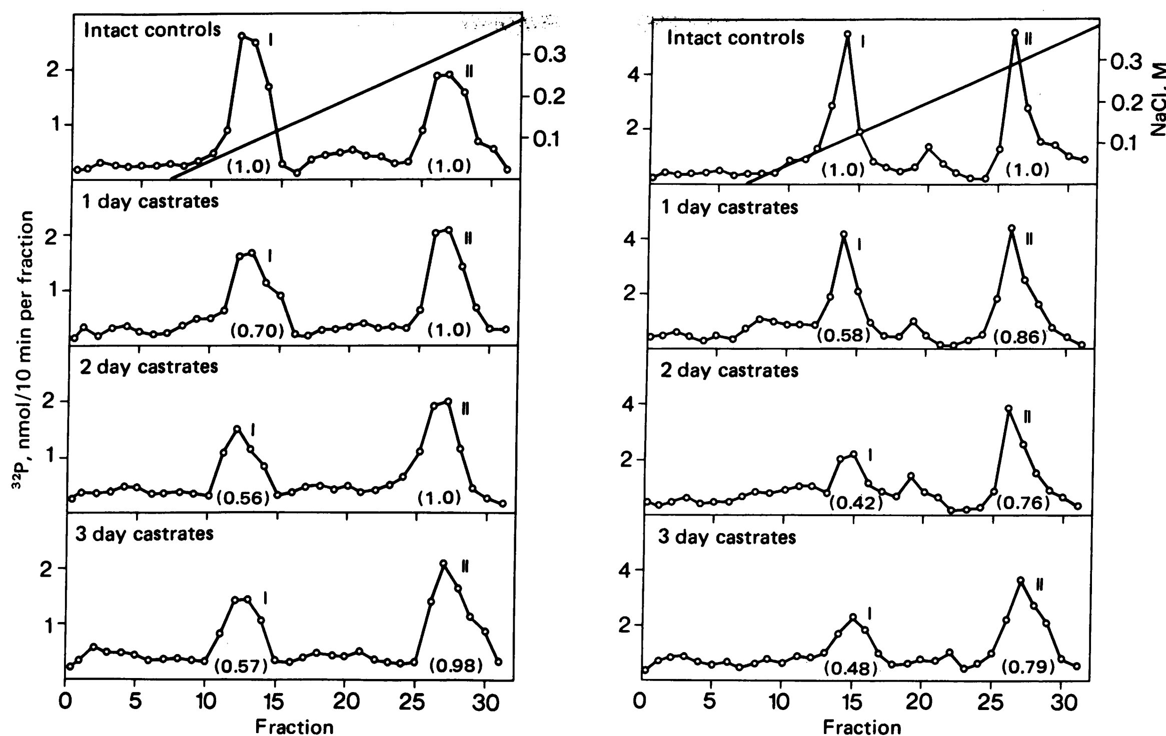

FIG. 1. Representative elution profiles after DEAE-cellulose chromatography of type I (I) and type II (II) cyclic AMP-dependent protein kinases of the ventral prostate. Chromatography was performed on sham-operated controls and at 1, 2, and 3 days after castration. (*Left*) Ventral prostate. Ten separate columns were run for each time point. Each column run was comprised of pooled tissue from three or four rats. The variation of chromatographs was less than 10%. (*Right*) Levator ani muscle. Five separate columns were run for each time point. Each column run was comprised of pooled tissue from three or four rats. The variation of chromatographs was less than 10%.

Type I and Type II Cyclic AMP-Dependent Protein Kinases in the Liver in Response to Castration. Type I protein kinase decreased to 69% of that in the intact controls within 3 days after castration (Fig. 2). There was not the rapid decline in type I kinase that was observed in steroid-responsive tissues, and there was no significant alteration in type II protein kinase after castration.

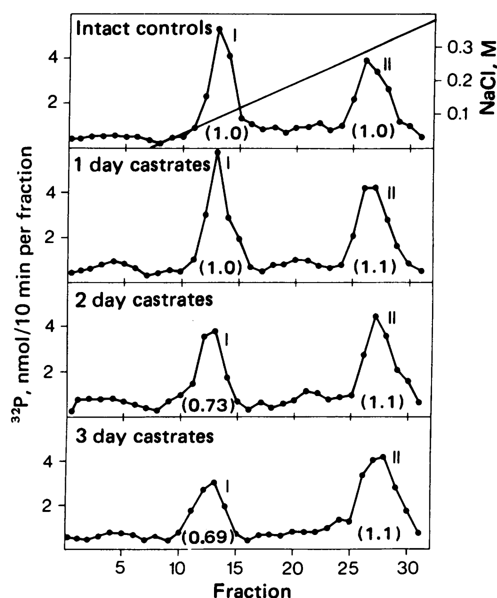

FIG. 2. Representative elution profiles after DEAE-cellulose chromatography of type I (I) and type II (II) cyclic AMP-dependent protein kinases of the liver. Chromatography of the liver was performed on sham-operated controls and at 1, 2, and 3 days after castration. Four separate columns were run for each time point. Each column run was comprised of pooled tissue from three or four rats. The variation of chromatographs was less than 10%.

Response of Cyclic AMP-Dependent Protein Kinases to Adrenalectomy and Hypophysectomy in Steroid-Responsive and Nonresponsive Tissues. In an attempt to determine whether the decrease in type I protein kinase that occurred following castration was directly related to the removal of steroid hormone or was mediated by alterations in adrenal and pituitary hormones, the distribution of the protein kinases in these tissues was studied after bilateral adrenalectomy and hypophysectomy. The tissues were removed from the animals 8 days after the operation. Bilateral adrenalectomy resulted in small decreases in type I and type II activity, to 90% and 94% of control levels, respectively, in the prostate (Fig. 3). The ventral prostate of hypophysectomized animals had atrophied to such a degree that no tissue could be recovered for analysis.

In the levator ani, there was no significant alteration in the distribution of type I and type II protein kinases after adrenalectomy compared to the sham-operated intact animals (Fig. 4 *left*). After hypophysectomy, however, there was a decrease of type I and type II activities to 42% and 88% of the controls, respectively. Reductions in type I and type II kinase activity were comparable to those observed in the levator ani of 2- and 3-day castrates (Fig. 2). Liver of adrenalectomized animals had decreased type I protein kinase (75% of controls, Fig. 4 *right*). Hypophysectomy also led to a decrease in type I protein kinase in the liver (64% of controls); the amount of type II protein kinase did not change after removal of either the adrenals or the pituitary.

Effect of Daily Dihydrotestosterone Maintenance on Protein Kinase Activity in the Ventral Prostate and Levator

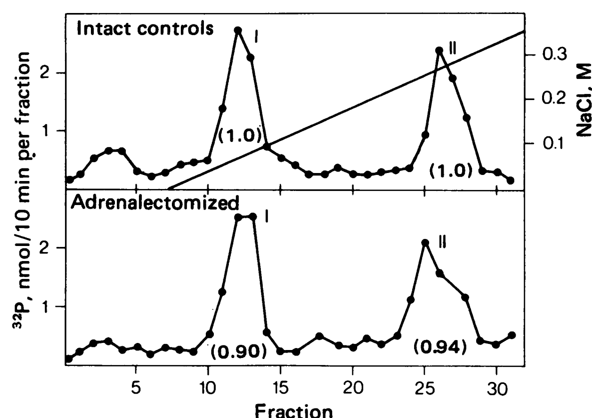

FIG. 3. Representative elution profiles after DEAE-cellulose chromatography of type I (I) and type II (II) cyclic AMP-dependent protein kinases in prostate of adrenalectomized rats and sham-operated controls. Chromatography was performed 8 days after adrenalectomy to ensure clearance of adrenal hormones. Ten separate columns were run for each time point. Each column run was comprised of pooled tissue from three or four rats. The variation of chromatographs was less than 10%.

Ani after Castration. At the time of castration, one group of rats was injected immediately with dihydrotestosterone and then daily for 2 days postoperatively in a maintenance regimen. In another group, the prostate was allowed to regress for 3 days after castration before daily injections (for 2 days) of dihydrotestosterone prior to sacrifice. Tissue from both groups was then analyzed for kinase isozyme activities. The amount of type I protein kinase activity did not decrease in the ventral prostate of castrated animals maintained on dihydrotestosterone, in contrast to the regression noted after complete androgen withdrawal for 3 days (Table 1). Treatment of 3-day castrates with dihydrotestosterone for 3 days resulted in the return of type I isozyme activity to a level comparable to that of the intact control animals.

Similar results were observed in the levator ani muscle (Table 1). A maintenance dose of dihydrotestosterone immediately after castration actually increased type I and type II activities to values greater than observed in tissue from control animals. However, although 3 days of daily injection of dihydrotestosterone given to 3-day castrates increased type I protein kinase activity from 48% to 93% of type I activity in intact animals, this treatment did not increase the amount of type II protein kinase activity.

Table 1. Effect of dihydrotestosterone (DHT) maintenance on type I and type II protein kinases of the ventral prostate and levator ani after castration

Tissue	Treatment	Protein kinase activity, % of intact control	
		Type I	Type II
Ventral prostate	Intact	100	100
	3-day castration	57	102
	Castration, maintained on DHT for 3 days	110	98
	3-day castration and DHT for 3 days	103	99
Levator ani	3-day castration	48	79
	Castration, maintained on DHT for 3 days	113	122
	3-day castration and DHT for 3 days	93	77

Animals were castrated via the scrotal route under light ether anesthesia. One group was allowed to regress for 3 days prior to subcutaneous injections of DHT daily for 3 days. The other group was placed on maintenance injections of DHT for 3 days starting at the time of operation. After sacrifice, the protein kinase isozyme profiles in the ventral prostate and levator ani of the two groups were determined by DEAE-cellulose chromatography.

DISCUSSION

The possibility that cyclic AMP-dependent protein kinases act as mediators of trophic stimuli has gained considerable substantiation in recent years. Indeed, the activation of these enzymes by cyclic AMP appears to be a more reliable measure of a cyclic AMP-mediated event than alterations in cyclic AMP levels (21). Several authors (22, 23) have reported their inability to stimulate proliferative responses in androgen target tissues, such as the ventral prostate, by the addition of cyclic AMP analogues and phosphodiesterase inhibitors and have concluded that the role of cyclic AMP in mediating androgen action does not include proliferation.

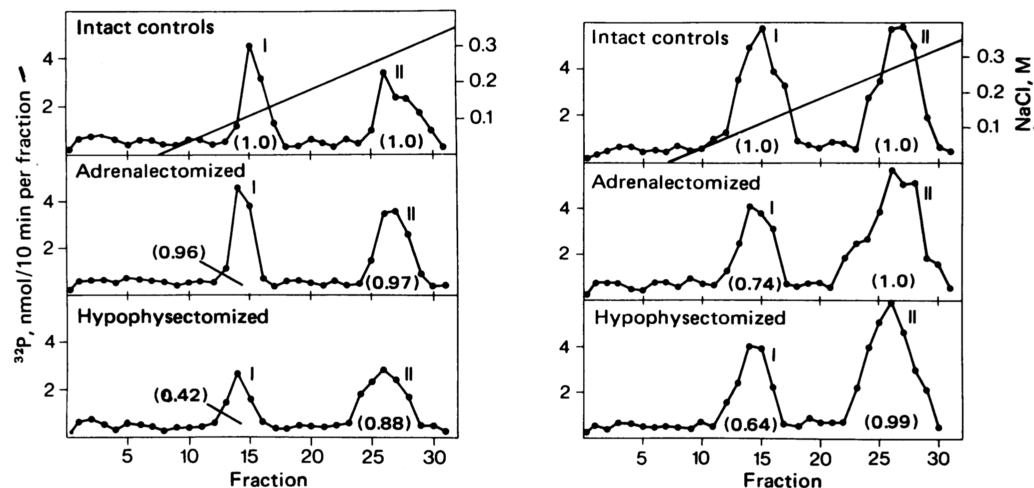

FIG. 4. Representative elution profiles after DEAE-cellulose chromatography of type I (I) and type II (II) cyclic AMP-dependent protein kinases in adrenalectomized, hypophysectomized, and sham-operated controls. Chromatography was performed 8 days after adrenalectomy or hypophysectomy. Three separate columns were run for each time point. Each column run was comprised of pooled tissue from three or four rats. The variation of chromatographs was less than 10%. (*Left*) Levator ani muscle. (*Right*) Liver.

Our data suggest that androgens may be working to mediate cyclic AMP events by modulating the amount of type I protein kinase activity in steroid target tissues. The failure of these authors to demonstrate responses to phosphodiesterase inhibitors and cyclic AMP may be explained by the decreased amount of type I protein kinase in the 7-day castrated rats used in their experiments. In our studies, the pool of type I isozyme decreased to 57% of control within 3 days.

In both the ventral prostate and the levator ani, tissues responsive to steroids, we observed a rapid decline of type I protein kinase activity which, in the case of the prostate, parallels the demonstrated post-castration decay of receptors, RNA synthesis, and dihydrotestosterone levels (24). Ornithine decarboxylase activity, present at high basal levels in prostate and also an integral part of the trophic response (2), declines to nondetectable levels within 3 days (unpublished data) and cannot be reïnduced in the 3-day castrates by phosphodiesterase inhibitors, but it can be stimulated in the intact prostate 3- to 4-fold by these compounds, supporting a role of the total amount of type I protein kinase activity dependent on testosterone maintenance in the ability of cyclic AMP to trigger a response.

The slower decline in the amount of type I protein kinase activity in liver after castration would suggest a different steroid control mechanism of nonandrogenic tissues. Indeed, the decline in type I isozyme activity in response to adrenalectomy and hypophysectomy, coupled with the failure of prostate and levator ani to respond to adrenalectomy, would support the concept of adrenal steroids contributing to the maintenance of kinase pools in the liver. A direct effect of androgens cannot be ruled out but work by other authors suggests that, although ACTH production in male rats increases after orchiectomy (25), castration has a direct effect on adrenocorticoid secretion by acting to increase 5α-reductase activity in the adrenal cortex, resulting in lowered levels of corticosterone and increased conversion to the 5α-reduced metabolites 5α-dihydrocorticosterone and $3\beta,5\alpha$-tetrahydrocortisone (26). Therefore, the change in adrenal metabolism may be the important component in the decrease in kinase pools seen on castration.

Although further studies are necessary, this study provides evidence linking steroid action to the ability of steroid-responsive tissues to maintain a substantial concentration of type I cyclic AMP-dependent protein kinase to be activated by cyclic AMP.

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1. Robison, G. A., Butcher, R. W. & Sutherland, E. W. (1971) *Cyclic AMP* (Academic Press, New York).
2. Russell, D. H., Byus, C. V. & Manen, C. A. (1976) *Life Sci.* **19**, 1297–1306.
3. Kuo, J. F. & Greengard, P. (1969) *Proc. Natl. Acad. Sci. USA* **64**, 1349–1355.
4. Singhal, R. L., Tsang, B. K. & Sutherland, D. J. (1976) in *Advances in Sex Hormone Research*, eds. Singhal, R. L. & Thomas, J. A. (University Park Press, Baltimore, MD), Vol. 2, pp. 325–424.
5. Tsang, B. K. & Singhal, R. L. (1976) *Pharmacologist* **18**, 164.
6. Wilson, M. J. & Ahmed, K. (1977) *Exp. Cell Res.* **106**, 151–157.
7. Liao, S. & Fang, S. (1969) *Vitam. Horm. NY* **27**, 17–90.
8. Corbin, J. D., Kelly, S. L., Soderling, T. R. & Park, C. R. (1975) in *Advances in Cyclic Nucleotide Research*, eds. Drummond, G. I., Greengard, P. & Robison, G. A. (Raven Press, New York), Vol. 5, pp. 265–279.
9. Rosen, O. M., Erlichman, J. & Rubin, C. S. (1975) in *Advances in Cyclic Nucleotide Research*, eds. Drummond, G. I., Greengard, P. & Robinson, G. A. (Raven Press, New York), Vol. 5, pp. 253–263.
10. Lee, P. D., Radloff, D., Schweppe, J. S. & Jungmann, R. A. (1976) *J. Biol. Chem.* **251**, 914–921.
11. O'Neill, J. P., Schröder, C. H., Riddle, J. C. & Hsie, A. W. (1976) *Exp. Cell Res.* **97**, 213–217.
12. Porter, K. R., Puck, T. T., Hsie, A. W. & Kelley, D. (1974) *Cell* **2**, 145–162.
13. Gharrett, A. J., Malkinson, A. M. & Sheppard, J. R. (1976) *Nature* **264**, 673–675.
14. Costa, M., Gerner, E. W. & Russell, D. H. (1976) *J. Biol. Chem.* **251**, 3313–3319.
15. Byus, C. V., Chubb, J. M., Huxtable, R. J. & Russell, D. H. (1976) *Biochem. Biophys. Res. Commun.* **73**, 694–702.
16. Byus, C. V., Klimpel, G. R., Lucas, D. O. & Russell, D. H. (1977) *Nature* **268**, 63–64.
17. Jungmann, R. A. & Russell, D. H. (1977) *Life Sci.* **20**, 1787–1798.
18. Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254.
19. Corbin, J. D. & Reimann, E. M. (1974) in *Methods in Enzymology*, eds. Hardman, J. G. & O'Malley, B. W. (Academic Press, New York), Vol. 38, pp. 287–290.
20. Corbin, J. D., Keely, S. L. & Park, C. R. (1975) *J. Biol. Chem.* **250**, 218–225.
21. Costa, M., Manen, C. A. & Russell, D. H. (1975) *Biochem. Biophys. Res. Commun.* **65**, 75–81.
22. Craven, S., Lesser, B. & Bruchovsky, N. (1974) *Endocrinology* **95**, 1177–1180.
23. Mangan, F. R., Pegg, A. E. & Mainwaring, W. I. P. (1973) *Biochem. J.* **134**, 129–142.
24. Liao, S. (1976) in *Receptors and Mechanism of Action of Steroid Hormones*, ed. Pasqualini, J. R. (Marcel Dekker, Inc., New York), Part I. pp. 159–214.
25. Coyne, M. D. & Kitay, J. I. (1971) *Endocrinology* **89**, 1024–1028.
26. Colby, H. D. & Kitay, J. I. (1972) *Endocrinology* **91**, 1523–1527.