Biochemical Pharmacology, Vol. 24, pp. 2097–2099. Pergamon Press. 1975. Printed in Great Britain.

SHORT COMMUNICATIONS

Relation of the pituitary gland to the actions of testosterone on hepatic ethylmorphine metabolism in rats

(*Received* 13 *December* 1974; *accepted* 28 *February* 1975)

The oxidative metabolism of many drugs and steroids by rat liver microsomes is sex-dependent [1–6]. Substrates such as hexobarbital, ethylmorphine and testosterone are metabolized far more rapidly by males than females. Differences in hepatic microsomal content of cytochrome P-450 cannot fully account for sex differences in drug and steroid oxidation [4–8]. However, liver microsomes from male rats have been shown to have a greater affinity for many substrates, as determined by both spectral and enzymatic methods, than those from females [4, 5, 9, 10]. Moreover, the magnitude of the type I spectral change produced by various substrates is greater in livers from males [4, 5] as is the substrate-stimulated rate of reduction of hepatic cytochrome P-450 [7, 8].

Sex differences in hepatic mixed-function oxidase systems have been attributed largely to the actions of testosterone. Orchiectomy lowers oxidative enzyme activity, while testosterone administration to female or castrated male rats increases metabolism [1–3, 6, 9–13]. However, androgens influence the secretion of many other hormones, particularly those of pituitary origin. Thus, the actions of testosterone on hepatic metabolism could be intertwined with those of other hormones. In fact, we have recently demonstrated [14] that the effects of gonadal hormones on hepatic steroid Δ^4-hydrogenase activity are not demonstrable in hypophysectomized animals, suggesting mediation by or interaction with (a) pituitary factor(s). The present studies were therefore carried out to determine if the pituitary gland was also required for androgenic effects on hepatic oxidative metabolism. The results indicate that the actions of testosterone on ethylmorphine oxidation are not manifested in the absence of the pituitary gland.

Sprague–Dawley rats, 60–75 days old (obtained from Zivic–Miller Laboratories, Pittsburgh, Pa.) were maintained at 22·0 ± 1·0° under standardized conditions of light (0600–1800) on a diet of Purina Laboratory Chow and water *ad lib.* Parapharyngeal hypophysectomies and orchiectomies were performed at approximately 60 days of age and hormonal replacement was initiated approximately 48 h later. Completeness of hypophysectomy was determined by visual inspection at autopsy and by a significant decline in adrenal weight. Testosterone was administered as a single subcutaneous depot preparation (Testosterone Cypionate) at a dose of 5 mg/100 g of body weight. ACTH was given as a daily subcutaneous injection (Cortrosyn Depot, Organon, 100 μg/kg). All rats were sacrificed after 10 days of hormonal replacement.

All animals were decapitated between 9 and 10 a.m. and livers quickly removed and homogenized in cold 1·15% potassium chloride. Homogenates were centrifuged at 9000 *g* for 20 min in a Sorvall refrigerated centrifuge. Aliquots of the supernatant were removed for enzyme assays and the rest was centrifuged at 105,000 *g* for 60 min in a Beckman preparative ultracentrifuge. The microsomal pellet was suspended in 1·15% potassium chloride containing 0·05 M Tris-HCl buffer, pH 7·4. Substrate-induced type I difference spectra (Δ O.D.$_{385\ 420}$) were obtained using a Cary 17 recqrding spectrophotometer at room temperature. Microsomal suspensions contained 3–4 mg protein/ml. Spectral dissociation constants were calculated by the method of Schenkman *et al.* [15]. Cytochrome P-450 was measured as described by Omura and Sato [16] and microsomal protein was determined by the method of Lowry *et al.* [17].

Reaction mixtures for drug metabolism assays contained either aniline (5 μmoles) or ethylmorphine (10 μmoles) and 0·5 ml liver 9000 *g* supernatant (200 mg/ml), glucose-6-phosphate (9 μmoles), $MgSO_4$ (24·2 μmoles), NADP (2·08 μmoles) and Tris-HCl buffer (0·02 M), pH 7·4, in a final volume of 3·0 ml. Semicarbazide-HCl (25 μmoles) served as a trapping agent for formaldehyde produced from ethylmorphine. Incubations were carried out in a Dubnoff metabolic incubator at 37° for 15 min under air. Formaldehyde [18] and *p*-aminophenol [3] were assayed by methods previously described. All samples were read against appropriate tissue blanks.

Body and liver weights were increased by testosterone administration to intact but not hypophysectomized female rats (Table 1). As previously noted [1–3, 9–13], treatment of normal females with testosterone increased the rate of ethylmorphine metabolism by hepatic microsomes (Table 1). The increment in ethylmorphine oxidation was independent of the mode of expression and was equally valid whether expressed per unit of tissue weight, microsomal protein or cytochrome P-450.

The lack of effect of testosterone on aniline hydroxylase activity (Table 1) is consistent with the absence of a sex difference in aniline metabolism [2, 11]. Cytochrome P-450 concentrations in hepatic microsomes also were not affected by testosterone. Some investigators [13] have noted an increase in cytochrome P-450 content after testosterone treatment, but the change observed was relatively small and probably related to both the dose of androgen employed and the duration of treatment. The amount of testosterone given in the present studies is one which we have previously found to maintain normal sex accessory tissue weights in castrated or hypophysectomized male rats.

The magnitude of the type I spectral change produced by ethylmorphine in hepatic microsomes was unaffected by testosterone administration to intact females (Table 1). However, the spectral dissociation constant (K_s) was significantly reduced, indicating an increased affinity of cytochrome P-450 for the substrate. The latter is in agreement with the observations of other investigators [9–13] and could provide a mechanism for the increase in ethylmorphine demethylation. However, the work of Gigon *et al.* [7, 8] indicates that an increase in the rate of substrate-stimulated cytochrome P-450 reduction contributes significantly to the increment in ethylmorphine metabolism in response to testosterone.

Removal of the pituitary gland produced a decline in ethylmorphine metabolism in female rats (Table 1). Accompanying the fall in enzyme activity was a decrease in K_s and increase in the magnitude of the ethylmorphine-induced spectral change. Since testosterone administration and hypophysectomy produced similar changes in K_s but opposite effects on demethylase activity, androgenic

Table 1. Effects of testosterone administration to intact and hypophysectomized female rats on hepatic oxidative metabolism*

	Intact female		Hypophysectomized	
	Control	Testosterone	Control	Testosterone
Body weight (g)	263·0 ± 7·3	289·6 ± 4·5†	198·9 ± 3·8	188·9 ± 5·7
Liver weight (g)	10·1 ± 0·4	11·3 ± 0·4‡	5·7 ± 0·2	5·7 ± 0·2
Microsomal protein (mg/g liver)	30·0 ± 0·8	33·0 ± 1·2	32·0 ± 0·8	30·1 ± 1·1
Cytochrome P-450 (nmoles/mg prot)	0·54 ± 0·04	0·50 ± 0·03	0·62 ± 0·05	0·57 ± 0·02
Ethylmorphine metabolism				
(nmoles/min/g liver)	142·2 ± 8·9	229·2 ± 26·5†	107·8 ± 11·3	99·0 ± 18·3
(nmoles/min/P-450)	9·1 ± 0·5	14·2 ± 0·7†	5·4 ± 0·6	5·7 ± 0·8
Aniline metabolism				
(nmoles/min/g liver)	31·5 ± 3·5	35·3 ± 3·0	34·0 ± 3·7	31·6 ± 4·8
(nmoles/min/P-450)	1·97 ± 0·20	2·42 ± 0·29	1·72 ± 0·15	1·82 ± 0·23
Ethylmorphine type I spectrum				
(Δ O.D./mg prot)10^3	7·0 ± 1·2	7·6 ± 0·9	12·6 ± 1·5	11·6 ± 0·3
$(K_s)\ 10^{-5}$	10·1 ± 2·5	3·8 ± 0·6†	4·5 ± 1·1	4·6 ± 0·6

* Values expressed as mean ± S.E.; six to ten animals per group.
† $P < 0·01$ (vs control group).
‡ $P < 0·05$ (vs control group).

actions on ethylmorphine metabolism cannot logically be attributed solely to changes in K_s. Additional studies are needed to define the specific hormonal factor(s) responsible for the changes related to ethylmorphine metabolism resulting from hypophysectomy. Aniline hydroxylation was unaffected by hypophysectomy.

In contrast to its actions in normal females, testosterone, when given to hypophysectomized rats, had no effect on the rate of ethylmorphine oxidation (Table 1). Similarly, the magnitude of the ethylmorphine type I difference spectrum and the spectral dissociation constant for ethylmorphine were unaffected by testosterone administration to hypophysectomized animals. The actions of testosterone on ethylmorphine metabolism in female rats, therefore, seem to require the presence of the pituitary gland.

Preliminary observations indicate a similar pituitary dependence for androgenic actions in males (Table 2). Orchiectomy decreased the rate of ethylmorphine demethylation, an effect fully reversed by testosterone replacement (Table 2). Removal of the pituitary gland in male rats lowered metabolism even more than castration. However, as in females, testosterone, when given to hypophysectomized males, was without effect on ethylmorphine oxidation (Table 2). Moreover, although ACTH administration to hypophysectomized male rats increased ethylmorphine metabolism (358·7 ± 19·2 vs 247·3 ± 16·7 nmoles/min/g of liver; $P < 0·01$), simultaneous treatment with ACTH and testosterone produced no additional increment (329·8 ± 20·3), excluding a dependence on or interaction with adrenal steroids. The observations of Ichii and Yago

Table 2. Effects of testosterone administration to orchiectomized and hypophysectomized male rats on hepatic ethylmorphine metabolism*

Group	Body weight (g)	Liver weight (g)	Ethylmorphine metabolism	
			(nmoles/min/g liver)	(nmoles/min/mg prot)
Intact (8)	381·3 ± 13·1	15·3 ± 0·9	698·0 ± 62·1	14·4 ± 1·1
Orchiectomy (9)	339·0 ± 10·8†	12·9 ± 0·6†	320·2 ± 38·4†	6·7 ± 0·8†
Orchiectomy + testosterone (9)	370·0 ± 10·6‡	15·2 ± 0·1‡	762·1 ± 58·4‡	14·8 ± 1·0‡
Hypophysectomy (9)	191·1 ± 4·7†	6·4 ± 0·3†	195·3 ± 14·8†	5·1 ± 0·5†
Hypophysectomy + testosterone (9)	197·8 ± 7·5†	5·8 ± 0·5†	198·7 ± 22·0†	4·9 ± 0·5†

* Values expressed as mean ± S.E.; number of animals per group indicated in parentheses.
† $P < 0·05$ (vs intact group).
‡ $P < 0·05$ (vs orchiectomy group).

[19] also indicate independent effects of gonadal and adrenal steroids on hepatic oxidative metabolism. These observations indicate that the effects of testosterone to promote hepatic demethylase activity are not the result of direct and independent actions on the liver. Thus, the mechanism of action of testosterone to enhance hepatic oxidative metabolism differs from that of several synthetic steroids, including cyproterone acetate and pregnenolone-16α-carbonitrile [20, 21], whose effects are fully manifested in hypophysectomized animals.

It is presently not known whether the effects of testosterone are mediated completely by the pituitary gland or if testosterone interacts with some pituitary-dependent factor at the hepatic cell. The results do, however, suggest an important role for the pituitary in the regulation of hepatic mixed-function oxidases. Studies are now in progress to identify the pituitary factor(s) involved and determine the mechanism(s) of interaction with testosterone.

Acknowledgements—The technical assistance of Marlene Pope is gratefully acknowledged. These investigations were supported by NSF Research Grant GB41215.

Departments of Physiology and Biophysics and Biochemistry, West Virginia University Medical Center, Morgantown, W. Va. 26506, U.S.A.

ROBERT E. KRAMER
JOHN W. GREINER
WILLIAM J. CANADY
HOWARD D. COLBY

REFERENCES

1. J. R. Gillette, D. C. Davis and H. A. Sasame, *A. Rev. Pharmac.* **12**, 57 (1972).
2. G. P. Quinn, J. Axelrod and B. B. Brodie, *Biochem. Pharmac.* **1**, 152 (1958).
3. R. Kato and J. R. Gillette, *J. Pharmac. exp. Ther.* **150**, 279 (1965).
4. D. S. Davies, P. L. Gigon and J. R. Gillette, *Life Sci.* **8**, 85 (1969).
5. J. B. Schenkman, I. Frey, H. Remmer and R. W. Estabrook, *Molec. Pharmac.* **3**, 516 (1967).
6. R. Kuntzman, R. Welch and A. H. Conney, *Adv. Enzyme Regulat.* **4**, 149 (1966).
7. P. L. Gigon, T. E. Gram and J. R. Gillette, *Biochem. biophys. Res. Commun.* **31**, 558 (1969).
8. P. L. Gigon, T. E. Gram and J. R. Gillette, *Molec. Pharmac.* **5**, 109 (1969).
9. R. Kato and K. Onoda, *Biochem. Pharmac.* **19**, 1649 (1970).
10. M. E. Hamrick, N. G. Zampaglione, B. Stripp and J. R. Gillette, *Biochem. Pharmac.* **22**, 293 (1973).
11. R. Kato and J. R. Gillette, *J. Pharmac. exp. Ther.* **150**, 286 (1965).
12. R. Kato, K. Onoda and M. Sasjima, *Jap. J. Pharmac.* **20**, 194 (1970).
13. S. E. Defrawy E. Masry and G. J. Mannering, *Drug Metab. Dispos.* **2**, 279 (1974).
14. H. D. Colby, J. H. Gaskin and J. I. Kitay, *Endocrinology* **92**, 769 (1973).
15. J. Schenkman, H. Remmer and R. W. Estabrook, *Molec. Pharmac.* **3**, 113 (1967).
16. T. Omura and R. Sato, *J. biol. Chem.* **239**, 2370 (1964).
17. O. H. Lowry, N. J. Rosebrough, A. O. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
18. T. Nash, *Biochem. J.* **55**, 416 (1953).
19. S. Ichii and N. Yago, *J. Biochem.* **65**, 597 (1969).
20. S. Szabo, K. Kovacs, B. D. Garg, B. Tuchweber and G. Lazar, *Hormone Metab. Res.* **5**, 109 (1973).
21. B. D. Garg, S. Szabo, J. D. Khandekar and K. Kovacs, *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak*, **269**, 7 (1971).

Biochemical Pharmacology, Vol. 24, pp. 2099–2100. Pergamon Press, 1975. Printed in Great Britain.

ATP-induced changes in microsomal Mg^{2+} levels and relationship to muscle contraction in isolated guinea-pig ileum

(*Received* 23 *January* 1975; *accepted* 7 *April* 1975)

ATP has either an excitatory (contractile) or inhibitory (relaxing) effect on smooth muscle depending on the tissue [1]. Daniel and Irwin [2] have suggested that the contractile effect of ATP may be due its ability to complex Mg^{2+} in the cell membrane, thereby favoring Ca^{2+} entry and contraction. The present study was performed in an attempt to test this hypothesis and to determine effects of adenine nucleotides on the Ca^{2+} and Mg^{2+} binding activity of the membrane fraction isolated from guinea-pig ileum.

Guinea-pigs, weighing 350–500 g, were sacrificed by a blow on the head, and immediately the muscle layer of the ileum was dissected by the method of Ambache [3]. The microsomal fraction was prepared by the method of Schneider and Hogeboom [4]. The homogeneity of the subcellular fractions was checked by electron microscopy and the activities of the following marker enzymes were measured; NADH-cytochrome *c* reductase [5], glucose-6-phosphatase [6] and 5′-nucleotidase [7]. Ca^{2+} and Mg^{2+} binding activity of the fraction was measured according to the procedure described by Carvalho and Leo [8]. Microsomes (1 mg/3 ml of medium) were incubated in medium containing 150 mM KCl, 5 mM $MgCl_2$, 0·1 mM $CaCl_2$ and Tris–HCl (pH 7·4) with or without nucleotides at 25° for 10 min. The suspension was centrifuged at 0–5° for 30 min at 105,000 *g*. Bound Ca^{2+} and Mg^{2+} were determined by atomic absorption spectrophotometry. Protein concentration was determined by the method of Lowry *et al.* [9].

The activities of the marker enzymes (Table 1) indicate a satisfactory purity of the microsomal fraction.

As shown in Fig. 1, the Mg^{2+} binding activity of the microsomal fraction was significantly decreased by ATP and ADP at concentrations greater than 1 mM, while the Ca^{2+} binding activity was significantly increased only by ATP at concentrations of 0·1–6 mM. 5′-AMP, 3′-AMP and adenosine did not have any effect on the binding activities. However, the decreasing effect of ATP on the Mg^{2+} binding activity was significantly potentiated by 5′-AMP, but not by 3′-AMP or adenosine (Fig. 1). Similarly the effect of ADP was potentiated by 5′-AMP (Fig. 1). However, the increasing effect of ATP on the Ca^{2+} binding activity was not affected by 5′-AMP, 3′-AMP or adenosine.

Previous studies [10, 11] in this laboratory demonstrated that among the adenine nucleotides only ATP and ADP produce contraction in isolated guinea-pig ileum and their contractile effects are specifically potentiated by 5′-AMP. It was suggested that this phenomenon may provide a clue to elucidate the contractile mechanism of ATP. The present study indicates that these pharmacological effects