Testosterone and 6-*N*,2'-*O*-Dibutyryladenine 3':5'-Cyclic Monophosphate Stimulate Protein and Lysosomal Enzyme Secretion in Rat Seminal Vesicle

By HAROLD KOENIG,*† CHUNG Y. LU* and ROY BAKAY*

*Neurology Service, Veterans Administration Lakeside Hospital, and †Department of Neurology, Northwestern University Medical School, Chicago, IL 60611, U.S.A.

(Received 6 May 1976)

Rat seminal-vesicle secretion was studied *in vitro* in a slice-incubation system. Seminal-vesicle slices were preincubated with $^{32}\text{P}_i$ for 15 min, rinsed, and incubated in an isotope-free 'chase' medium for up to 4 h. Gland slices spontaneously discharged protein, three lysosomal hydrolases and trichloroacetic acid-insoluble ^{32}P into the medium in a time- and temperature-dependent manner. Testosterone ($10\text{ }\mu\text{M}$) and dibutyryl cyclic AMP (1 mM) stimulated the discharge of protein, acid hydrolases and trichloroacetic acid-insoluble ^{32}P , and also stimulated the incorporation of $^{32}\text{P}_i$ into trichloroacetic acid-insoluble components. The acid phosphatase and β -*N*-acetylhexosaminidase isoenzymes were separated by isoelectric focusing. These hydrolases were secreted into the medium as acidic isoenzymes, presumably contained within primary lysosomes, whereas they occurred largely as less acidic and basic isoenzymes in the glandular tissue.

The seminal vesicles are androgen-dependent accessory sex glands which in most mammals contribute a protein-rich secretion containing fructose, citric acid, ascorbic acid, choline, glycerophosphorylcholine and prostaglandins to the seminal plasma (Mann, 1964). Biochemical studies of the seminal vesicle have dealt largely with the androgenic stimulation of metabolic and synthetic processes (Singhal & Sutherland, 1975) and the uptake and metabolism of androgens (Tveter *et al.*, 1975). However, the molecular and biochemical mechanisms of secretion in this gland have received little or no attention.

We previously found that testosterone *in vivo* rapidly decreased the equilibrium density of lysosomes and caused their enlargement in the rat seminal vesicle (Koenig *et al.*, 1975). A cytological study showed that testosterone also stimulated the discharge of basophilic secretory granules and acid phosphatase-reactive lysosomes into the gland lumen, apparently by exocytosis (merocrine secretion) (Koenig & Hughes, 1975). In the present paper we report that testosterone and dibutyryl cyclic AMP stimulate the rat seminal vesicle to secrete protein and several lysosomal hydrolases *in vitro*. These hormones also stimulate the formation and the secretion of phosphorylated constituents in this gland.

Experimental

Materials

Chemicals and substrates were obtained from sources previously described (Goldstone & Koenig, 1974a). The hormones were purchased from Sigma

Chemical Co., St. Louis, MO, U.S.A. and $^{32}\text{P}_i$ as $\text{Na}_3^{32}\text{PO}_4$ from ICN Pharmaceuticals, Irvine, CA, U.S.A.

Methods

Incubation procedures. The seminal vesicles were removed from decapitated adult male Sprague-Dawley rats (180–250 g), cut into slices ($\sim 0.5\text{ mm}$) and rinsed in cold buffer. The incubation medium was a Krebs-Ringer buffer with a full complement of amino acids (Eagle, 1959) in a ratio of 1 g of tissue to 100 ml of medium. The pooled slices from three rats were preincubated for 15 min in the medium with $^{32}\text{P}_i$ (50 μCi per 100 ml of medium) with constant shaking in an atmosphere of $\text{O}_2 + \text{CO}_2$ (95:5). The slices were removed by centrifugation, rinsed in the same medium without isotope, and distributed equally between four flasks in the isotope-free medium equilibrated with $\text{O}_2 + \text{CO}_2$ (95:5). Flasks containing dibutyryl cyclic AMP (1 mM) and testosterone ($10\text{ }\mu\text{M}$) and a control flask without added hormone were incubated at 37°C with continuous shaking for 4 h. A fourth flask was kept at 0°C . Samples of the incubation medium were removed at intervals for analysis. At the end of the incubation, the tissue and medium were separated by centrifugation (1000 *g* for 10 min) and stored at -20°C .

Analytical procedures. Protein was determined by the method of Lowry *et al.* (1951), after precipitating with 10% (w/v) trichloroacetic acid. Acid phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2) was assayed with a *p*-nitrophenyl phosphate substrate (Andersch & Szczypinski,

1947); β -N-acetylhexosaminidase (β -2-acetamido-2-deoxy-D-glucoside acetamidodeoxyglucosylhydrolase, EC 3.2.1.30) with a 4-methylumbelliferyl- β -D-glucosaminide substrate (Robinson *et al.*, 1967); and arylsulphatase (arylsulphate sulphohydrolase, EC 3.1.6.1) with a *p*-nitrocatechol substrate (Roy, 1958). For measurement of the trichloroacetic acid-insoluble ^{32}P , proteins were precipitated and washed with 10% trichloroacetic acid, taken up in 0.5 M-NaOH, 10ml of Aquasol (New England Nuclear

Corp., Boston, MA, U.S.A.) was added and the samples were counted for radioactivity in a Beckman LS-250 liquid-scintillation system.

Isoelectric focusing. The enzymes in the incubation medium were concentrated by pressure dialysis against 0.05 M-glycine/NaOH buffer, pH 8.7, in an Amicon ultrafiltration cell with a UM 10 membrane (Amicon Corp., Lexington, MA, U.S.A.). The tissues were sonicated in 0.1 M-glycine/NaOH buffer, pH 9, in 0.2% Triton X-100, and the extracts were clarified

Fig. 1. Effect of temperature, testosterone and dibutyryl cyclic AMP on the release of protein (a), lysosomal acid hydrolases (b, c and d) and trichloroacetic acid-insoluble ^{32}P (e) by rat seminal-vesicle slices during incubation *in vitro*

Seminal-vesicle slices from intact rats were preincubated with $^{32}\text{P}_i$ for 15 min, rinsed and incubated at 37°C in an isotope-free 'chase' medium for 4 h in the presence of 10 μM -testosterone (Δ), 1 mM-dibutyryl cyclic AMP ($\square$), or without added hormone ($\blacksquare$). A fourth flask was maintained at 0°C ($\bullet$). Supernatant samples were removed at stated intervals for analysis. The results are means $\pm$ S.E.M. for three experiments. * $P < 0.05$; ** $P < 0.01$ compared with the 37°C control.

Table 1. *Effect of temperature, testosterone and dibutyl cyclic AMP on the formation and secretion of trichloroacetic acid-insoluble ^{32}P by the rat seminal vesicle*

Seminal-vesicle slices from male rats were pulse-labelled with $^{32}\text{P}_i$ for 15 min and incubated in a 'chase' medium for 4 h as described in the legend to Fig. 1 and in the text. The results are means $\pm$ S.E.M. of three experiments. The percentage of the 0°C control value is given in parentheses. * $P < 0.05$; ** $P < 0.01$ as compared with the 37°C control.

Experiment	$10^{-4} \times$ Trichloroacetic acid-insoluble radioactivity (c.p.m./g of tissue)		
	In tissue slice	Secreted into medium	Total (slice & medium)
0°C control	1.27 $\pm$ 0.06* (100%)	2.18 $\pm$ 0.12* (100%)	3.45 $\pm$ 0.13* (100%)
37°C control	2.44 $\pm$ 0.33 (192%)	3.01 $\pm$ 0.03 (138%)	5.45 $\pm$ 0.33 (158%)
Dibutyl cyclic AMP (1 mM)	3.23 $\pm$ 0.23 (254%)	6.21 $\pm$ 0.24** (285%)	9.44 $\pm$ 0.33** (274%)
Testosterone (10 μM)	3.01 $\pm$ 0.26 (237%)	7.39 $\pm$ 0.38** (339%)	10.40 $\pm$ 0.46** (301%)

by centrifuging at 100000g for 60 min. Isoelectric focusing was done in a pH 10–3 ampholine gradient in an LKB model 7100 column at 4°C for 24 h as previously described (Goldstone & Koenig, 1947a).

Results

During incubation at 37°C without added hormones, control slices spontaneously discharged protein, enzymes and trichloroacetic acid-insoluble ^{32}P into the medium (Fig. 1). The spontaneous discharge of protein and hydrolases began at once at a maximum rate and then slowed down, but was still progressing after 4 h, at which time 13–17% of the protein and hydrolases had been released. The discharge of trichloroacetic acid-insoluble ^{32}P attained a maximum value of 55% by 30 min of incubation and then stopped. The spontaneous discharge of protein, hydrolases and trichloroacetic acid-insoluble ^{32}P was substantially decreased by incubating slices at 0°C, indicating that secretion is dependent oxidative metabolism. Testosterone and dibutyl cyclic AMP produced a significant increase in the rate of discharge of protein and acid hydrolases that was greatest during the first 30 min of incubation, but produced a maximum increase in the rate of discharge of labelled material between 30 and 60 min of incubation. After 4 h of incubation in the presence of testosterone and dibutyl cyclic AMP, 66 and 71% of the trichloroacetic acid-insoluble ^{32}P appeared in the medium, the total secretion amounting to 246 and 206% respectively of the 37°C controls (Table 1). The hormones also elicited a 173–191% increase in incorporation of $^{32}\text{P}_i$ into trichloroacetic acid-insoluble material.

Fig. 2 compares the isoelectric-focusing pattern of the acid phosphatase and β -N-acetylhexosaminidase in the vesicular tissue with that of the enzymes discharged into the medium by seminal-vesicle slices

incubated in the presence of 1 μM -5 α -dihydro-testosterone for 1 h. This androgenic steroid stimulated the discharge of protein and hydrolases by 23–46% over the control slices. Both enzymes occurred in multiple molecular forms with pI values ranging between 4.1 and 8.4. However, the acidic forms of these hydrolases predominated in the incubation medium, whereas in the glandular tissue, the less acidic and the basic forms predominated. Similar results were obtained with the enzymes that were released spontaneously into the medium by control tissue slices. The isoelectric-focusing profile of the E_{280} of the incubation medium and the tissue extract also differed substantially, indicating differences in their protein composition (results not shown).

Discussion

Our results show that rat seminal-vesicle slices spontaneously discharged protein, three lysosomal hydrolases and acid-insoluble ^{32}P into the medium in a time- and temperature-dependent fashion during incubation *in vitro*. Testosterone and the dibutyl cyclic AMP analogue stimulated the discharge of all these constituents. Non-specific leakage of cytoplasmic constituents from damaged cells can be excluded as a significant factor in these incubation experiments by the finding that the isoelectric focusing patterns of proteins and lysosomal enzymes in the incubation medium and the tissue were sharply divergent. It is therefore reasonable to conclude that the spontaneous and hormone-evoked discharge of proteins and lysosomal enzymes *in vitro* is a valid representation of secretory activity.

Our experiments appear to provide the first demonstration that androgens exert an immediate, direct secretagogue action on the gland. The finding

Fig. 2. Isoelectric-focusing patterns of (a) acid phosphatase and (b) β -N-acetylhexosaminidase of rat seminal-vesicle tissue and the incubation medium

The incubation medium was collected after seminal-vesicle slices had been incubated in the presence of $1\mu\text{M}$ -5 α -dihydrotestosterone for 1 h at 37°C , desalted and concentrated by pressure dialysis. The tissue enzymes were extracted from unincubated seminal-vesicle slices. The isoelectric points (pI) of the fractions are given on the abscissa. Additional details are given in the text. Enzymes in incubation medium ($\circ$); in tissue ($\bullet$).

that dibutyryl cyclic AMP mimicked the androgenic steroids in stimulating vesicular secretion suggests that the secretagogue action of androgens may be mediated by the adenyl cyclase/cyclic AMP system. Cyclic AMP appears to be involved as an 'intracellular messenger' in mediating several other androgenic effects in the rat seminal vesicle (Singhal & Sutherland, 1975).

Our experiments indicate that phosphorylation of cytoplasmic components may play an important role

in vesicular secretion. Thus when seminal vesicle slices were pulse-labelled with $^{32}\text{P}_i$ for 15 min and then transferred to an isotope-free 'chase' medium for the incubation experiment, control slices spontaneously discharged a secretion product that was markedly enriched (about ninefold on a protein basis) in ^{32}P -labelled components, as shown by the fact that 55% of the trichloroacetic acid-insoluble ^{32}P was released into the medium, together with 6% of the protein, after 30 min of incubation. Testosterone and dibutyryl

cyclic AMP produced an approximately twofold increase in the synthesis and the secretion of ^{32}P -labelled components. Preliminary experiments (C. Y. Lu & H. Koenig, unpublished work) showed that the trichloroacetic acid-insoluble ^{32}P radioactivity in control vesicular slices was present in phospholipid (59%), nucleic acid (RNA + DNA) (33%) and phosphoprotein (8%); 5 α -dihydrotestosterone increased the incorporation of $^{32}\text{P}_i$ into these components to a similar extent.

We infer from the findings in the control slices that $^{32}\text{P}_i$ must have been incorporated into secretory constituents shortly before the latter were packaged in secretory granules or lysosomes or both and released into the incubation medium. The phosphorylation of phosphoproteins of milk (the caseins) (Bingham *et al.*, 1972) and tooth dentin (Weinstock & Leblond, 1973) is known to occur in the Golgi apparatus. It is therefore likely that the phosphorylation of secretory constituents in the seminal vesicle also takes place in the Golgi apparatus. The secretory protein in the rat seminal vesicle is known to be transported through the Golgi apparatus for packaging in secretory granules before discharge (Flickinger, 1974). The lag period that preceded the hormone stimulated discharge of ^{32}P -labelled components probably reflects the time required for the intracellular transport, packaging and secretion of the hormone-induced components.

It is noteworthy that the acid phosphatase and β -N-acetylhexosaminidase were secreted into the incubation medium as acidic isoenzymes, whereas these hydrolases occurred largely as basic isoenzymes in the glandular tissue. We have previously shown that the nascent lysosomal hydrolases are sialylated in the Golgi apparatus (Nayyar *et al.*, 1972; Goldstone & Koenig, 1972, 1973; Needleman *et al.*, 1975) and the completed hydrolases are packaged in primary lysosomes as acidic sialoglycoproteins, whereas secondary lysosomes contain both acidic and basic forms of these enzymes (Goldstone & Koenig, 1974a,b; Sanghavi & Koenig, 1975). The acidic nature of the secreted vesicular hydrolases is consistent with the view that these enzymes, after undergoing sialylation in the Golgi apparatus, are packaged for secretion in primary lysosomes. We may therefore conclude that the acid phosphatase-reactive particles previously found to be released into the gland lumen *in vivo* (Koenig & Hughes, 1975) are in fact primary lysosomes.

It is evident from our work that the lysosomal hydrolases are important constituents of the vesicular secretion. Therefore it seems reasonable to infer that certain small-molecular-weight constituents (e.g., glycerophosphorylcholine, amino acids, prostaglandins) in the glandular secretion may be produced by lysosomal digestion of macromolecular components of the secretory granules and lysosomes such as lipoproteins and glycoproteins within the gland lumen.

R. B. participated in this research during his free quarter as a Senior Medical Student. We thank Dr. Alfred Goldstone for helpful discussions. This research was supported in part by NIH grants NS 06838, NS 05509 and HD 19729.

References

- Andersch, M. A. & Szczypinski, A. J. (1947) *Am. J. Clin. Pathol.* **17**, 571–574
- Bingham, E. W., Farrell, Jr., H. M. & Basch, J. J. (1972) *J. Biol. Chem.* **247**, 8193–8194
- Eagle, H. (1959) *Science* **130**, 432–437
- Flickinger, C. J. (1974) *Anat. Rec.* **180**, 407–426
- Goldstone, A. & Koenig, H. (1972) *Life Sci.* **11**, 511–523
- Goldstone, A. & Koenig, H. (1973) *Biochem. J.* **132**, 267–282
- Goldstone, A. & Koenig, H. (1974a) *Biochem. J.* **141**, 527–535
- Goldstone, A. & Koenig, H. (1974b) *FEBS Lett.* **39**, 176–181
- Koenig, H. & Hughes, C. (1975) *J. Cell Biol.* **67**, 221
- Koenig, H., Knight, R., Nayyar, R. & Hughes, C. (1975) *J. Cell. Biol.* **67**, 220A
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Mann, T. (1964) *The Biochemistry of Semen and of the Male Reproduction Tract*, John Wiley and Sons, New York
- Nayyar, R., Goldstone, A., Koenig, H. & Hughes, C. (1972) *Proc. Int. Congr. Histochem. Cytochem.* **4th** p. 335
- Needleman, S. B., Koenig, H. & Goldstone, A. (1975) *Biochim. Biophys. Acta* **379**, 57–73
- Robinson, D., Price, R. G. & Dance, N. (1967) *Biochem. J.* **102**, 525–532
- Roy, A. B. (1958) *Biochem. J.* **68**, 519–528
- Singhal, R. L. & Sutherland, D. J. B. (1975) *Adv. Sex Horm. Res.* **1**, 225–282
- Sanghavi, P. & Koenig, H. (1975) *Trans. Am. Soc. Neurochem.* **6**, 217
- Tveter, K. J., Hansson, V. & Unhjem, O. (1975) *Adv. Sex Horm. Res.* **1**, 17–26
- Weinstock, M. & Leblond, C. P. (1973) *J. Cell Biol.* **56**, 838–845