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STEROIDOGENESIS ACTIVATOR POLYPEPTIDE (SAP) IN THE RAT OVARY AND TESTIS

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Summary—A steroidogenesis activator polypeptide (SAP) has previously been identified in the rat adrenal cortex (Pedersen and Brownie, *Proc. natn. Acad. Sci. U.S.A.* **80** (1983) 1882–1886). This factor apparently facilitates the association of mitochondrial cholesterol with the cholesterol side-chain cleavage cytochrome P-450, a reaction which is generally regarded as rate-controlling in the steroid biosynthetic pathway. The same preparative techniques have now been applied in a search for this material in other rat tissues. Among those investigated, the ovary and testis demonstrate significant concentrations of a factor which is biologically and chromatographically similar to adrenal SAP. In the immature ovary the activator becomes manifest after priming with PMSG and rises dramatically during hCG-stimulated luteinization, an increase which can be blunted with cycloheximide. In the adult rat testis it is increased acutely by treatment with hCG or dibutyryl cAMP and is diminished in response to hypophysectomy or cycloheximide. At approximately equivalent concentrations (10^{-7} M), preparations of the activator from the adrenal cortex, the testis, and the superovulated ovary each enhance the activity of cholesterol side-chain cleavage in adrenocortical mitochondria by 5- to 6-fold over basal controls. We conclude that steroidogenic organs share a similar or identical intracellular modulator of cholesterol → pregnenolone conversion which is under pituitary control.

INTRODUCTION

The synthesis of steroid hormones from cholesterol is controlled in part at the formation of pregnenolone, the common, committed step in the various biosynthetic pathways [1–3]. This mitochondrial reaction, catalyzed by the cholesterol side-chain cleavage (cholesterol scc) cytochrome P-450, is under pituitary-dependent endocrine regulation; in a cAMP-dependent manner [4–15], ACTH and LH stimulate the association of substrate with the cytochrome active site in the adrenal cortex and gonads, respectively.

This reaction is distinguished by its acute sensitivity to inhibitors of protein synthesis [16–28], and by way of explanation, a labile, intracellular protein activator of the reaction has been postulated [23, 29–33]. Recently, this laboratory has identified a potential candidate for such a role [34]. The factor, steroidogenesis activator polypeptide (SAP), was purified to apparent homogeneity from the adrenocortical cytosol of rats implanted with an ACTH-secreting tumor. Given the similarities between the adrenal cortex and the gonads in the mode of cytochrome $P\text{-}450_{scc}$ regulation, we sought a comparable activator in those and other rat tissues. Our results suggest that this material, or something quite similar, can be detected in the testis and ovary but is not present, at least in a biologically active form, in various non-steroidogenic organs.

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EXPERIMENTAL

Animal experimentation

For the isolation of ovarian SAP, immature (24-day old) Sprague–Dawley (Holtzman) rats were used. Ovaries were primed and superluteinized according to Parlow [35] and the schedule in Table 1. Pregnant mares' serum gonadotropin (PMSG; Sigma) and hCG (Organon) were given by i.v. injection in saline. Cycloheximide (Sigma) was administered i.p. in 0.2 ml ethanol–saline (1:3, v/v). Decapsulated testicular tissue was harvested from adult (60-day old) Sprague–Dawley rats and applicable drug treatments were as described above. In separate experiments, various other tissues were collected from adult male Sprague–Dawley rats at 10 min after i.v. administration of dibutyryl cAMP (Bt_2cAMP; Sigma) in 0.2 ml saline. All animals were killed by decapitation.

Isolation of SAP

For each organ or treatment group, tissue was rapidly trimmed of fat and transferred to chilled saline. Pooled tissue was homogenized in an acidic extraction medium (10 ml/g) [34, 36] and centrifuged at 9,000 **g** for 30 min. The supernatant was concentrated on pre-equilibrated C_{18}-silica Sep-Pak® cartridges (Millipore) and a cut of 15 → 75% acetonitrile in aqueous trifluoroacetic acid (0.1%) was then collected, split into 2 unequal fractions, and dried under vacuum. Protein content was determined on the minor (5%) fraction by the method of Bradford [37], using standards of bovine serum albumin (BSA).

The major fraction was subjected to size exclusion chromatography on a column of Superose 12 (Pharmacia) using a Varian 5500 high performance liquid chromatography system (SEC-HPLC). Separations were carried out under denaturing conditions with 20% acetonitrile in 0.1 N HCl (0.5 ml/min). One-min fractions were collected and those in the region of interest (~3 kDal; 26–30 min elution time) were pooled, adjusted to pH 2 by dropwise addition of triethylamine, and subjected to reverse phase HPLC on a column (0.4 × 15 cm; 3 micron packing) of C_{18}-silica (MicroPak-SP®, Varian). A 60-min gradient (29 → 35% acetonitrile in 0.25 M triethylammonium formate, pH 3.0) was run at 0.5 ml/min and the u.v. absorbance was monitored at 215 nm (Isco V_4 detector). One-min fractions (0.5 ml) were collected.

To identify biologically active fraction(s) of interest, an 0.2 ml aliquot of each reverse phase HPLC fraction derived from ovarian or testicular tissue was tested in the cholesterol scc assay (described below) with adrenal mitochondria. Only material eluting between 32 and 34 min, associated with a discrete u.v. absorbance peak on the chromatograms, displayed a detectable stimulatory activity. The retention time of this peak coincided with that for adrenocortical SAP and the quantitations in Tables 1 and 2 were thus arrived at by peak integration and conversion to units of mass, using adrenal SAP as a standard. For other tissues (Table 4), fractions eluting in a slightly larger window (31–35 min) were pooled, dried, and assayed for cholesterol scc activity.

Mitochondrial cholesterol scc activity assay

The assay measures the rate of pregnenolone formation from endogenous cholesterol when adrenocortical mitochondria are provided with a means for generating reducing equivalents [34, 38]. The mitochondria are poised for optimal response by ensuring a low basal rate of cholesterol scc activity in the presence of substantial available substrate. This can be achieved by treating hypophysectomized donor rats with cycloheximide (15 mg, i.p.) and $ACTH_{1-24}$ (Cortrosyn®, Organon; 100 ng, i.v.) at 45 min and 10 min, respectively, prior to tissue collection. The transport of adrenocortical cholesterol into the outer mitochondrial membrane [22, 28] is thereby stimulated while the level of endogenous SAP [34] and the rate of steroidogenesis are greatly diminished.

Two animals were used for each assay. After adhering to the protocol above, the pairs of rat adrenals were enucleated *in situ* to eliminate capsular tissue and mitochondria were then prepared by differential centrifugation as described previously [39]. Washed mitochondria were resuspended (1 mg protein/ml) in reaction buffer (0.10 M Tris–HCl, 0.05 M sucrose, 0.08 M NaCl, 5 mM $MgCl_2$, and 5 mM KCl) containing 10 μM cyanoketone (Upjohn) to inhibit pregnenolone metabolism and then aliquoted (20 μl) into polypropylene tubes containing 50 μl of sample or buffer. The tubes were warmed at 37°C for 10 min and the reaction was started by adding 10 μl of isocitrate (Sigma; 10 mM, final concentration) in reaction buffer. The reaction was terminated after 60 s by plunging each tube into liquid N_2. Two freeze/thaw cycles were carried out on the samples, 2 ml of gelatin–phosphate RIA buffer were added to each tube, and 100 μl aliquots were then taken for direct assay of pregnenolone by RIA [40]. Controls included incubations stopped at t_0; or carried out without added test substance (basal controls), with test substance but without isocitrate, and with exogenous NADPH (10 mM) substituting for isocitrate. (NADPH cannot cross the membranes of intact mitochondria and thus an enhanced cholesterol scc in its presence indicates a loss of mitochondrial integrity and respiratory control.)

RESULTS

As the results in Table 1 indicate, sham-treated immature rats (group A) had no detectable level of SAP-like material in extracts of their ovarian tissue. On the other hand, rats pre-treated with PMSG and hCG (B) displayed substantial quantities of ovarian

Table 1. Estimated concentration of SAP in the immature rat ovary.

Group	Treatment				Activator	
	Day 0	Day 2	Day 9		(pmol/pair ovaries	(pmol/mg protein)
			−45 min	−5 min		
A	Saline[a]	Saline[a]	Saline[b]	Saline[c]	<0.5*	<3*
B	PMSG[d]	hCG[e]	Saline	Saline	2.6	34
C	PMSG	hCG	CH[f]	Saline	0.7	9
D	PMSG	hCG	Saline	hCG[g]	20.0	124
E	PMSG	hCG	CH	hCG	7.5	52

The activator polypeptide was isolated from pooled ovarian tissue (6 pair per treatment group) and quantitated as described in Experimental. *Detection limit. [a]0.2 ml, s.c.; [b]0.2 ml, i.p.; [c]0.2 ml, i.v.; [d]50 I.U. in 0.2 ml saline, s.c.; [e]25 I.U. in 0.2 ml saline, s.c.; [f]20 mg cycloheximide in 0.2 ml ethanol–saline (1 : 3, v/v), i.p.; [g]10 I.U. in 0.2 ml saline, i.v.

Table 2. Estimated concentration of SAP in the rat testis.

Group	Treatment			Activator (pmol/pair testes)
	−24 h	−45 min	−10 min	
A	Hypox[a]	Saline[b]	Saline[c]	<0.5*
B	Hypox	Saline	hCG[d]	3.7
C	—	Saline	Saline	1.6
D	—	CH[e]	Saline	<0.5*
E	—	Saline	hCG	9.8
F	—	CH	hCG	2.1
G	—	Saline	Bt_2cAMP[f]	4.1

The activator polypeptide was isolated from pooled, decapsulated testicular tissue (5 pair per treatment group) and quantitated as described in Experimental. *Detection limit. [a]Hypophysectomized; [b]0.2 ml, i.p., [c]0.2 ml, i.v.; [d]10 I.U. in 0.2 ml saline, i.v.; [e]20 mg cycloheximide in 0.2 ml ethanol–saline (1:3, v/v), i.p.; [f]10 mg in 0.2 ml saline, i.v.

SAP and these levels could be further enhanced by a second dose of hCG given 10 min before tissue isolation (D). In both instances, the prior administration of cycloheximide partially blocked this rise (C and E).

In the testes of hypophysectomized (A) or intact sham-treated (C) adult male rats, the apparent concentration of a SAP-like material was also low to undetectable (Table 2). However, in both instances there was a positive response when hCG was administered (B and E). The concentration of this material in intact animals was also increased upon i.v. injection of Bt_2-cAMP (G). Cycloheximide decreased the testicular content of SAP to undetectable levels in rats untreated with exogenous gonadotropin (D) and blunted its rise when the hormone was given (F).

Using a cholesterol side-chain cleavage assay employing rat adrenocortical mitochondria, the SAP-like materials isolated from the ovaries of superovulated, luteinized females and from the testes of hCG-stimulated males were analyzed for biological activity. As a basis for comparison, authentic SAP from the rat adrenal cortex was also tested. At approximately equivalent concentrations of protein (10^{-7} M), material prepared from each of the tissues significantly ($P < 0.01$) stimulated pregnenolone formation by a factor of 5- to 6-fold over controls receiving buffer alone (Table 3). As the data in Table 4 illustrate, comparable chromatographic fractions derived from a variety of non-steroidogenic tissues did not contain significant amounts of a cholesterol scc-stimulating activity.

Table 3. Effect of activators on adrenal cholesterol scc activity.

Addition (~0.1 μM)	Cholesterol SCC activity (nmol pregnenolone/ min/mg mito protein) ($\bar{X} \pm$ SEM)
Buffer only	0.16 ± 0.03
+SAP	
Adrenal cortex	0.80 ± 0.04*
Luteinized ovary	0.76 ± 0.06*
Testis	0.95 ± 0.04*

Activator fractions were prepared and assayed for biological activity as described in Experimental. The data are the mean ± SEM obtained from 3 cholesterol side-chain cleavage experiments. *$P < 0.01$ vs control (buffer only), as determined by Student's t-test.

DISCUSSION

The rapid onset of steroid formation in response to pituitary stimulation and the acute sensitivity of this response to inhibitors of protein synthesis have been the basis for proposals implicating a labile protein mediator of cholesterol metabolism in the regulation of steroidogenesis. Although much of the work in support of this hypothesis has been carried out with the adrenal cortex (for a review, see [41]), there are parallel data for the ovary and the testis. For example, gonadotropin-stimulated ovarian cholesterol scc is blocked by puromycin or

Table 4. Effect on adrenal cholesterol scc activity of appropriate pooled chromatographic fractions from various tissues harvested from Bt_2cAMP-treated rats.

Tissue of origin	Cholesterol SCC activity (nmol pregnenolone/ min/mg mito protein)
—	0.33
Brain	0.30
Cardiac muscle	0.40
Kidney	0.45
Liver	0.31
Lung	0.27
Pancreas	0.42
Pituitary	0.32
Spleen	0.36
Thymus	0.29
Adrenal cortex	1.81

Appropriate chromatographic fractions from the pooled tissues of 10 rats pretreated with 10 mg of Bt_2cAMP (−10 min, i.v.) were prepared and assayed for cholesterol side-chain cleavage activity as described in Experimental. The data are the mean of two experiments.

cycloheximide [19, 42] at a locus distal to cAMP generation and mitochondrial cholesterol accumulation. Experiments by Hall and Eik-Nes[16] with the rabbit testis demonstrated that puromycin inhibited the metabolism of radiolabeled acetate to androgens, and Moyle *et al.*[20] found a similar sensitivity to the drug by LH-stimulated Leydig cell tumor tissue. LH promotes a rapid increase in testosterone production by rat Leydig cells which can be inhibited by either puromycin or cycloheximide [11, 24], and van der Molen and colleagues have suggested a "labile" protein model similar to that invoked for the adrenal cortex to explain these data [31, 32]. Therefore, an extension to the gonads of our recent work characterizing such a polypeptide factor (SAP) in the adrenal cortex [34] seemed warranted.

The results support the presence of a material in the rat ovary and testis which is chromatographically indistinguishable from adrenocortical SAP. It is important to emphasize that a common identity has not yet been unequivocally established. However, changes in the tissue content of this gonadal factor in response to gonadotropin and cycloheximide (Tables 1 and 2) are consistent with the expected behavior of a putative regulatory factor, and with adrenocortical SAP under circumstances that are endocrinologically analogous. The similar biological potencies of fractions isolated from the testis, ovary, and adrenal in the adrenal mitochondria assay (Table 3) are additional evidence for an activator common to each venue. Also, recent studies suggest that the corresponding polypeptide in a Leydig cell tumor is active in an homologous mitochondrial cholesterol scc assay [43]. Further characterization of these isolates to confirm their identity is clearly warranted.

The mechanism by which SAP facilitates cholesterol association with cytochrome P-450$_{scc}$ is still unknown. Its concentration is responsive to cAMP (Table 2 and [34]), suggesting that some uncharacterized phosphorylation event is implicated in its generation or activation. There is no evidence, however, that SAP itself is phosphorylated. From a functional standpoint, various potential modes of action remain mostly speculative and have been reviewed elsewhere [41]. Nevertheless, any model must take into account the site of cycloheximide sensitivity—the interaction of cholesterol with its cytochrome P-450 binding site—and evidence [34] that SAP promotes an association of mitochondrial cholesterol with the enzyme. The results of experiments designed to address these questions will give us a greater insight into the mechanism by which steroidogenesis is regulated, and in a broader context, of how intracellular polypeptides can serve as second and third messengers of signal transduction.

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