In vitro Effect of Acetylcholine on Adrenocortical Secretion of Incubated Rat Adrenals.* (26325)

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Acetylcholine (ACh) appears capable of stimulating adrenocortical secretion both *in vivo* and *in vitro*. *In vivo* evidence suggests increased cortical function following parenteral administration of ACh(1) or following splanchnic nerve stimulation wherein increased quantities of ACh might be assumed to be released(2,3). Rosenfeld(4) has demonstrated direct stimulation of corticosteroidogenesis in perfused calf adrenals by ACh and other cholinergic drugs as evidenced by increased corticoid production and atropine inhibition of this effect. Results of these studies indicate, furthermore, that ACh stimulation of corticosteroidogenesis may be mediated *via* regulation of 17-hydroxylation as indicated chromatographically by a primary increase in 17-hydroxylated corticoids. The present investigation has explored possible species differences in *in vitro* stimulation of adrenocortical secretion by ACh which might be linked to its action in regulating 17-hydroxylation. We have used an indirect approach to the problem employing the incubated rat adrenal which is known to secrete primarily corticosterone(5) and in which 17-hydroxylation probably does not occur.

Methods. Sprague-Dawley CD (caesarean delivered) or Wistar male albino rats weighing 150-300 g each and maintained on tap water and Purina chow *ad libitum* were used. Both adrenals were removed under sodium pentobarbital anaesthesia, trimmed of periadrenal tissue, quartered or cut into sixths, and the pieces distributed equally among 4 to 6 sectors in a Petri dish humidor kept on ice. Thus, each sector received a representative piece of adrenal from each rat. Pooled adrenal tissue from each sector at least equivalent in weight to one adrenal was weighed on a torsion microbalance and transferred to a vessel containing 2.0 ml ice-cold Krebs - Ringer - bicarbonate - glucose medium (pH 7.4). Vessels were flushed for 10 minutes with 95% O_2—5% CO_2, sealed, and incubated at 38°C in a Dubnoff metabolic incubator. After pre-incubation for 30 minutes, incubation for 2 hours was carried out in fresh medium with adrenocorticotropin (ACTH, Armour ACTHar, LalA, Blend 2H, 1.39 ± 0.12 I.U./mg) in maximally effective doses (500 mμ/100 mg tissue) added to one flask of each series, and ACh bromide or chloride (Eastman organic chemicals, Lot Nos. 26A and 374245) in varying doses added to other flasks. In these series one flask received no additive. In one series acetylcholinesterase (AChase) (Sigma Chemicals Lot. No. 28-059) was added at variable time intervals following initial stimulation with effective doses of either ACTH or ACh. Upon completion of incubation, the medium was extracted with methylene dichloride, duplicate measurements made on unpurified, dried extract residues with blue tetrazolium (BT) and by measurement of peak absorption in the ultraviolet at 240 mμ (UV), and output rates calculated as μg Corticosterone equivalents/100 mg adrenal (wet wt)/2 hours incubation as previously described(6). ACTH, ACh, or AChase did not contribute significantly to BT or UV values.

Results. Stimulation of adrenocortical output by ACh bromide in doses ranging from 0.0001 to 10,000 μg/100 mg adrenal was investigated using Sprague-Dawley rat adrenals. Table I shows that a maximally effective dose of ACTH significantly increases adrenal output as measured both by BT and UV methods, whereas ACh at all doses tested above 0.0001 μg/100 mg adrenal apparently increases only output of BT reducing substances. Furthermore, the magnitude of increased output achieved with a maximally effective dose of ACh is only approximately

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TABLE I. Adrenocortical Secretion of Incubated Male Sprague-Dawley (CD) Rat Adrenals in Presence of ACh Bromide or ACTH.

Additive	Dose/100 mg adrenal	BT μg/100 mg/2 hr	BT Change μg/100 mg/2 hr	BT Change %	UV μg/100 mg/2 hr	UV Change μg/100 mg/2 hr	UV Change %
None		10.02 ± 4.30* (10)†			20.84 ± 4.10		
ACTH	500 mu	39.30 ±13.22 (12)	+29.28	+292	52.53 ±15.24	+31.69	+152
ACh	.0001 μg	8.44 ± 2.84 (6)	− 1.58	− 15.7	16.30 ± 4.31	− 4.54	− 21.7
"	.01 "	13.40 ± 3.14 (7)	+ 3.38	+ 33.7	19.40 ± 4.47	− 1.44	− 6.89
"	.1 "	15.43 ± .83 (4)	+ 5.41	+ 54.0	14.72 ± 9.38	− 6.12	− 27.3
"	1 "	16.10 ± 6.26 (4)	+ 6.08	+ 60.6	22.90 ± 5.32	+ 2.06	+ 9.84
"	1000 "	16.83 ±10.81 (5)	+ 6.81	+ 67.9	17.16 ± 7.74	− 3.68	− 17.6
"	10,000 "	15.80 ± 1.08 (4)	+ 5.78	+ 57.6	22.04 ± 3.72	+ 1.20	+ 5.74

* Mean ± S.D. † No. of experiments.

1/5 that achieved with ACTH. Although ACh at a dose of 0.0001 μg/100 mg adrenal does not increase adrenal output of BT reducing substance, a relationship apparently exists between dose and response at levels between 0.01 to 1000 μg/100 mg adrenal with the minimal dose requisite for maximal response between 1 and 1000 μg/mg adrenal.

To determine whether adrenocortical stimulation by ACh might be related to strain of rat or to ACh derivative, investigations were carried out using Wistar rat adrenals and ACh chloride. For comparison ACh bromide and ACTH were tested simultaneously. Two doses of ACh were tested, one known to produce measurable but submaximal stimulation and the other maximal stimulation of BT reducing substances. The data in Table II compared to those of Table I illustrate similar findings with respect to ACTH and ACh effects on adrenal output regardless of ACh derivative or strain of rat used. The

TABLE II. Comparison of *In Vitro* Effect of ACh Bromide, ACh Chloride and ACTH on Adrenocortical Secretion of Male Wistar Rat Adrenals.

Additive	Dose/100 mg adrenal	BT μg/100 mg/2 hr	BT Change μg/100 mg/2 hr	BT Change %	UV μg/100 mg/2 hr	UV Change μg/100 mg/2 hr	UV Change %
None		8.65 ± 2.23* (3)†			9.20 ± 4.89		
ACTH	500 mu	43.07 ± 5.65 (3)	34.42	+398	52.2 ± 9.11	43.00	+468
ACh Br	1000 μg	16.00 ± 2.82 (4)	7.35	+ 85.0	11.36 ± 9.41	2.16	+ 23.5
Idem	.1 "	12.95 ± 3.74 (4)	4.30	+ 49.8	8.41 ± 5.38	− .79	− 8.54
ACh Cl	1000 "	12.00 (1)	3.35	+ 38.8	7.66	−1.54	− 16.8
Idem	.1 "	10.90 (1)	2.25	+ 26.0	11.30	+2.10	+ 22.9

* Mean ± S.D. † No. of experiments.

TABLE III. *In Vitro* Effect of Acetylcholinesterase on Adrenocortical Secretion of Male Sprague-Dawley (CD) Rat Adrenals Following Initial Stimulation with ACh Bromide or ACTH.

Additive	Dose/100 mg adrenal	BT			UV		
		μg/100 mg/2 hr	Change μg/100 mg/2 hr	Change %	μg/100 mg/2 hr	Change μg/100 mg/2 hr	Change %
None		10.02 ± 4.30* (10)†			20.84 ± 4.10		
ACTH + AChase‡	500 mu 1000 μg	48.70 ±21.11	+36.68	+366	65.30 ±28.72	+44.46	+213
ACh + AChase‡	1 μg 2 "	6.11 ± 4.35 (5)	- 3.91	- 39.0	10.78 ± 4.89	-10.04	- 48.2
ACh + AChase§	1 " 2 "	14.40 ± 2.02 (4)	+ 4.38	+ 43.7	22.27 ± 2.65	+ 1.43	+ 6.86
ACh	1 "	16.10 ± 6.26 (4)	+ 6.08	+ 60.6	22.90 ± 5.32	+ 2.06	+ 9.84
AChase	2 "	8.05 ± 3.13 (4)	- 1.97	- 19.6	18.62 ± 2.64	- 2.22	- 10.7

* Mean ± S.D. † No. of experiments. ‡ Added 30 min. after initial stimulation.
§ Added 60 min. after initial stimulation.

lower BT response obtained with ACh chloride as compared to ACh bromide cannot be explained. This applies as well to the apparently significant UV response observed with the low dose of ACh chloride.

To demonstrate whether increased output of BT reducing substances is attributable directly to ACh, inhibition of this effect was attempted with AChase. In this series appropriate amounts of AChase (at least 2× the dose of ACTH or ACh) were added to the incubation medium at 30 or 60 minutes after initial addition of ACTH or ACh bromide in doses previously shown to be effective (Table I). The effect of AChase alone in doses comparable to those used to inhibit ACh was also tested in similar manner. Table III summarizes results which include for comparison average output rates in absence of additive or in the presence of ACh alone obtained previously under similar conditions. These data show that whereas AChase *per se* does not substantially alter adrenal BT output and does not inhibit ACTH, it does inhibit ACh. Addition of AChase 30 minutes after addition of ACh did not increase BT output, whereas AChase added after 60 minutes increased the output to a level below that following stimulation with ACh alone for 2 hours. This finding justifies the conclusion that the adrenal tissue was initially responsive to ACh and that AChase inhibition did occur.

Discussion. Our findings that ACh *in vitro* does not increase rat adrenal output of substances measurable by UV are in disagreement with those previously reported for perfused calf adrenals(4). This may reflect differences in species or in the *in vitro* methods employed. Demonstration by us that ACh stimulates release of BT reducing substances without concomitant increase in substances measurable by UV suggests that the former are not Δ^4-3 ketosteroids. Furthermore, it has been shown that adrenals of several mammalian species may release significant quantities of non-α ketolic BT reducing substances under the influence of serotonin(7). Although the BT substances released by ACh have not yet been identified, it is possible that they are not α ketol-Δ^4-3 ketosteroids. Since the rat adrenal presumably does not possess a 17-hydroxylating system it may be inferred that if ACh stimulates steroidogenesis it is primarily by regulating 17-hydroxylation as previously suggested. Our finding that ACh stimulation of BT reducing substances is inhibited by AChase further demonstrates that the effect is specific for ACh. Although it is not valid on the basis of these data to attribute a greater activity to the ACh bromide as compared to ACh chloride, our findings are highly suggestive.

Since adrenal output in presence of ACh is approximately 1/5 that achieved with ACTH and since AChase does not inhibit ACTH action, it would appear that ACTH action is not mediated *via* an ACh controlled mechanism.

Summary. The *in vitro* effect of acetylcholine on adrenocortical secretion of rat adrenals was investigated by direct methods of analysis. ACh increases rat adrenal output of BT reducing substances without corresponding increase in substances measurable by UV regardless of strain of rat or type of ACh derivative used. A relationship between dose and response was observed with ACh doses between 0.01 to 1000 μg/100 mg adrenal. Output achieved with a maximally effective dose of 1000 μg/100 mg adrenal was approximately 1/5 that observed following maximal ACTH stimulation. Specificity of the ACh effect was demonstrated by inhibition with acetylcholinesterase.

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Enzymic Hydrolysis of Stilbestrol Diphosphate *in vitro* and *in vivo*. (26326)

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Stilbestrol diphosphate is an inactive "transport" form of stilbestrol currently employed in treatment of prostatic cancer. By virtue of the high level of phosphatase activity in malignant prostatic tissue(1) active stilbestrol is presumed to be preferentially released within the tumor and its metastases(2, 3). The absence of gynecomastia and other feminizing effects in patients treated with stilbestrol diphosphate is adduced as further evidence for target specificity of the substance. Recent studies with C^{14}-labelled stilbestrol diphosphate in prostatic carcinoma patients have shown the occurrence of considerable localization of free stilbestrol in the prostate; no localization occurred when stilbestrol was administered(4). However, in view of the abundant distribution of acid and alkaline phosphatases in animal tissues it might be expected that the hydrolysis of stilbestrol diphosphate would not be restricted to prostatic tissues, malignant or otherwise. In fact, it has been reported(5), without supporting data, that the compound is dephosphorylated by various rat tissues and fresh human prostatic tissue. In the present study, the phosphatase activity of several rat tissues towards stilbestrol diphosphate has been examined. Also, an attempt has been made to ascertain the extent of this activity *in vivo* by measurement of stilbestrol plasma levels following administration of the disphosphate ester.

Materials and methods. Tissue preparations. Homogenates of rat spleen, prostate, kidney, liver and of brain were prepared by grinding the tissues in a Potter-Elvehjem type homogenizer with 0.154 M KCl in the proportion of 1:20. To 1.0 ml of homogenate was added 0.5 ml of 0.2 M buffer solution and the mixture placed in a water bath at 37° for 5 minutes; 0.5 ml of 4×10^{-3} M stilbestrol diphosphate (disodium salt), dissolved in the same buffer, was then added to start the reaction. Tris (hydroxymethyl) aminomethane buffer was used to provide a pH of 7.4; acetate for pH 5.0, and borate for pH 9.0. A tissue blank was run with each experimental vessel. After the desired incubation time, which varied from 5 minutes to 2 hours, 5 ml of 6% trichloracetic acid was