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# NICOTINE-INDUCED STIMULATION OF STEROIDOGENESIS IN ADRENOCORTICAL CELLS OF THE CAT

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1 The effect of nicotine on steroid production and release from trypsin-dispersed cat adrenocortical cells was investigated.

2 Nicotine, like adrenocorticotrophin (ACTH), elicited a dose-dependent increase in steroidogenesis, which depended upon the presence of calcium in the medium.

3 Augmented steroid production evoked by submaximal concentrations of ACTH, monobutyryl cyclic adenosine 3′,5′-monophosphate (AMP), or prostaglandin $E_2$ was further enhanced by steroidogenic concentrations of nicotine.

4 These results are discussed in relation to the possible mode of action of nicotine on cortical cells and to the potential consequences of smoking during stress.

## Introduction

The diverse pharmacological effects of nicotine on biological systems have been most extensively studied in electrically excitable tissues such as nerve and muscle. The depolarization of autonomic ganglia is the most characteristic action of nicotine (Paton & Perry, 1953), and related to this effect, nicotine augments catecholamine release from the homologous chemosensitive adrenomedullary chromaffin cells by promoting an influx of extracellular calcium (Douglas & Rubin, 1961; Rubin & Miele, 1968). The depolarizing activity of nicotine extends to nerve terminals where it enhances neurotransmitter release (Lee & Shideman, 1959; Bhagat, 1966) and has been reported to augment vasopressin release from neurosecretory nerve terminals in the neurohypophysis (Burn, Truelove & Burn, 1945; Beardwell, 1971), although this latter finding was not corroborated *in vitro* (Thorn, 1966).

In contrast to the existing evidence regarding the action of nicotine on secretory organs of neuronal origin, much less is known of the effects of nicotine on secretory tissues of non-neuronal origin. The present studies focus on the action of nicotine on cells of the adrenal cortex. Knowledge of the effects of this alkaloid on the gland which plays such a key role in the organism's response to stress is of considerable interest and importance in the light of its widespread use.

Although recent work done in our laboratory using the *in situ* perfused cat adrenal gland has yielded a great deal of evidence concerning the mechanism of ACTH action (Jaanus, Rosenstein & Rubin, 1970; Jaanus & Rubin, 1971; Carchman, Jaanus & Rubin, 1971; Jaanus, Carchman & Rubin, 1972), the cat isolated adrenocortical cell preparation was selected for this investigation because of the homogeneity of its cell population and the ability to obtain readily dose-response relationships which cannot be so easily accomplished with the intact gland (Jaanus, *et al.*, 1970).

## Methods

### *Preparation of the cortical cell suspension*

Trypsin-dispersed cat adrenocortical cells were prepared according to a modification of the method of Sayers, Swallow & Giordano (1971) for dispersing rat adrenocortical cells. Male cats were anaesthetized with an intraperitoneal injection of pentobarbitone sodium (50 mg/kg). The adrenal glands were quickly removed and placed in cold modified Krebs solution, containing Eagle's Minimal Essential Medium (MEM) plus glutamine (2 mM). The Krebs MEM was equilibrated with a gas mixture of 95% $O_2$ and 5% $CO_2$ and had a pH

of 7.4. The glands were cut into 8 $mm^3$ cubes, placed in siliconized trypsinizing flasks containing 10 ml Krebs MEM plus trypsin (0.5%) and bovine serum albumin (0.25%), and incubated at room temperature in a metabolic shaker. After 90 min, the temperature was raised to 37°C, and the rate of shaking increased from 120-400 rev/minute. After 1 h the undigested material was removed and the suspension incubated for an additional 20 min at 120 rev/min with sufficient trypsin inhibitor to block further proteolytic activity. The amount of inhibitor added depended upon the quantity of trypsin present and the activity of the trypsin inhibitor. The cell suspension was washed once with Krebs MEM and finally resuspended in Krebs MEM containing bovine serum albumin (0.5%) and trypsin inhibitor (0.2%).

Two types of cortical cells could be distinguished microscopically, larger cells (17 $\mu$m in diameter) with densely speckled cytoplasm and slightly smaller cells (15 $\mu$m) with a lighter appearance. The cortical cells were about four times larger and had a darker cytoplasm than erythrocytes, which were the major contaminant in the system. Contamination by other tissue components, such as chromaffin cells, adipose tissue and cell fragments, was minimized since they were either digested by the trypsin (Malamed, Sayers & Swallow, 1970) or they remained suspended during centrifugation, while the cortical cells formed a pellet. The cortical cells were counted with a haemocytometer and the cell concentration adjusted to approximately 250,000 cells per ml (equivalent to the final incubation volume). The cells were incubated at 37°C for 2 h in normal or modified Krebs MEM in the absence or presence of stimulating agent. At the end of the incubation, 1 ml of Krebs MEM was added to rinse the beakers, and the suspension was centrifuged to separate the cells from the supernatant.

*Steroid analysis*

Corticosteroids, mainly in the form of hydrocortisone, were extracted from the supernatant with 5 ml methylene chloride and assayed by competitive protein binding using transcortin from human plasma as the binding agent (Jaanus *et al.*, 1972). The calculated steroid values were expressed either in ng/250,000 cells per 2 h (ng $ml^{-1}$ 2 $h^{-1}$) or as percent increase relative to unstimulated control cells.

*Drugs and reagents used*

Trypsin and lima bean trypsin inhibitor, (Worthington); bovine serum albumin and minimal Eagle's medium, (Schwarz-Mann); monobutyryl cyclic adenosine 3′,5′-monophosphate (AMP), (Sigma); nicotine base, (Eastman); [$^3$H]-corticosterone (42 Ci/mM), (New England Nuclear); $\beta$ 1-24 adrenocorticotrophin (ACTH) (Ciba); prostaglandin $E_2$ (Upjohn) and NPS-ACTH (from Dr J. Ramachandran, University of California at San Francisco).

## Results

*Basal and ACTH-stimulated steroid levels*

Cells incubated in Krebs solution for 2 h in the absence of ACTH manifested a fairly constant, low level of steroid production and release, with an average of 32 ng $ml^{-1}$ 2 $h^{-1}$ (Table 1). The addition of microunit concentrations of ACTH to the medium for 2 h caused a dose-related increase in steroidogenesis (Figure 1). Although responsiveness to ACTH varied from preparation to preparation, ACTH concentrations of 1-2 $\mu$u/ml generally produced discernible increases in steroid production. The $ED_{50}$ appeared to be approximately 50 $\mu$u/ml and a plateau was achieved at or above 500 $\mu$u/ml.

*Effects of nicotine on steroid production*

The stimulant effect of various nicotine concentrations on basal steroid production is shown in Table 1. Nicotine, in a concentration of 6 $\mu$M, produced a small but inconsistent increase in steroid production, whereas 60 $\mu$M nicotine

**Table 1** Effect of nicotine on basal and adrenocorticotrophin (ACTH)-induced steroid release from isolated adrenocortical cells

| *Experiment* | *No. of experiments* | *Steroid release (ng $ml^{-1}$ 2 $h^{-1}$)* |
|---|---|---|
| Control | 11 | 32 ± 3.5 |
| Nicotine | | |
| 6 $\mu$M | 4 | 35 ± 7.0 |
| 60 $\mu$M | 9 | 53 ± 8.0[a] |
| 600 $\mu$M | 9 | 79 ± 8.0[a] |
| ACTH 25 $\mu$u/ml | 9 | 77 ± 9.0 |
| ACTH + nicotine | | |
| 6 $\mu$M | 4 | 83 ± 17 |
| 60 $\mu$M | 10 | 110 ± 12[b] |
| 600 $\mu$M | 9 | 121 ± 16[b] |

[a] Significantly greater than control by Student's *t* test ($P < 0.05$).
[b] Significantly greater than ACTH control by Student's *t* test ($P < 0.05$).

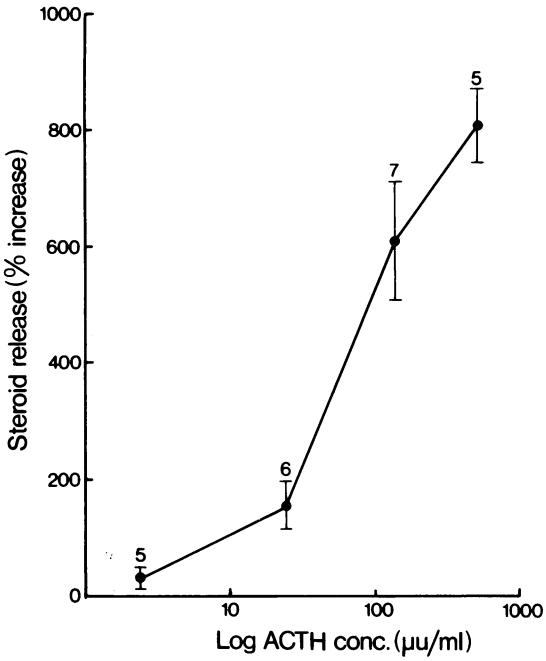


**Figure 1** Dose-response relationship of the steroidogenic effect of adrenocorticotrophin (ACTH) on trypsin-dispersed cat adrenocortical cells. Following trypsinization (see methods section) the cells were incubated for 2 h in fortified Krebs solution which also contained 0.5% bovine serum albumin, 0.2% lima bean trypsin inhibitor, plus varying concentrations of ACTH. The cells were centrifuged and the supernatant assayed for steroid. The values represent average steroid production calculated from the number of experiments indicated in parentheses. Vertical bars show s.e. mean.

augmented steroid production by more than 65%. A 150% augmentation in steroid production was observed with the highest nicotine concentration used (600 $\mu$M). Higher nicotine concentrations were not tested due to the potential danger of alterations in the pH of the medium which might be produced by the alkaloid.

When nicotine was added to the medium containing a submaximal concentration of ACTH (25 $\mu$u/ml), an additional increase in steroid release was observed (Table 1). However, the increase produced by nicotine in the presence of ACTH was clearly observed only at those concentrations (60 and 600 $\mu$M) which by themselves were steroidogenic. Thus, ACTH, in the absence and presence of 6 $\mu$M nicotine increased steroidogenesis by 140 and 160%, respectively; whereas ACTH plus nicotine 60 and 600 $\mu$M increased steroidogenesis by 3-4 fold, 250 and 278%, respectively (Table 1).

Additional insight into the nature of the interaction between ACTH and nicotine was obtained in an experiment in which a particular cell preparation was quite unresponsive to ACTH (25 $\mu$u/ml), steroidogenesis increasing only from 50 to 53 ng/ml. In this preparation steroid production elicited by 600 $\mu$M nicotine in the absence and presence of ACTH was 130 and 110 ng/ml, respectively. In another experiment when a threshold concentration (250 $\mu$u/ml) of the weak agonist, NPS-ACTH, failed to increase steroid production above the basal level of 19 ng/ml, steroidogenesis was increased by nicotine (600 $\mu$M) to 64 and 53 ng/ml in the absence and presence of NPS-ACTH, respectively.

*Interaction between nicotine and cyclic AMP and prostaglandin $E_2$.*

Cyclic AMP and prostaglandins are two putative mediators of ACTH-induced steroidogenesis; therefore, the effect of nicotine on the steroidogenic action of these agents was also tested. Monobutyryl cyclic AMP was used in place of its parent compound because its greater lipid solubility may enhance its penetration into cells. Prostaglandin $E_2$ was used since it was the most potent prostaglandin in augmenting steroid production in rat adrenal tissue (Flack, Jessup & Ramwell, 1969).

Although complete dose-response relationships were not ascertained in the present study, both monobutyryl cyclic AMP and prostaglandin $E_2$ were observed to have similar threshold (0.02-0.05 mM) and plateau (0.4-0.5 mM) concentrations. To test the effects of nicotine on the steroidogenic responses to these two agents, concentrations of the cyclic nucleotide (0.25 mM) and prostaglandin $E_2$ (0.1 mM) were selected which enhanced steroid production to approximately the same extent as 25 $\mu$u/ml ACTH. Table 2 shows that both the lower and higher concentrations of nicotine greatly enhanced the steroidogenic action of butyryl cyclic AMP, but only the higher alkaloid concentration clearly enhanced the action of prostaglandin $E_2$.

*The effect of calcium deprivation on nicotine induced steroidogenesis*

The steroidogenic action of ACTH in the intact perfused cat adrenal gland requires the presence of calcium (Jaanus *et al.*, 1970). Similarly, incubation of isolated adrenocortical cells of the cat in a calcium-deprived medium containing (ethylenebis-(oxyethylenenitrilo))-tetraacetic acid (EGTA)

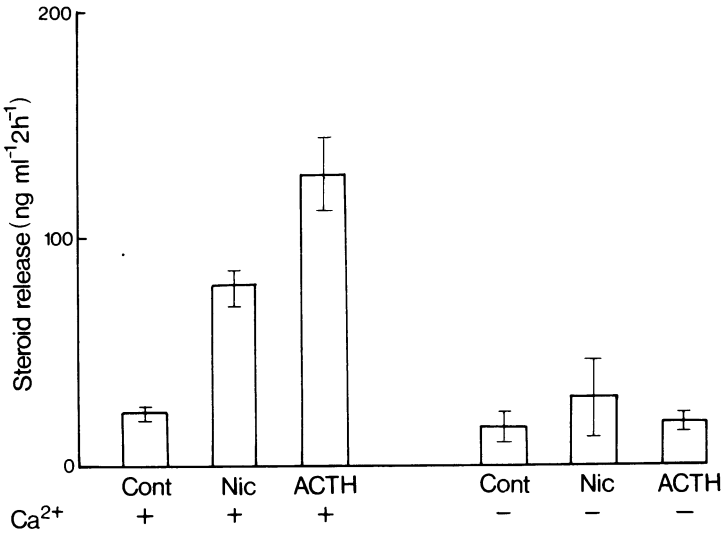


**Figure 2** The effect of calcium deprivation on the steroidogenic response to adrenocorticotrophin (ACTH) and nicotine (Nic). Equal numbers of cells were incubated in the normal incubation medium (see methods section), or in calcium-free medium plus EGTA (1 mM) with ACTH (500 μu/ml) or nicotine (600 μM). The columns represent mean steroid values, calculated from at least three experiments. Vertical bars show s.e. mean.

(1 mM), although not discernibly affecting basal steroid production, completely depressed the steroidogenic response to a maximum stimulating ACTH concentration (500 μ/ml) (Figure 2). Steroid production induced by a high nicotine concentration (600 μM) was depressed by calcium deprivation by 70% (Figure 2). In these experiments the average steroid production of 96 ng/ml elicited by nicotine was reduced to 29 ng/ml in the same cell preparations after calcium deprivation.

**Table 2** Effect of nicotine on steroid release induced by monobutyryl cyclic AMP (bcAMP) or prostaglandin $E_2$ ($PGE_2$)

| *Experiment* | *No. of experiments* | *Steroid release (% increase)* |
|---|---|---|
| $PGE_2$ | 4 | 78 ± 8 |
| $PGE_2$ + nicotine | | |
| 60 μM | 3 | 88 ± 38 |
| 600 μM | 4 | 408 ± 137 |
| bcAMP | 4 | 90 ± 38 |
| bcAMP + nicotine | | |
| 60 μM | 3 | 291 ± 38 |
| 600 μM | 3 | 508 ± 185 |

Mean values with s.e. mean are expressed as % increase over the basal value of a given experiment.

## Discussion

Previous investigators have observed, both in experimental animals and in human subjects, a rise in plasma corticosteroids after the administration of nicotine (see, Larson & Silvette, 1971); this stimulation of adrenocortical activity was ascribed to an indirect action resulting from enhanced ACTH release from the adenohypophysis (Kershbaum, Pappajohn, Bellet, Hirabayashi & Shafiha, 1968; Suzuki, Ikeda & Narita, 1973).

The present studies have clearly shown that nicotine is capable of directly stimulating isolated adrenocortical cells of the cat. The stimulant effect is observable in micromolar concentrations, is dose-dependent and like the physiological stimulus, ACTH, depends upon the presence of calcium. Not only is nicotine able to stimulate cortical cells directly but it enhances the steroidogenic effect of ACTH. The interaction between nicotine and ACTH appears to be additive rather than synergistic. This conclusion is based upon the fact that the steroid reponse to ACTH plus nicotine approached the sum of their separate responses. Moreover, nicotine was unable to potentiate a threshold response to ACTH or to NPS-ACTH, a weak ACTH analogue.

The mechanism by which nicotine causes the cortical cells to produce and release steroids cannot be defined at present. It is clear, however, that the stimulation of cortical cells by nicotine,

like its action on medullary chromaffin cells (Douglas & Rubin, 1961) requires the presence of calcium. Nicotine, like acetylcholine, appears to stimulate the medulla to secrete by depolarizing the chromaffin cell membrane (Douglas, Kanno & Sampson, 1967) allowing the transmembrane flux of calcium, which in turn triggers the release of preformed catecholamine stored in secretory granules (Douglas, 1968; Rubin, 1970).

However, one must be circumspect about extending the sequence of events thought to exist in the medulla to the cortex; for there appears to be no correlation between steroidogenic activity and the depolarization of cortical cells (Matthews & Saffran, 1967; Jaanus *et al.*, 1970). Moreover, in contrast to the medulla where transmembrane calcium flux is responsible for initiating evoked secretion, in the cortex stimulation by ACTH appears to trigger a cellular translocation of calcium (Jaanus & Rubin, 1971).

Thus, on the basis of these data it is premature and perhaps misleading to ascribe the stimulant effect of nicotine to a membrane depolarizing action on cortical cells. Nicotine can traverse the cell membranes of secretory tissue by a passive diffusion process (Putney & Borzelleca, 1972) and in skeletal muscle may release calcium from cellular sites (Weiss, 1968), so that an intracellular mobilization of calcium could be responsible for nicotine-induced steroidogenesis.

Although calcium has a key role in the stimulant action of nicotine, other proposed modulators of steroidogenesis such as cyclic AMP and prostaglandins may also participate in the events culminating in steroid production and release. The observations that nicotine enhances the steroidogenic action of exogenous cyclic nucleotide and prostaglandin $E_2$, just as it enhances the action of ACTH, might suggest that these proposed mediators indeed play key roles; however, more conclusive evidence implicating cyclic AMP and/or prostaglandins in nicotine-induced steroid production would be the detection of changes in tissue levels of these proposed mediators during stimulation. These experiments are now in progress.

In regard to the obvious practical implications of the present findings, the ability of nicotine to enhance corticosteroid output may be salutary or detrimental. In individuals having an inadequate response to stress, smoking may enhance their ability to cope with stressful situations by augmenting steroid output. Conversely, nicotine-induced stimulation of cortical cells may cause aberrations in the metabolic and electrolyte balance of the organism, especially in stressful situations where steroid levels are already high.

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