Acetylcholine stimulates steroidogenesis in isolated frog adrenal gland through muscarinic receptors: evidence for a desensitization mechanism

M. Benyamina, F. Leboulenger, I. Lirhmann, C. Delarue, M. Feuilloley and H. Vaudry

Groupe de Recherche en Endocrinologie Moléculaire, UA CNRS 650, Unité Alliée à l'INSERM, Faculté des Sciences, Université de Rouen, 76130 Mont-Saint-Aignan, France

RECEIVED 25 September 1986

ABSTRACT

The effect of cholinergic agonists on glucocorticoid and mineralocorticoid production by frog interrenal (adrenal) tissue was studied *in vitro* by means of continuous perifusion. Acetylcholine, at doses ranging from 1 to 100 μmol/l, stimulated both corticosterone and aldosterone output in a dose-dependent manner, with a half-maximal effective dose of 2·5 μmol/l. Corticosteroid production was also stimulated by muscarine (10 μmol/l). In contrast, neither nicotine nor nicotine bitartrate (1–100 μmol/l) enhanced corticosteroid biosynthesis. The kinetics of the response of adrenal cells to acetylcholine and muscarine were similar to those observed during angiotensin II stimulation. In particular, a significant reduction (20–40%) in the spontaneous level of corticosteroid production was recorded after the initial infusion of muscarinic agents, but no further decrease in the basal level occurred after a second cholinergic administration. The effect of acetylcholine was blocked by the muscarinic receptor antagonist atropine (10 μmol/l). These results indicate that acetylcholine can stimulate frog adrenocortical cells through muscarinic receptors. Repeated 20-min pulses of acetylcholine (50 μmol/l) or muscarine (10 μmol/l), given at one pulse per 130 min, resulted in a marked reduction in the secretory response to the second pulse. No reduction in the stimulatory effect of acetylcholine or muscarine was observed when a 6·5-h interval separated two 20-min infusions of the secretagogue. In contrast with these findings, iterative pulses of the muscarinic agonist pilocarpine (in the range 1–100 μmol/l) did not cause any desensitization.

These data show that the neurotransmitter acetylcholine can modulate frog adrenocortical function and suggest that, in addition to more conventional regulators, i.e. ACTH and angiotensin II, the cholinergic endings of the splanchnic nerve might participate in the regulation of corticosteroid secretion, at least under some physiological conditions such as neurogenic stress.

J. Endocr. (1987) **113**, 339–348

INTRODUCTION

In mammals the production of aldosterone by zona glomerulosa cells is controlled mainly by two corticotrophic peptides, adrenocorticotrophic hormone (ACTH) and angiotensin II, and by the extracellular concentration of potassium (Fredlung, Saltman, Kondo *et al.* 1977). Besides these three regulators, several biogenic amines such as serotonin (Farese, Larson, Sabir & Gomez-Sanchez, 1983), dopamine (McKenna, Island, Nicholson & Liddle, 1979) and adrenaline (De Léan, Racz, McNicoll & Desrosiers, 1984) may be involved in the control of aldosterone production from zona glomerulosa cells.

Cholinergic nerve terminals in the adrenal cortex have been reported in various animals including sheep (Robinson, Perry, Hardy *et al.* 1977), hamsters (Unsicker, 1969), birds (Unsicker, 1973) and reptiles (Unsicker, 1974). In an earlier study, Rosenfeld (1955) showed that infusion of acetylcholine in isolated calf adrenal glands induced a significant stimulation of steroid secretion. Other studies, however, failed to show any effect of acetylcholine on adrenal steroidogenesis *in vivo* (Blair-West, Coghlan, Denton *et al.* 1962) or *in vitro* (Unsicker, 1984). More recently, Hadjian, Ventre & Chambaz (1981) demonstrated the presence of cholinergic receptors in bovine adrenocortical tissue. These investigators have shown that acetylcholine is a

  Printed in Great Britain
0022–0795/87/0113–0339 $02.00/0

powerful stimulator of cortisol secretion by isolated cells from the bovine zona fasciculata (Hadjian, Guidicelli & Chambaz, 1982). In addition, Kojima, Kojima, Shibata & Ogata (1986) provided evidence for the involvement of the calcium messenger system in the mechanism of stimulation of aldosterone production in bovine zona glomerulosa cells by acetylcholine.

In the present study we have used a perifusion technique to examine the in-vitro effect of acetylcholine on corticosteroid secretion from frog adrenal (interrenal) tissue. The results support the existence of muscarinic receptors stimulating adrenal steroidogenesis in amphibia.

MATERIALS AND METHODS

Animals

Adult male frogs (*Rana ridibunda* Pallas) of about 40 g body weight originating from Yugoslavia, were obtained from Couétard (St Hilaire de Riez, France). The animals were maintained under running water with lights on from 06.00 to 18.00 h in a temperature-controlled room (8 ± 1 °C). The animals were killed by decapitation between 08.00 and 09.00 h, the kidneys removed and the interrenal tissue was dissected and minced as described previously (Leboulenger, Delarue, Tonon *et al.* 1978). The interrenal fragments were incubated for 15 min in 5 ml amphibian culture medium prepared by Eurobio (Paris) according to the method of Wolf & Quimby (1964). The medium was gassed for 15 min with O_2/CO_2 (95:5) before use.

Perifusion of adrenal glands

The interrenal fragments were washed with 5 ml medium to reduce the level of corticosteroids present in the medium. The perifusion system was set up as described previously (Leboulenger *et al.* 1978). Briefly, siliconized glass columns (9 mm internal diameter) were prepacked with Bio-Gel P-2 beads (Bio-Rad Laboratories, Richmond, CA, U.S.A.; 500 µl/column) and minced interrenal tissue from eight frogs was layered between several 2 mm beds of Bio-Gel. The columns were connected via polyethylene tubing (Pharmacia, Bois d'Arcy, France) to a reservoir bottle containing gassed medium. Medium was delivered to columns at a flow rate of 200 µl/min by a multichannel peristaltic pump. The temperature of the column was kept constant (24 ± 0·1 °C) with a water jacket. After perifusion for 2 h to allow steroid release to stabilize, the perifusion medium was either unchanged (control) or changed to medium containing secretagogues. The effluent perifusate from each column was collected in 5-min fractions which were stored frozen until assayed.

Test substances (secretagogues)

Acetylcholine chloride, DL-muscarine chloride, pilocarpine hydrochloride, nicotine and nicotine bitartrate (Sigma, St Louis, MO, U.S.A.) were dissolved in medium just before use. Atropine (Sigma) was initially dissolved in absolute ethanol. The final dilution was made up in medium so that the ethanol concentration was 0·5%. We have previously established (Delarue, Perroteau, Leboulenger *et al.* 1981) that ethanol concentrations up to 1% have no effect on corticosteroid production by perifused frog interrenal tissue.

Steroid radioimmunoassays

Corticosterone and aldosterone concentrations were determined by radioimmunoassay (RIA) as described previously (Leroux, Delarue, Leboulenger *et al.* 1980; Leboulenger, Delarue, Bélanger *et al.* 1982) in 30 µl aliquots of each fraction of effluent perifusate collected. The working ranges of the assays were 58–14 430 fmol for corticosterone and 14–1776 fmol for aldosterone. The specificity of the antibodies has been evaluated by determining their cross-reactivities with 22 different steroids and related compounds. Corticosterone antibodies showed significant cross-reaction with progesterone (19%), 11-deoxycorticosterone (17%) and 21-deoxycortisol (8%) but the cross-reactivity was much lower with all other steroids tested, especially aldosterone (0·6%) and testosterone (1·8%). The aldosterone antibodies exhibited very weak cross-reactivity (< 0·005%) with all compounds tested. None of the secretagogues interfered in the corticosterone and aldosterone assays. Preliminary experiments were conducted in which effluent medium was submitted to Sephadex LH-20 chromatography before RIA. These studies showed that only corticosterone and aldosterone were measured in their respective assays and therefore these two steroids could be assayed directly in the perifusate without prior extraction (Delarue *et al.* 1981). The detection limits of the assays (5% reduction in antibody-bound counts) were 72 fmol for corticosterone and 14 fmol for aldosterone. The intra-assay reproducibility was lower than 3% and the between-assay precision 6% in both assays. Steroid concentrations were calculated from the parameters of standard curves linearized by means of logit-log transformation.

Calculations

Each perifusion pattern was established as the mean (± S.E.M.) profile of corticosteroid production calculated over three or four independent experiments. In the perifusion profiles, corticosterone and aldosterone production at any time was expressed as a percentage of the basal value. Reference levels of corticosteroid production (100% basal level) were calculated for

each experiment as the mean secretion rate of corticosteroid during the 40-min period (eight consecutive fractions) just preceding the onset of the infusion of the first test substance. Student's *t*-test for variation analysis was employed to compare the means of different values within the same set of experiments.

RESULTS

Steroidogenic effect of acetylcholine

Figure 1*a* shows the changes in corticosterone and aldosterone secretion by frog interrenal tissue in response to different concentrations of acetylcholine (1, 10 and 100 μmol/l). A 20-min infusion of acetylcholine induced a dose-related increase in corticosteroid production. The minimum dose of acetylcholine used in this study (1 μmol/l) led to stimulation of corticosterone and aldosterone output by 10 and 9% respectively. A dose of 100 μmol acetylcholine/l induced a 52% rise in corticosterone and a 44% rise in aldosterone. The lag period of the responses was 10–15 min and the maximum effect on steroid output always occurred 30 min after the onset of administration of the secretagogue. After termination of exposure to acetylcholine, the spontaneous secretion of corticosteroid always stabilized at a level consistently lower (20–40%) than the initial level (100% basal level).

Series of stimulations similar to those presented in Fig. 1*a* were carried out with different concentrations of acetylcholine. The corresponding dose–response curves for corticosterone and aldosterone are shown in Fig. 1*b*. Each perifusion column received only a single dose of acetylcholine in these experiments because of the desensitization phenomenon shown later. The half-maximal effective dose was 2·5 μmol/l and the maximum response was obtained at a concentration of 50 μmol acetylcholine/l, for both corticosterone and aldosterone. Acetylcholine, regardless of dose, stimulated a greater release of corticosterone than of aldosterone.

Characterization of cholinergic receptors involved in the steroidogenic response to acetylcholine

Muscarine (10 μmol/l) also stimulated steroid secretion from frog interrenal tissue (Fig. 2*a* and *c*). The time-course of the response was identical to that observed with acetylcholine. Nicotine (50 μmol/l) did not, however, affect corticosterone or aldosterone secretion (Fig. 2*b* and *d*). Various concentrations (ranging from 1 to 100 μmol/l) of nicotine and nicotine bitartrate were tested and all found to be totally ineffective in modifying corticosteroid production (data not shown).

Acetylcholine-induced stimulation of corticosteroid output was totally abolished by previous infusion of the muscarinic antagonist atropine at 10 μmol/l (Fig. 3).

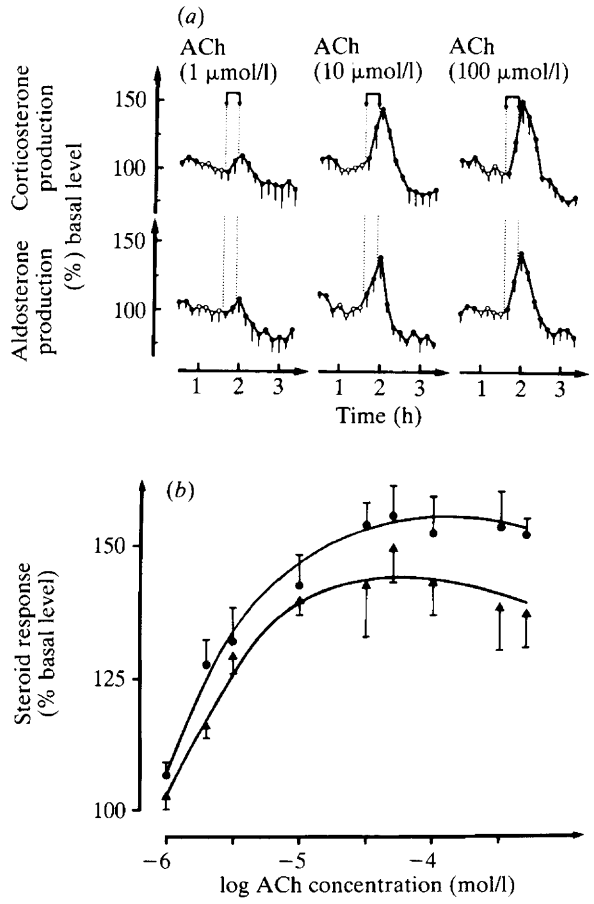

FIGURE 1. Effect of graded concentrations of acetylcholine (ACh) on corticosterone production by frog interrenal tissue. (*a*) Time-course of the stimulatory effect of three different doses (1, 10 and 100 μmol/l) of ACh. After a 100-min equilibration period, ACh was administered for 20 min and the interrenal tissue then allowed to stabilize for another 90-min period. For each dose of ACh the profile represents the mean (± S.E.M.) secretion pattern of three independent perifusion experiments. Each point is the mean corticosteroid production (expressed as a percentage of spontaneous steroid output) in two consecutive fractions collected during 5 min. The spontaneous level of steroid release (100% basal level) was calculated as the mean of eight consecutive fractions (40 min) (○) just preceding the infusion of ACh. The mean secretion rates of corticosterone in these experiments were 54·3 ± 2·9, 69·3 ± 3·5 and 76·8 ± 3·8 fmol/interrenal gland per min, and the mean secretion rates of aldosterone were 45·8 ± 2·5, 41·3 ± 1·7 and 62·7 ± 3·0 fmol/interrenal gland per min for 1, 10 and 100 μmol ACh/l respectively. (*b*) Semilogarithmic plot comparing the dose–response curves for ACh on corticosterone (●) and aldosterone (▲) production. Experimental values were calculated from perifusion data similar to those shown in *a*. Each point represents the maximum amplitude of stimulation of corticosteroid secretion induced by ACh (peak height) compared with the mean corticosteroid levels observed just before infusion of each dose of secretagogue (100% basal level).

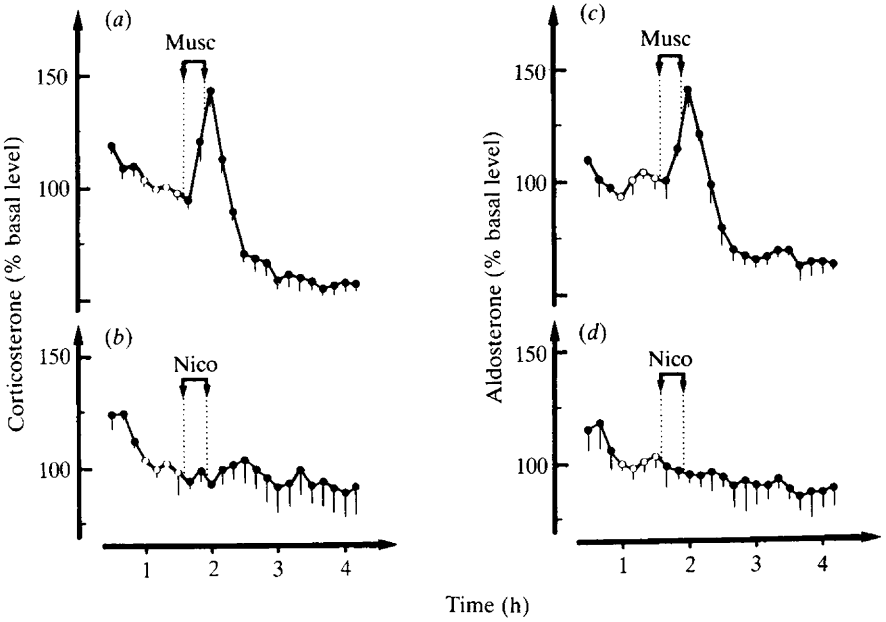

FIGURE 2. Comparative effects of muscarine (Musc; 10 μmol/l) and nicotine (Nico; 50 μmol/l) on the production of (*a* and *b*) corticosterone and (*c* and *d*) aldosterone by frog interrenal tissue. See legend to Fig. 1 for further details. Mean basal levels of corticosterone were 55·4 ± 1·4 and 81·4 ± 2·9 fmol/interrenal gland per min for *a* and *b* respectively; mean basal levels of aldosterone were 47·2 ± 1·9 and 60·8 ± 2·5 fmol/interrenal gland per min for *c* and *d* respectively.

Evidence for a desensitization phenomenon

When 20-min pulses of acetylcholine (50 μmol/l) or muscarine (10 μmol/l) were administered at 130-min intervals, a marked reduction in the steroidogenic response was recorded after the second pulse of acetylcholine (Fig. 4*a* and *c*) or muscarine (Fig. 5*a* and *c*). When increasing doses of acetylcholine (1–100 μmol/l) were administered at 130-min intervals, the same refractoriness was observed (data not shown). Conversely, when two identical pulses of acetylcholine (Fig. 4*b* and *d*) or muscarine (Fig. 5*b* and *d*) were administered 6·5 h apart, no reduction in the amplitude of the response occurred.

Such a desensitization phenomenon did not develop when 20-min pulses of the muscarinic agonist pilocarpine at doses ranging from 1 to 100 μmol/l were infused at 100-min intervals (Fig. 6). Similarly, infusion of a 20-min pulse of pilocarpine (10 μmol/l) did not inhibit the response of the interrenal glands to a 20-min pulse of acetylcholine (10 μmol/l) delivered 120 min later (Fig. 7). Conversely, infusion of another 20-min pulse of pilocarpine 120 min after the pulse of acetylcholine resulted in reduced peaks of corticosterone and aldosterone compared with those recorded with the first pulse of pilocarpine (Fig. 7).

In order to ascertain that acetylcholine did not affect the secretory response of adrenal cells to other corticotrophic factors, we compared the effect of three consecutive doses of acetylcholine (50 μmol/l) with consecutive stimulations by acetylcholine (50 μmol/l), ACTH(1–39) (1 nmol/l) and angiotensin II (100 nmol/l). The results of these studies clearly demonstrate that acetylcholine induced only self-desensitization (Fig. 8*a*) and not heterologous desensitization (Fig. 8*b*). Similar results were obtained whatever the order of the stimuli applied (data not shown).

As already shown in Fig. 1, a significant lowering of the spontaneous corticosteroid level was observed after acetylcholine stimulation. This phenomenon was also recorded with muscarine and pilocarpine. However, no further decrease in basal output of corticosteroids occurred after any second stimulation by muscarinic agents (Figs 4, 5, 6 and 8).

DISCUSSION

We have found in the present study that acetylcholine stimulates corticosteroid production by frog interrenal tissue *in vitro*. Despite the evidence that a cholinergic discharge of splanchnic nerve terminals is generally

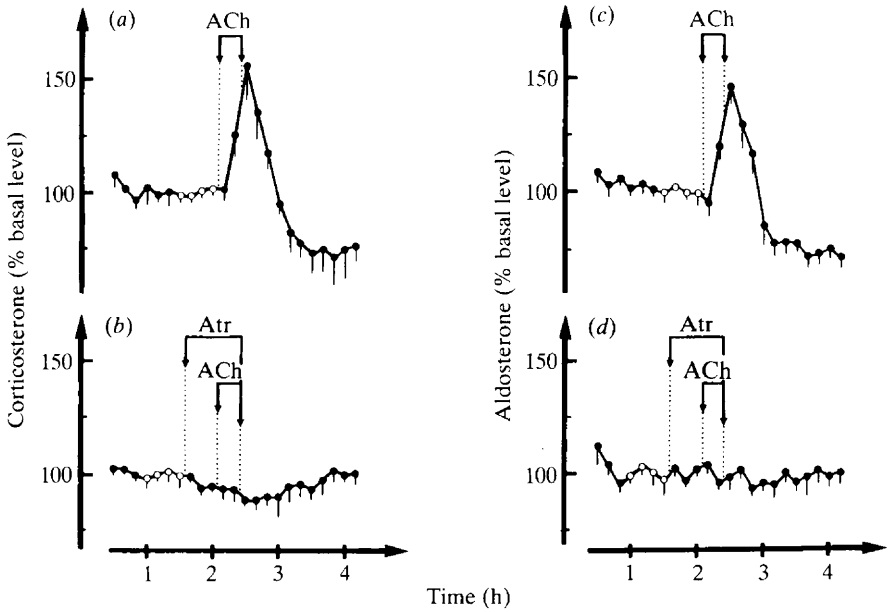

FIGURE 3. Comparative effects of acetylcholine (ACh; 50 μmol/l) administered (*a* and *c*) alone or (*b* and *d*) during infusion of atropine (Atr; 10 μmol/l) on production of (*a* and *b*) corticosterone and (*c* and *d*) aldosterone by frog interrenal tissue. For each series of experiments, interrenal fragments from 32 frogs were pooled together and distributed into four perifusion columns. Two columns received a single dose of ACh (20 min) alone while the two other columns received the same dose of ACh during prolonged (50 min) infusion of Atr. Each profile represents the mean of six independent experiments. See legend to Fig. 1 for further details. Mean basal levels of corticosterone were $65{\cdot}5 \pm 2{\cdot}6$ and $54{\cdot}3 \pm 2{\cdot}0$ fmol/interrenal gland per min for *a* and *b* respectively; mean basal levels of aldosterone were $45{\cdot}2 \pm 1{\cdot}9$ and $28{\cdot}3 \pm 1{\cdot}1$ fmol/interrenal gland per min for *c* and *d* respectively.

accompanied by an increase in corticosteroid production, surprisingly few investigations have been carried out to determine whether acetylcholine can influence corticosteroidogenesis directly. While Hadjian *et al.* (1982) showed that acetylcholine acutely stimulates cortisol production by bovine fasciculata cell suspensions other authors have not found any direct effect of acetylcholine on steroid production in the dog (Yamashita, Shimizu, Mieno & Yamashita, 1978).

As emphasized by Tait, Schulster, Okamoto *et al.* (1970), the perifusion model used in the present study is particularly well adapted to provide detailed information about the kinetics of the response of interrenal tissue to acetylcholine. The time-course of the effect of acetylcholine on steroid production was similar to that reported in bovine cells (Hadjian *et al.* 1982) and identical to those of other corticotrophic factors in the frog (Leboulenger *et al.* 1978; Delarue *et al.* 1981; Leboulenger, Leroux, Delarue *et al.* 1983; Delarue, Netchitaïlo, Leboulenger *et al.* 1984; Perroteau, Netchitaïlo, Homo-Delarche *et al.* 1984; Lihrmann, Netchitaïlo, Leboulenger *et al.* 1985). Since cellular products are being eliminated in this open-loop system, the effects of secretagogues can be examined without the interference exerted by cell products. Thus the inhibition of the spontaneous level of corticosteroid observed after acetylcholine administration cannot be ascribed to the feedback of corticosteroids released into the medium. The perifusion system is well adapted to reveal the biphasic effect (stimulation followed by inhibition) of acetylcholine. Such a biphasic response of the interrenal tissue, which has also been observed during stimulation by angiotensin II (Delarue *et al.* 1984), would have been concealed in a static incubation system. In addition, the perifusion model is the most appropriate one with which to study the effect of pulse frequency on the responsiveness of the tissue to repeated doses of secretagogues. The decline in the response which was observed when pulses of acetylcholine were administered at a frequency of one pulse per 130 min is indicative of a desensitization process induced by the neurotransmitter. Such a desensitization phenomenon is consistent with the findings of Kojima *et al.* (1986) who showed that following a 40-min stimulation of bovine glomerulosa cells by carbachol, a prompt increase in calcium efflux occurred and that this efflux response was terminated within 10 min of the beginning

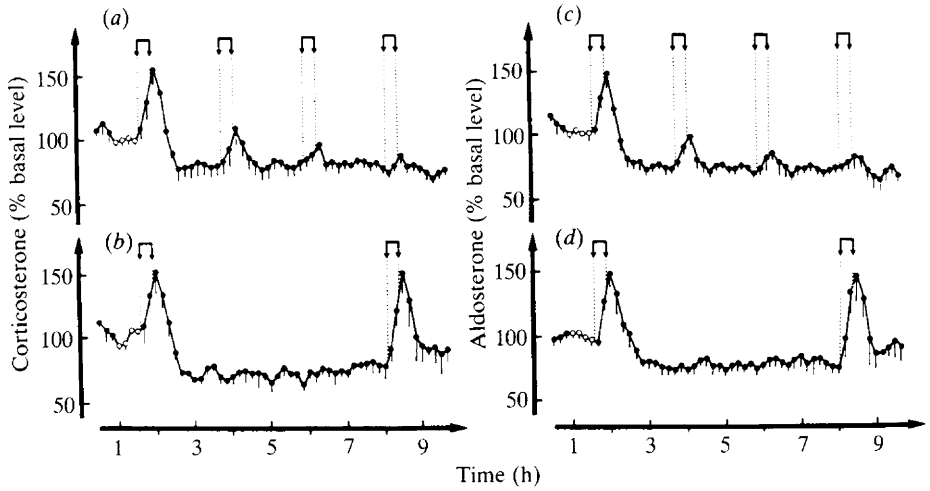

FIGURE 4. Influence of the interval between two consecutive doses of acetylcholine (50 μmol/l) on (*a* and *b*) corticosterone and (*c* and *d*) aldosterone production by frog interrenal tissue. For each series of experiments, four perifusion columns were conducted in parallel. Two of them received iterative 20-min pulses of acetylcholine given at 130-min intervals, while the other two received only two 20-min pulses of acetylcholine 6·5 h apart. Each profile represents the mean of six independent experiments. See legend to Fig. 1 for further details. Mean basal levels of corticosterone were 71·9 ± 1·2 and 71·0 ± 2·6 fmol/interrenal gland per min for *a* and *b* respectively; mean basal levels of aldosterone were 52·7 ± 1·1 and 42·7 ± 1·7 fmol/interrenal gland per min for *c* and *d* respectively. Arrows indicate periods of administration of acetylcholine.

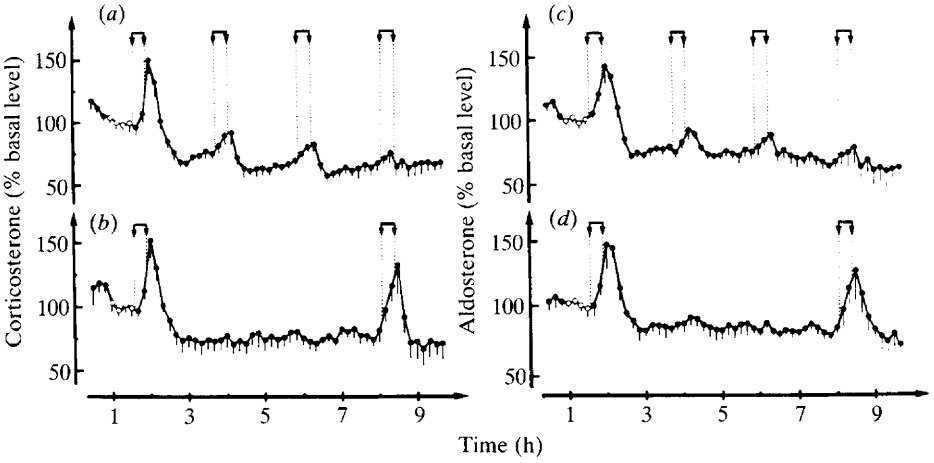

FIGURE 5. Influence of the time allowed between consecutive 20-min infusions of muscarine (10 μmol/l) on (*a* and *b*) corticosterone and (*c* and *d*) aldosterone secretion by frog interrenal tissue. The experimental procedure was similar to that described in Fig. 4. See legend to Fig. 1 for other details. Mean secretion rates of corticosterone under basal conditions were 70·4 ± 0·6 and 71·3 ± 2·0 fmol/interrenal gland per min in *a* and *b* respectively; mean secretion rates of aldosterone were 30·5 ± 0·6 and 45·5 ± 2·5 fmol/interrenal gland per min in *c* and *d* respectively. Arrows indicate periods of administration of muscarine.

of administration of the cholinergic agonist. In the frog the desensitization phenomenon did not result from general cellular deterioration, since the cells were still able to respond to other corticotrophic stimuli during the desensitized period. As expected for a physiological desensitization process, the cell responsiveness recovered in 6 h. Such a desensitization mechanism has never been investigated in acetylcholine-

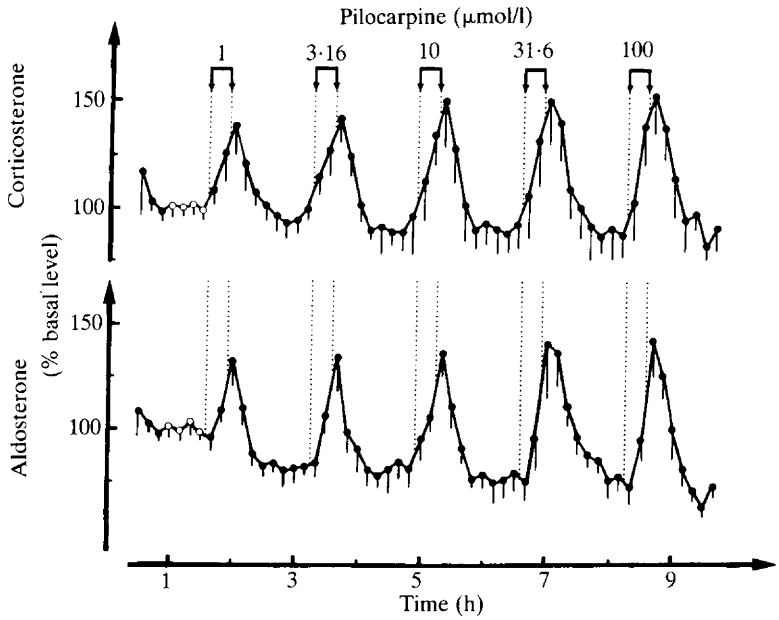

FIGURE 6. Effect of increasing concentrations of pilocarpine (from 1 to 100 μmol/l) on corticosteroid output from frog interrenal tissue. Interrenal fragments were perifused for 20 min with graded doses of pilocarpine delivered at 100-min intervals. See legend to Fig. 1 for further details. Mean secretion rates of corticosterone and aldosterone under basal conditions were $65{\cdot}2 \pm 0{\cdot}6$ and $48{\cdot}8 \pm 0{\cdot}8$ fmol/interrenal gland per min respectively.

stimulated adrenocortical cells, though desensitization to acetylcholine is well documented in other target cells (Higuchi, Uchida & Yoshida, 1985). In addition, we have never observed such desensitization of frog adrenal tissue during repeated pulses of other corticotrophic stimuli (Leboulenger *et al.* 1978; Delarue *et al.* 1981; Delarue *et al.* 1984; Perroteau *et al.* 1984; Lihrmann *et al.* 1985), although desensitization has been reported previously in superfused rat adrenocortical cells submitted to repeated doses of ACTH (Schulster, Rafferty & Williams, 1984). Surprisingly, the muscarinic agonist pilocarpine did not induce desensitization. This may indicate that while acetylcholine and muscarine induced both receptor stimulation and receptor internalization, pilocarpine stimulated muscarinic receptors without inducing clustering and internalization. The reasons for such a difference between the action of two agonists on the same receptor type remain unclear and require further investigations.

The nature of the receptors involved in acetylcholine-induced steroid production has been determined in two mammalian species. Bovine adrenocortical cells possess typical muscarinic receptors as shown by binding studies on adrenocortical membranes (Hadjian *et al.* 1981) and by measurement of steroidogenic activity of various cholinergic agonists (Hadjian *et al.* 1982). This has been confirmed by recent studies on the effect of acetylcholine on corticosteroidogenesis in primary culture of bovine adrenocortical cells (Kawamura, Yonezawa, Tanaka *et al.* 1985). Apparently, nicotinic receptors are found in cat adrenocortical cells (Rubin & Warner, 1975). This latter study, however, was conducted with adrenocortical preparations which were slightly contaminated by chromaffin cells. The effect of nicotine on corticosteroid production in the cat could thus possibly be exerted indirectly through chromaffin cells. The recent demonstration that adrenaline stimulates adrenal steroid biosynthesis (De Léan *et al.* 1984) supports this hypothesis. Our present studies clearly indicate that, in the frog, acetylcholine-induced stimulation of adrenocortical steroids is mediated by typical muscarinic receptors.

Kojima *et al.* (1986) observed a rapid increase in [^{3}H]inositol triphosphate formation during carbachol-induced aldosterone secretion by bovine glomerulosa cells. In the frog adrenal gland, stimulation of aldosterone production by muscarinic agonists is always preceded by a marked enhancement of the formation of arachidonic acid metabolites (Delarue, Leboulenger, Homo-Delarche *et al.* 1986). All these data are consistent with the hypothesis of Hadjian, Cultey & Chambaz (1984) who indicated that cholinergic stimulation of corticosteroid production in adrenal cells is mediated by an increase in phosphatidylinositol turnover.

Since, in amphibians, adrenocortical cells are inti-

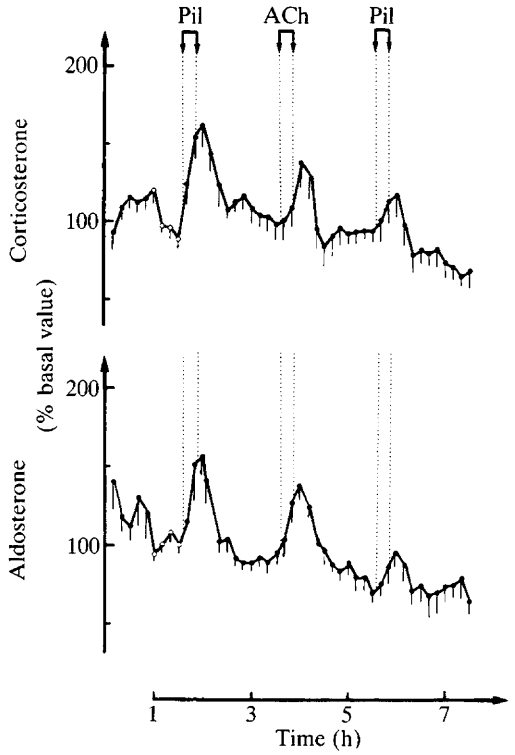

FIGURE 7. Comparison between corticosteroid responses by frog interrenal tissue to 20-min pulses of pilocarpine (Pil; 10 μmol/l), acetylcholine (ACh; 10 μmol/l) and pilocarpine (10 μmol/l) again, infused successively at 120-min intervals. See legend to Fig. 1 for further details. Mean basal levels of corticosterone and aldosterone were 72·2 ± 3·5 and 51·3 ± 1·9 fmol/interrenal gland per min respectively.

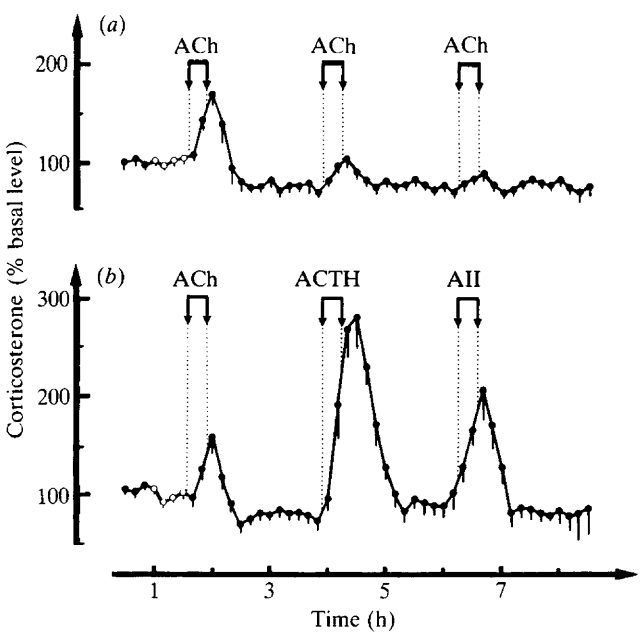

FIGURE 8. Comparison between corticosteroid responses by frog interrenal tissue (*a*) to three iterative 20-min infusions of acetylcholine and (*b*) to consecutive 20-min infusions of acetylcholine (ACh), ACTH and angiotensin II (AII). Series of perifusion experiments were conducted in parallel. Frog interrenal fragments received either (*a*) three pulses of ACh (50 μmol/l) or (*b*) successively one pulse of acetylcholine (50 μmol/l), human ACTH(1–39) (1 nmol/l) and [Sar^1,Val^5]-AII (100 nmol/l), delivered at 140-min intervals. See legend to Fig. 1 for further details. Mean basal levels of corticosterone were 62·6 ± 2·6 and 52·7 ± 1·9 fmol/interrenal gland per min for *a* and *b* respectively.

mately associated with chromaffin tissue (Leboulenger *et al.* 1983), whether acetylcholine acts directly on adrenocortical cells or indirectly through chromaffin cells remains questionable. In fact, we have recently shown that frog chromaffin cells contain, among another neuropeptides, vasoactive intestinal peptide (VIP) which acts as a paracrine factor to stimulate corticosteroid production (Leboulenger *et al.* 1983; Leboulenger, Perroteau, Netchitaïlo *et al.* 1984). Several lines of evidence, however, suggest that acetylcholine-induced stimulation of steroid biosynthesis results from a direct effect on adrenocortical cells. First, the lag period between the onset of stimulation and the response of the gland is identical with VIP (Leboulenger *et al.* 1983, 1984) and acetylcholine; a longer latency would be expected if acetylcholine was acting indirectly via chromaffin cells. Secondly, we have observed that supramaximal concentrations of acetylcholine (50 μmol/l) and VIP (10 μmol/l) gave additive effects (M. Benyamina & F. Leboulenger, unpublished data); exogenous acetylcholine would not enhance maximal stimulation by VIP if acetylcholine-induced steroidogenesis was mediated by release of VIP from chromaffin cells. Thirdly, it is generally accepted that cholinergic stimulation of adrenal catecholamine secretion is mediated by nicotinic receptors (Viveros, 1975), although muscarinic receptors have also been documented in the adrenal medulla (Role & Perlman, 1983); stimulation of steroidogenesis by nicotinic agonists would be expected if acetylcholine was acting via chromaffin cells. In addition, acetylcholine-induced corticosteroid production cannot be accounted for by catecholamines released by chromaffin cells, since exogenous adrenaline (50 μmol/l) did not stimulate steroid production by frog adrenocortical cells and the β-adrenergic antagonist propranolol did not block the stimulatory effect of acetylcholine in our model (F. Leboulenger & M. Benyamina, unpublished data).

Our results therefore indicate that the adrenocortical tissue of amphibians is under cholinergic control. The fact that cholinergic nerve endings have been observed in contact with adrenocortical cells of mammals (Unsicker, 1969) and lower vertebrate species (Unsicker, 1974) supports a physiological role for

acetylcholine in the control of corticosteroid production. Thus, under conditions of stress, both the hypothalamic-pituitary axis and direct cholinergic innervation may be involved in the stimulation of corticosteroid production.

ACKNOWLEDGEMENTS

This work was supported by grants from the Centre National de la Recherche Scientifique (UA 650 and 9-84-46), the Fondation de Recherche en Hormonologie (76-85-009) and the University of Rouen. We are indebted to Drs R. Andreatta and K. Scheibli (Ciba-Geigy Laboratories, Basle) for their generous gift of synthetic human ACTH. [Sar^1-Val^5]-angiotensin II was kindly supplied by Dr E. Escher (University of Sherbrooke, Quebec, Canada). We also gratefully acknowledge Mrs V. Franc for her skilful technical assistance and Ms I. Ogez for typing the manuscript.

REFERENCES

Blair-West, J. R., Coghlan, J. P., Denton, D. A., Goding, J. R., Munro, J. A., Peterson, R. E. & Wintour, M. (1962). Hormonal stimulation of adrenal cortical secretion. *Journal of Clinical Investigation* **41,** 1606–1627.

Delarue, C., Leboulenger, F., Homo-Delarche, F., Benyamina, M., Lihrmann, I., Perroteau, I. & Vaudry, H. (1986). Involvement of prostaglandins in the response of frog adrenocortical cells to muscarinic receptor activation. *Prostaglandins* **32,** 87–91.

Delarue, C., Netchitaïlo, P., Leboulenger, F., Perroteau, I., Escher, E. & Vaudry, H. (1984). *In vitro* study of frog (*Rana ridibunda* Pallas) interrenal function by use of a simplified perifusion system. VII. Lack of effect of somatostatin. *General and Comparative Endocrinology* **54,** 333–338.

Delarue, C., Perroteau, I., Leboulenger, F., Netchitaïlo, P., Leroux, P., Tonon, M. C. & Vaudry, H. (1981). *In vitro* effect of prostaglandins on corticosterone and aldosterone production by frog interrenal gland. *Biochemical and Biophysical Research Communications* **100,** 769–777.

De Léan, A., Racz, K., McNicoll, N. & Desrosiers, M. L. (1984). Direct β-adrenergic stimulation of aldosterone secretion in cultured bovine adrenal subcapsular cells. *Endocrinology* **115,** 485–492.

Farese, R. V., Larson, R. E., Sabir, M. A. & Gomez-Sanchez, C. E. (1983). Effects of angiotensin II, K^+, adrenocorticotropin, serotonin, adenosine 3′,5′-monophosphate, guanosine 3′,5′-monophosphate, A 23187, and EGTA on aldosterone synthesis and phospholipid metabolism in the rat adrenal zona glomerulosa. *Endocrinology* **113,** 1377–1386.

Fredlung, P., Saltman, S., Kondo, T., Douglas, J. & Catt, K. J. (1977). Aldosterone production by isolated glomerulosa cells: modulation of sensitivity to angiotensin II and ACTH by extracellular potassium concentration. *Endocrinology* **100,** 481–485.

Hadjian, A. J., Cultey, M. & Chambaz, E. M. (1984). Stimulation of phosphatidylinositol turnover by acetylcholine, angiotensin II and ACTH in bovine adrenal fasciculata cells. *Biochimica et Biophysica Acta* **804,** 427–433.

Hadjian, A. J., Guidicelli, C. & Chambaz, E. M. (1982). Cholinergic muscarinic stimulation of steroidogenesis in bovine adrenal cortex fasciculata cell suspensions. *Biochimica et Biophysica Acta* **714,** 157–163.

Hadjian, A. J., Ventre, R. & Chambaz, E. M. (1981). Cholinergic muscarinic receptors in bovine adrenal cortex. *Biochemical and Biophysical Research Communications* **98,** 892–900.

Higuchi, H., Uchida, S. & Yoshida, H. (1985). Recovery of the muscarinic cholinergic receptor from its down-regulation in cultured smooth muscle. *European Journal of Pharmacology* **109,** 161–172.

Kawamura, M., Yonezawa, Y., Tanaka, Y., Imagawa, N., Tomita, C. & Matsuba, M. (1985). Corticoidogenic effect of acetylcholine in bovine adrenocortical cells. *Endocrinologia Japonica* **32,** 17–20.

Kojima, I., Kojima, K., Shibata, H. & Ogata, E. (1986). Mechanism of cholinergic stimulation of aldosterone secretion in bovine adrenal glomerulosa cells. *Endocrinology* **119,** 284–291.

Leboulenger, F., Delarue, C., Bélanger, A., Perroteau, I., Netchitaïlo, P., Leroux, P., Tonon, M. C. & Vaudry, H. (1982). Direct radioimmunoassays for plasma corticosterone and aldosterone in frog. I. Validation of the methods and evidence for daily rhythms in a natural environment. *General and Comparative Endocrinology* **46,** 521–532.

Leboulenger, F., Delarue, C., Tonon, M. C., Jégou, S. & Vaudry, H. (1978). *In vitro* study of frog (*Rana ridibunda* Pallas) interrenal function by use of a simplified perifusion system. I. Influence of adrenocorticotropin upon corticosterone release. *General and Comparative Endocrinology* **36,** 327–338.

Leboulenger, F., Leroux, P., Delarue, C., Tonon, M. C., Charnay, Y., Dubois, P. M., Coy, D. H. & Vaudry, H. (1983). Co-localization of vasoactive intestinal peptide (VIP) and enkephalins in chromaffin cells of the adrenal gland of amphibia. Stimulation of corticosteroid production by VIP. *Life Sciences* **32,** 375–383.

Leboulenger, F., Perroteau, I., Netchitaïlo, P., Lihrmann, I., Leroux, P., Delarue, C., Coy, D. H. & Vaudry, H. (1984). Action of vasoactive intestinal peptide (VIP) on amphibian adrenocortical function, *in vitro*. *Peptides* **4,** 299–303.

Leroux, P., Delarue, C., Leboulenger, F., Jégou, S., Tonon, M. C., Vaillant, R., Corvol, P. & Vaudry, H. (1980). Development and characterization of a radioimmunoassay technique for aldosterone. Application to the study of aldosterone output from perifused frog interrenal tissue. *Journal of Steroid Biochemistry* **12,** 473–478.

Lihrmann, I., Netchitaïlo, P., Leboulenger, F., Delarue, C. & Vaudry, H. (1985). Effect of calcium on corticosteroid secretion by isolated frog interrenal gland. *Journal of Steroid Biochemistry* **23,** 169–175.

McKenna, T. J., Island, D. P., Nicholson, W. E. & Liddle, G. W. (1979). Dopamine inhibits angiotensin-stimulated aldosterone biosynthesis in bovine adrenal cells. *Journal of Clinical Investigation* **64,** 287–291.

Perroteau, I., Netchitaïlo, P., Homo-Delarche, F., Delarue, C., Lihrmann, I., Leboulenger, F. & Vaudry, H. (1984). Role of exogenous and endogenous prostaglandins in steroidogenesis by isolated frog interrenal gland: evidence for dissociation in adrenocorticotropin and angiotensin action. *Endocrinology* **115,** 1765–1773.

Robinson, P. M., Perry, R. A., Hardy, K. J., Coghlan, J. P. & Scoggins, B. A. (1977). The innervation of the adrenal cortex in the sheep, *Ovis ovis*. *Journal of Anatomy* **124,** 117–129.

Role, L. W. & Perlman, R. L. (1983). Both nicotinic and muscarinic receptors mediate catecholamine secretion by isolated guinea-pig chromaffin cells. *Neuroscience* **10,** 979–985.

Rosenfeld, G. (1955). Stimulative effect of acetylcholine on the adrenocortical function of isolated perfused calf adrenals. *American Journal of Physiology* **183,** 272–278.

Rubin, R. P. & Warner, W. (1975). Nicotine-induced stimulation of steroidogenesis in adrenocortical cells of the cat. *British Journal of Pharmacology* **53,** 357–362.

Schulster, D., Rafferty, B. & Williams, B. (1984). Corticotropin-

induced desensitization: steroidogenic and cyclic AMP responses in superfused adrenocortical cells. *Molecular and Cellular Endocrinology* **36,** 43–51.

Tait, S. A. S., Schulster, D., Okamoto, M., Flood, C. & Tait, J. F. (1970). Production of steroids by *in vitro* superfusion of endocrine tissue. II. Steroid output from bisected whole, capsular and decapsulated adrenals of normal intact, hypophysectomized and hypophysectomized-nephrectomized rats as a function of time of superfusion. *Endocrinology* **86,** 360–382.

Unsicker, K. (1969). Zur Innervation der Nebennierenrinde vom Goldhamster. *Zeitschrift für Zellforschung und Mikroscopische Anatomie* **95,** 608–619.

Unsicker, K. (1973). Fine structure and innervation of the avian adrenal gland. V. Innervation of interrenal cells. *Zeitschrift für Zellforschung und Mikroscopische Anatomie* **146,** 403–416.

Unsicker, K. (1974). Innervation of adrenal cells in the lizards *Lacerta dugesi* and *Lacerta pityusensis*. *General and Comparative Endocrinology* **24,** 409–412.

Unsicker, K. (1984). Innervation of endocrine tissues. In *Ultrastructure of Endocrine Cells and Tissues*, pp. 321–332. Ed. P. M. Motta. Boston: Martinus Nijhoff Publishers.

Viveros, O. H. (1975). Mechanism of secretion of catecholamines from adrenal medulla. In *Handbook of Physiology*, section 7, *Endocrinology*, vol. VI, pp. 389–426. Ed. American Physiological Society. Baltimore: Waverly Press.

Wolf, K. & Quimby, M. C. (1964). Amphibian cell culture: permanent cell line from the bullfrog (*Rana catesbeiana*). *Science* **144,** 1578–1580.

Yamashita, K., Shimizu, T., Mieno, M. & Yamashita, E. (1978). Effects of exogenous acetylcholine upon adrenal 17-hydroxysteroid secretion of intact and head X-irradiated dogs. *Neuroendocrinology* **27,** 39–45.