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ELECTRICALLY-EVOKED CATECHOLAMINE RELEASE FROM CAT ADRENALS

ROLE OF CHOLINERGIC RECEPTORS

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Abstract—Catecholamine (CA) release from perfused cat adrenal glands was continuously monitored using an on-line system coupled to an electrochemical detector. This highly sensitive procedure allowed the detection of small changes in the rate of secretion, even using short trains of electrical stimulation or brief acetylcholine (ACh) pulses. CA release was linear with increasing strength of ACh, transmural or splanchnic nerve stimulation. By using specific blockers, the contribution of nicotinic or muscarinic receptors to the overall secretory response to various stimuli could be established. That nicotinic receptors play a major role in mediating the secretory response to all stimuli is shown by the clear inhibition of the response with mecamylamine (10 μM). In contrast, atropine (1 μM) halved secretion evoked by ACh or nerve stimulation but had little effect on the response to trains of transmural electrical stimulation. When transmural electrical stimulation was applied continuously (instead of in trains), increasing the frequency in a step-wise manner, a bell-shaped curve was obtained; secretion reached a peak at 8 Hz and then declined sharply at 16 and 32 Hz. With this stimulation pattern, atropine decreased by 50% the secretion response at the higher frequencies (4–32 Hz). Very few studies are available which define the role of receptors and ionic channels in mediating electrically-evoked CA release. These stimulation patterns have not been used previously and are likely to mimic more closely than those used in earlier studies the physiological firing pattern of splanchnic nerves innnervating adrenomedullary cells.

Adrenal chromaffin cells release catecholamine (CA) into the blood stream as a physiological response to stress. This response is mediated by splanchnic nerve activity. The splanchnic branches release acetylcholine (ACh) which then stimulates nicotinic and muscarinic receptors present on the surface of chromaffin cells. The role of both types of receptors in the response to stress is still unclear and probably varies from one species to another (for review see Ref. 1).

The discharge of ACh from motor nerves is believed to occur in brief bursts [2]. If this is also true at the splanchnic–chromaffin cell synapse, the available studies on CA secretion do not reproduce this physiological pattern. Prolonged exposure (min) of the tissue gland to solutions containing cholinergic drugs are usually reported [3–5]. This pattern of stimulation leads to the inactivation of the secretory response, probably due to desensitization processes taking place at the acetylcholine receptors, the ionic channels carrying Ca^{2+} ions or the secretory machinery itself [6, 7]. In addition, exogenous cholinomimetic drugs are unlikely to mimic fully the endogenous transmitter release because of the co-release of other endogenous substances like neuropeptides which are known to modulate CA secretion [8–10].

The secretory response of the perfused glands evoked by transmural electrical stimulation *in vitro* can be inhibited by a combination of nicotinic and muscarinic receptor blockers, suggesting that transmural stimulation releases CA by prior release of ACh from splanchnic nerve terminals [9, 11, 12]. For this reason, transmural stimulation has been used as a model for splanchnic release studies due to the difficulty in dissecting glands together with splanchnic nerves.

The lack of sensitivity and time-course resolution of the techniques used to quantify CA release from perfused cat adrenals, evoked by low concentrations or brief pulses (few sec) of cholinergic agonists, have been overcome recently by the application of online electrochemical detection techniques [13]. However, in this earlier work, a systematic analysis of the role of nicotinic or muscarinic cholinergic receptors in mediating the secretory response was not performed.

The aim of this study was to examine the nicotinic and muscarinic components of the response to brief electrical stimulation of the splanchnic nerves innervating the cat adrenal medullary chromaffin cells, or to ACh infusion using a short stimulation pattern, in order to imitate the physiological event and to prevent receptor desensitization. We present here the first concentration–response study comparing ACh-evoked release with the splanchnic nerve-mediated CA secretion elicited by direct or transmural electrical stimulation.

MATERIALS AND METHODS

Forty cats of either sex were anaesthetized by i.p.

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administration of sodium pentobarbital (40 mg/kg). Both adrenal glands were excised, together with 6–8 mm of splanchnic nerve branches going to the adrenal tissue. They were perfused retrogressively *in vitro* through the adrenal vein, at room temperature, with Kreb's bicarbonate buffer of the following composition (mM): NaCl, 119; KCl, 4.7; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $CaCl_2$, 2.5; $NaCO_3H$, 25; glucose, 11; equilibrated at pH 7.4 with 95% O_2/5% CO_2. An LKB microperspex peristaltic pump perfused each gland at 1 mL/min.

CA release was monitored continuously following the technique described previously [13]. Briefly, glands were cleaned from the surrounding tissue and each one was placed in a hermetic plastic chamber. A bipolar stainless steel electrode was placed around the splanchnic nerve. The chambers had two helical silver electrodes for transmural stimulation of the glands.

Electrical stimulation was applied using a Harvard stimulator at supramaximal voltage (50 V) and 0.5 msec pulse duration at increasing frequencies. The stimulus was applied every 5 min for 5 sec. Antagonists were applied using a three-way valve located just before the peristaltic pump.

In the experiments using exogenous ACh, the system was modified in order to reduce the dead space. The peristaltic pump was removed and the Krebs solution perfused by pressure. Buffer reservoirs were pressurized with 95% O_2/5% CO_2 to provide a constant flow of 1 mL/min. An electrically-driven 5% CO_2 to three-way valve (General Valve Co., Fairfield, U.S.A.) was placed close to each gland in order to apply ACh pulses (5 sec) with precision. Under these conditions, the estimated dead space was about 100 μL.

The perfusate from the glands was passed through a Bio Analytical Systems LC-4b electrochemical detector. An oxidation current of +0.65 V was applied to a glassy carbon working-electrode vs a Ag/AgCl reference-electrode. The oxidation current was continuously recorded onto a Houston Omniscribe polygraph. The detector was calibrated by perfusion with noradrenaline and adrenaline standards. The responses were quantified by measuring the peak height on the chart recorder (Fig. 1).

Two concentration– or stimulation–response curves were made for each gland, separated by a resting period of 60 min to allow recovery of the glands. As is observed in many isolated tissue preparations, there were quantitative differences between first and second stimulus–response curves (see Figs 1–3). In order to obviate desensitization phenomena, blocking drugs were tested on the second curve and compared with the second curve of untreated glands.

Chemicals used were reagent grade and all drugs were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.)

RESULTS

Secretion of catecholamines evoked by brief stimuli

Figure 1 shows typical secretion traces obtained by direct electrical stimulation of the splanchnic nerves using trains of stimuli of 5 sec duration every 5 min. When transmural electrical stimulation or infusion of exogenous ACh were applied, similar secretory patterns were obtained.

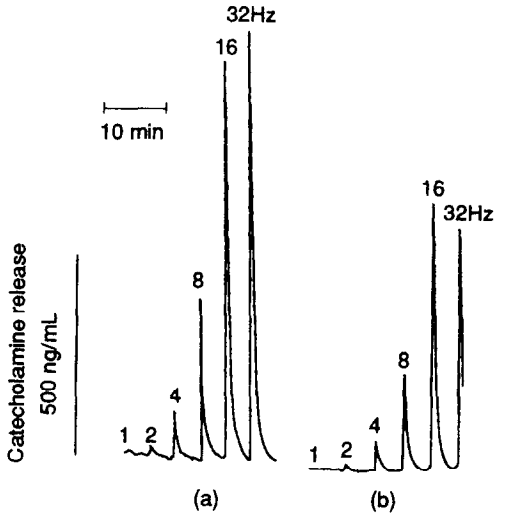

Fig. 1. On-line monitoring of catecholamine release from perfused cat adrenals. The figure shows typical CA oxidation current traces. Pulses of 0.5 msec duration at 50 V were applied for 5 sec every 5 min at the frequencies shown on top of each trace. Oxidation currents were converted to ng of total CA using known concentrations of adrenaline and noradrenaline for calibration. A second stimulus–response curve (b) was obtained after a 60 min resting period, in order to have proper controls for the later study of cholinergic blockade. Both traces were obtained using the same gland (see Materials and Methods).

There were quantitative differences between the three methods of stimulation used. Exogenous ACh-evoked responses were four times higher than those evoked by either direct or transmural electrical stimulation (Fig. 2).

The BAS electrochemical detector used was saturated when the catecholamine concentration passing through it exceeded 4 μg/mL, therefore, ACh concentrations higher than 10^{-3} M were not tested further (Fig. 2a). Moreover, larger concentrations of ACh or stimulation over 32 Hz caused strong desensitization of the responses observed in the second curve.

Under the experimental conditions used we got a reduction of dead space to approximately 100 μL, including the gland. An ACh pulse of 5 sec, at the perfusion rate of 1 mL/min, had a volume of 83 μL. It was, however, difficult to estimate the real concentration of drug reaching the surface of chromaffin cells.

The effect of nicotinic receptor blockade on brief stimulation pulses of adrenal glands

To block nicotinic receptors, the ganglionic agent mecamylamine (10 μM) was present in the perfusion buffer for 20 min before and during the recording of the second stimulus–response curve. This concentration reduced the secretory response evoked

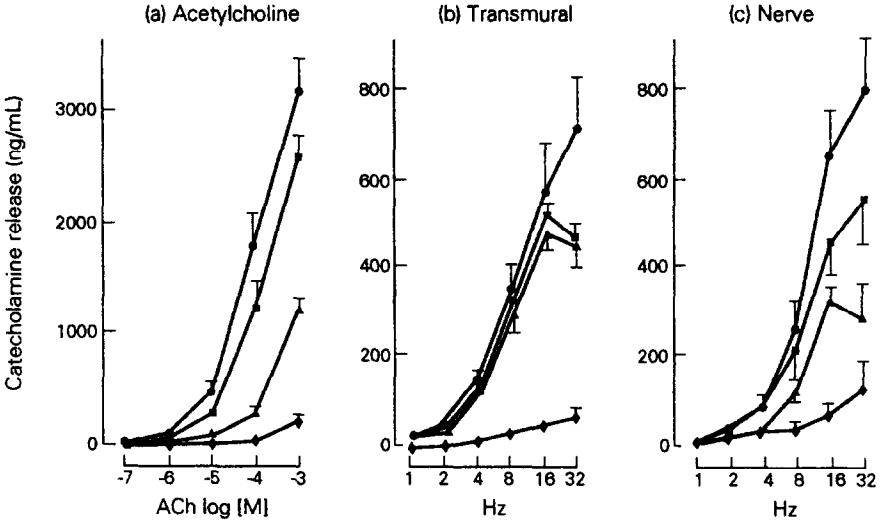

Fig. 2. Catecholamine release in response to stimuli of 5 sec duration. (a) ACh was applied for 5 sec every 5 min. The concentration–response curve (●) was repeated after 60 min in the absence (■) or presence (▲) of 1 μM atropine or 10 μM mecamylamine (◆). (b) Transmural electrical stimulation (0.5 msec at 50 V) was applied for 5 sec every 5 min; the frequency of stimulation was doubled at each step (see Fig. 1); the conditions and symbols are as described in a. (c) The same stimulation protocol as described in (b) was carried out, except that the stimulation was applied through electrodes placed on splanchnic nerve branches. The abscissae show ACh concentrations or frequencies of stimulation. Data are means ± SEM of 4–13 experiments.

by any of the three methods of stimulation used. However, nicotinic blockade was more efficient for secretion evoked by exogenous acetylcholine. Direct stimulation of the splanchnic nerve was less affected by the drug (Fig. 2).

The effect of muscarinic receptor blockade on brief stimulation pulses of adrenal glands

Atropine (1 μM) was applied in the same way as described for mecamylamine. Its effect on secretion was far more complex. Atropine caused an inhibition of 50% on the chemically-evoked CA release (Fig. 2) but hardly affected the response of transmural stimulation (compare atropine curves for both stimuli in Fig. 2a and b). Using direct stimulation of splanchnic nerves, atropine displaced the frequency–response curve to the right and markedly depressed particularly the secretory responses evoked by the highest frequencies of stimulation (32 Hz).

Effects of cholinergic blockade on secretory responses evoked by continuous transmural stimulation

In order to test whether the duration of stimulation modified the action of cholinergic blockade on the secretory responses, we performed a group of experiments using continuous electrical stimulation. Figure 3 shows the effect of atropine and mecamylamine when continuous transmural electrical stimulation is applied to the gland. Glands were stimulated continuously at 50 V, 0.5 msec; the frequency of stimulation was doubled when the secretory response reached a plateau or began to decrease. On the left panel, a typical trace of the secretory profile is shown. The CA secretion response reached a maximum at 8 Hz, while higher frequencies of stimulation caused a transient response followed by large inactivation. Perfusion with atropine (1 μM) caused a 40% reduction at the maximum CA output (8 Hz) but did not modify the inactivation pattern. Mecamylamine (10 μM) reduced the release of CA by 90%. No reduction on CA output was observed over a stimulation frequency of 8 Hz.

Concentration-dependence of the atropine and mecamylamine blockade of secretion evoked by direct stimulation of the splanchnic nerve

The secretory response evoked by a 5 sec stimulus was reproducible if the frequency of stimulation did not exceed 8 Hz. We used this frequency to study the inhibition on the secretion caused by atropine and mecamylamine. Pulses of 0.5 msec duration at 50 V, were applied repeatedly to splanchnic nerves at a frequency of 8 Hz for 5 sec every 5 min. When the response became reproducible, glands were perfused with increasing concentrations of antagonist. The IC_{50} value was close to 5×10^{-8} M for mecamylamine and 10^{-7} M for atropine (Fig. 4). The fact that a "pure" muscarinic antagonist such as atropine can block almost completely the secretory response at high concentrations may explain why 1 μM atropine caused a 40% inhibition of the secretory response evoked by the exogenous application of ACh or splanchnic nerve stimulation (Fig. 2). It seems therefore, that atropine blocks nicotinic receptors at micromolar concentrations.

DISCUSSION

The results presented here show that the

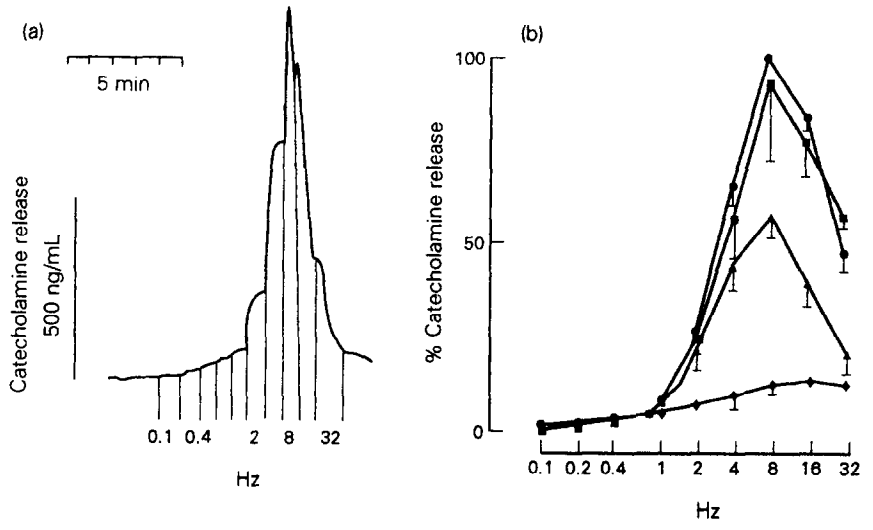

Fig. 3. Catecholamine release in response to continuous transmural electrical stimulation. An initial frequency of 0.1 Hz (0.5 msec at 50 V) was applied continuously to the gland; this frequency was doubled successively when the secretion reached a *plateau* or started to decrease. (a) A typical trace. (b) The protocol was repeated 30 min after the first curve (●) in the absence (■) or in the presence of 1 μM atropine (▲), or 10 μM mecamylamine (◆). The abscissae show the frequency of electrical stimulation. Data are means ± SEM of 4–12 different experiments; they are normalized as the percentage of the first curve at the maximum release point (8 Hz).

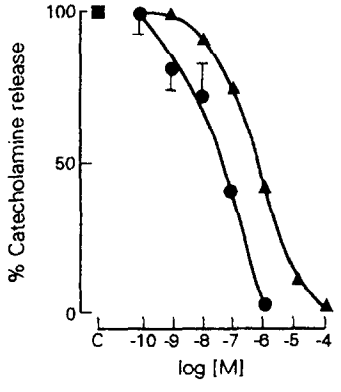

Fig. 4. Concentration-dependent blocking effect of mecamylamine and atropine on the secretory response to direct splanchnic nerve stimulation. A pulse of 50 V, 0.5 msec duration at 8 Hz was applied for 5 sec every 5 min. When the response became stable, the gland was perfused with increasing concentrations of atropine (▲) or mecamylamine (●) and the stimulation was repeated at each concentration. No appreciable desensitization phenomena were observed when the stimulation was repeated over 10 times. Data were normalized as a percentage of the response obtained before drug application and are means ± SEM from 3–4 different experiments.

monitoring of the CA release responses of perfused cat adrenal glands after brief stimulation periods, is technically feasible. The use of brief stimuli promotes reproducible secretory responses and reduces desensitization, a phenomenon seen currently with repetitive nictonic stimulation of the glands, even with long intervals between stimuli [3, 10, 14]. In contrast, under the present conditions of repetitive stimulation at 5 min intervals, using short pulses of ACh or short trains of transmural or splanchnic nerve stimulation, little or no desensitization of the secretory response occurred. The high sensitivity and time resolution of the on-line electrochemical detection of released amines allowed these studies to be carried out [13].

An additional technical improvement was the use of electronically-driven valves located close to the glands to change buffer solutions containing ACh. This procedure reduced the dead space and permitted accurate stimulation with brief pulses of ACh promoting reproducible responses, in order to imitate the stimulation pattern used for electrical pulses.

It is also worth noting that by careful dissection of the splanchnic nerve branches of both glands, we were able to extract the gland together with a piece of nerve long enough to enable us to stimulate the gland directly *in vitro*. Direct splanchnic nerve stimulation *in situ* has been reported in cats but there are technical difficulties to this approach in the collection of samples and it exhibits a poor time resolution [10, 15, 16]. With our approach we could, under the same conditions, compare the effects on secretion of exogenous applied ACh and endogenous ACh, released either by direct stimulation of splanchnic nerves or by the field electrical stimulation of their terminal branches.

Although the pharmacological properties of the adrenal medulla have been studied extensively, little is known about the involvement of ACh receptors in the adaptive response of chromaffin cells to

stress. Quantitatively, exogenous ACh-evoked CA secretion was almost four-fold higher than the release evoked by electrical stimulation. One possible explanation for this phenomenon might be that neither direct splanchnic nerve stimulation nor transmural stimulation could recruit all the splanchnic fibers; in fact, dissection of all the splanchnic branches which reach the adrenal gland was difficult because the major splanchnic nerve is accompanied by two to five minor branches which are not always easy to identify [17]. Thus, it is likely that not all the splanchnic fibers innervating adrenal chromaffin tissue were dissected and stimulated. On the other hand, transmural electrical stimulation gave an even smaller response, probably because the area covered by the two field electrodes was small.

In the experiments carried out to separate the muscarinic and nicotinic components of the responses to endogenous and exogenous ACh, we used 1 μM atropine and 10 μM mecamylamine, respectively. These concentrations are used frequently in this kind of secretion study [4, 11, 16]. In previous experiments [18], we found an IC_{50} value for atropine of 3×10^{-10} M against the pure muscarinic agonist methacholine. Upon direct splanchnic nerve stimulation, concentrations of atropine above 10^{-7} M caused complete inhibition of secretion. The experiment shown in Fig. 4 suggests a loss of atropine specificity as a pure muscarinic antagonist, when it was used at concentrations higher than 10^{-7} M. This fact should be taken into account when the muscarinic component is studied.

The results shown in Fig. 2 indicate only a very small secretory response in the presence of the nicotinic antagonist, mecamylamine. Stimulation of cat adrenal glands with micromolar concentrations of pure muscarinic agonists promotes a CA release response higher than those obtained here, either by evoked ACh or electrical stimulation, after a nicotinic blockade [19]. In addition, the abolition of secretion by 1 μM mecamylamine (Fig. 4) also suggests that at high concentrations, this drug loses its specificity as a pure nicotinic blocking agent and affects the muscarinic or other intracellular mechanism mediating CA secretion.

Although, physiologically, the ACh discharge from splanchnic nerves appears to occur in brief bursts, all the studies carried out to date have used long electrical stimulation periods. The results obtained here, using this method, show an increase in the catecholamine output reaching a maximum at 8 Hz followed by a rapid desensitization. Cholinergic blockade differed between atropine, with which only a quantitative difference could be observed, and mecamylamine, with which the responses appeared to reach a steady state (Fig. 3). Careful analysis of these curves showed no effect of cholinergic blockers at stimulation frequencies below 1 Hz. It may be that at these low frequencies, the splanchnic nerves release a non-adrenergic, non-cholinergic substance [9].

The differences observed between the effects of atropine on the two kinds of electrical stimulation (Fig. 2b and c) cannot be easily explained. Atropine did not affect the secretory response to a brief transmural stimulation but reduced the release evoked by direct stimulation of the splanchnic nerves. In rat adrenal glands, Wakade and Wakade [20] found a residual secretory component after cholinergic blockade of the early response to transmural stimulation, whereas the later response was almost completely inhibited. This bi-phasic secretory profile has also been observed in the cat [13] and could explain why atropine reduces the CA release evoked by continuous electrical transmural stimulation, when the late component is involved in the release. This inhibition was in the same order of magnitude as that, caused by atropine, of CA release in response to the short stimulation pattern using exogenous ACh or direct splanchnic nerve stimulation (compare atropine effect in Fig. 3 with that in Fig. 2a and c). Another possible explanation is that direct splanchnic stimulation acts only on splanchnic nerves, whereas transmural stimulation affects other complex interneuronal connections in which the roles of muscarinic receptors have not yet been established.

In conclusion, this paper presents the analysis of ACh evoked CA release from the cat adrenal gland using three different stimuli and on-line recording of released amines. These conditions allow mild repetitive stimulation of secretion by the adrenal medulla without causing its desensitization. The use of cholinergic receptor antagonists to separate different components of the secretory response leads to the conclusion that, at higher than micromolar concentrations, the antagonists might not act specifically on their cholinergic receptor subtypes. This conclusion should be considered in further studies on the secretory phenomena in the adrenal medulla.

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