Does Acetylcholinesterase Inhibition Affect Catecholamine Secretion by Adrenomedullary Cells?

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Abstract

Background: Splanchnic nerve stimulation evokes adrenomedullary catecholamine secretion via acetylcholine release and occupation of nicotinic cholinergic receptors on chromaffin cells.

Objectives: To assess whether among cultured adrenomedullary cells there exists a population that tonically secretes acetylcholine. If so, then blockade of enzymatic breakdown of acetylcholine by addition of a cholinesterase inhibitor to the medium would increase occupation of nicotinic receptors by endogenous acetylcholine and thereby induce catecholamine release.

Methods: Primary cultures of bovine adrenomedullary cells in 24-well plates (1 million cells per well) were incubated after 48–72 hours with fresh incubation medium (control), medium with added secretagogues (nicotine, angiotensin II, or K^+) or the acetylcholinesterase inhibitor, edrophonium (10^{-7} to 10^{-3} M), for 1–20 minutes. Fractional release rates of epinephrine, norepinephrine and dopamine were compared to a control. We also examined whether co-incubation with edrophonium enhanced the effects of the secretagogues. All experiments were performed in quadruplicate and repeated three times.

Results: Nicotine, angiotensin II, and K^+ each elicited time-related release of epinephrine, norepinephrine and dopamine by up to fourfold compared to the control. At all tested concentrations, edrophonium had no such effect. Co-incubation with edrophonium also failed to augment the secretory responses to nicotine, angiotensin II, or K^+.

Conclusion: Bovine adrenomedullary cells in primary culture do not include a population of tonically active cholinergic cells.

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Adrenomedullary chromaffin cells possess cell membrane nicotinic receptors, which when stimulated induce catecholamine secretion. In physiologic settings, splanchnic preganglionic nerves mediate cholinergic regulation of adrenal catecholamine secretion. Thus, adrenomedullary chromaffin cells have been used frequently as an analog of sympathetic postganglionic neurons.

The abundant expression of nicotinic receptors on adrenomedullary chromaffin cells might also suggest regulation by a local population of intrinsic cholinergic cells. Myocardial tissue possesses cholinergic neurons [1–4]. Furthermore, acetylcholinesterase has been shown to be present in the adrenal medulla; however, its expression has been thought to be related to protection or differentiation of chromaffin cells, not inactivation of acetylcholine as a neurotransmitter.

Sensitive biochemical assays are able to detect low levels of epinephrine without apparent sympathetic activation. In clinical practice various plasma catecholamine levels are seen, and elevated baseline levels of catecholamines are not rare. Therefore, theoretically, it is possible that this type of secretion is locally regulated as in other neurohormonal systems. Schneider et al. [5] examined the effect of eserine and neostigmine on catecholamine secretion in response to acetylcholine in bovine adrenomedullary cells. The results were not entirely consistent – eserine showed no effect while neostigmine did, although the effect was minimal.

The present study assessed whether among cultured bovine adrenomedullary cells there exists a population that tonically secretes acetylcholine. If so, then blockade of enzymatic breakdown of acetylcholine by addition of an acetylcholinesterase inhibitor to the medium would increase occupation of nicotinic receptors by endogenous acetylcholine and induce catecholamine release. We also considered whether acetylcholinesterase inhibition enhances responses to known secretagogues – nicotine, angiotensin II, and potassium ion.

Materials and Methods

Cell preparation

Bovine adrenal glands obtained from steers immediately after slaughter were delivered fresh on ice to the laboratory. Cultured chromaffin cells were prepared using procedures previously described [6,7] with a few modifications. The glands were perfused with Lockes 1X buffer and then with 4–5 ml of 0.2% collagenase P solution, followed by incubation for 10 minutes at 37°C twice in succession. Each gland was then dissected and the medullary tissue was removed, minced, and incubated for 30 min at 37°C in 0.2% collagenase P solution. The cells were filtered through 250 μm sterile nylon mesh and again through a 100 μm cell strainer into 50 ml tubes that were centrifuged for 10 min at 1,500 rpm. The supernatant was removed and the pellet was resuspended in Lockes 1X buffer. Red blood cells were destroyed by adding 10–12 ml of a lysis buffer and the medullary cells were resuspended for 3 minutes. Cold phosphate-buffered saline (25 ml) was added to cease erythrocyte lysis. The cells were filtered through a 40 μm cell strainer and centrifuged. The pellet was resuspended in DMEM/F12 (Gibco) and plated on glass Petri dishes for 24 hours of differential plating. The chromaffin cells were then placed in 24-well trays at a

density of 10^6 cells per well and incubated at 5% CO_2 atmosphere for 24–72 hours before the release experiments.

Catecholamine secretion experiments

The secretion experiments were performed using previously described procedures [8,9]. The chromaffin cells were washed with 1 ml of balanced salt solution 1X (HBSS, Mediatech, Inc., USA) and incubated for 10 minutes. The HBSS was drawn off and fresh HBSS added, with or without secretagogues and with or without increasing concentrations of edrophonium (10^{-7} to 10^{-3} M), for 1–20 min. The various secretagogues that were used were 50 mM of KCl, 10^{-6} M of angiotensin II and 50 μM of nicotine. At the end of the stimulation period, the HBSS was removed and centrifuged at 15,000 rpm for 5 min. The supernatant was drawn off and placed in 10 μl of 50% acetic acid. The HBSS solution was frozen at -70°C. The cells were lysed in the wells with 10% acetic acid and frozen at -70°C.

Catecholamine measurements

Catecholamine (norepinephrine, epinephrine and dopamine) concentrations in the medium and cells were assayed using high pressure liquid chromatography with electrochemical detection [10]. Catecholamine concentrations were measured in the medium and the cells from the same wells. Catecholamine secretion was calculated by dividing the catecholamine concentration in the medium by the total concentration in that well (medium + cells).

Data analysis

Catecholamine secretion after addition of secretagogues or edrophonium to the wells was expressed as a percent of that in the control wells. Results are expressed as means ± SE. Differences between dose-response curves of the various secretagogues were assessed by two-way repeated measure analyses of variance. The effects of added edrophonium to these secretagogues were assessed by independent means *t*-tests.

Results

The cell preparations yielded about $1x10^6$ chromaffin cells per well in the 24-well plates used for the various experiments.

The fractional secretion of epinephrine, norepinephrine and dopamine increased as a function of the time of incubation with all the known secretagogues [Figure 1]. Nicotine elicited a rapid increase in catecholamine secretion and was the most potent of the secretagogues. Potassium ion and angiotensin II were next in order in both rate and amplitude of catecholamine secretion. In contrast, pre-incubation with edrophonium at three different doses resulted in no increase in catecholamine secretion above the control.

Co-incubation of the cells with or without edrophonium in addition to the standard secretagogues did not augment catecholamine secretion [Table 1].

Discussion

The adrenal medulla plays a key role in homeostatic responses to many metabolic, hemodynamic and emotional stressors. The main determinant of adrenomedullary secretion of catecholamines is

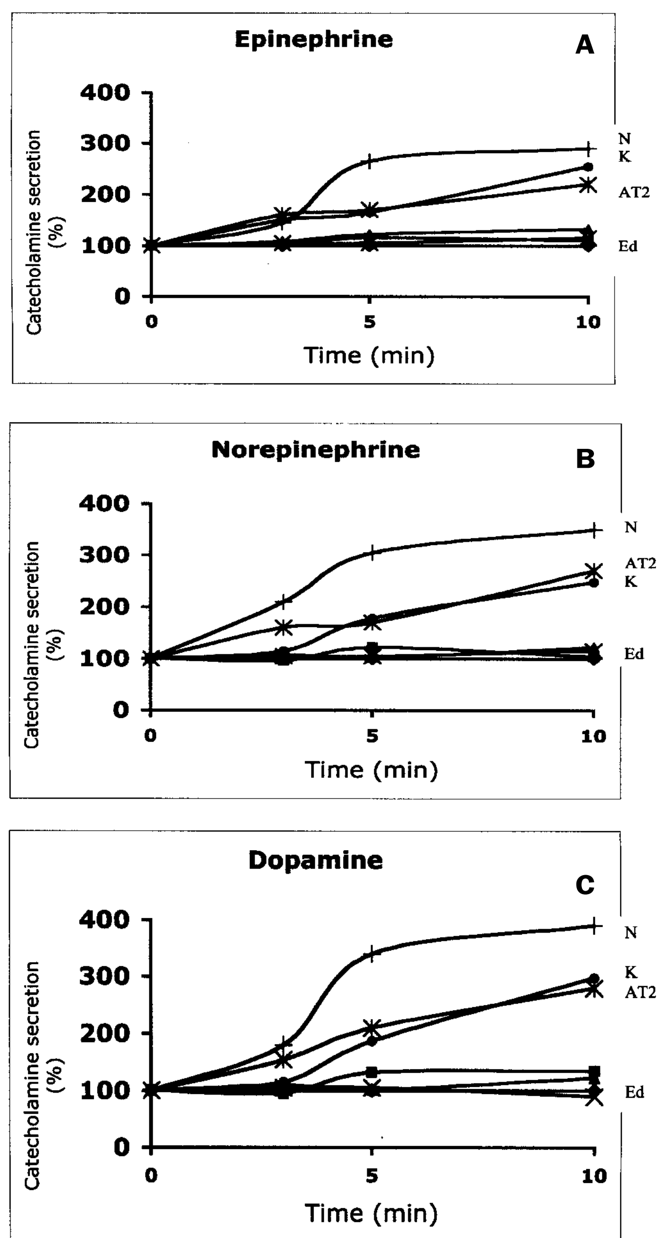

Figure 1. Time-response curve of the fractional excretion of **[A]** epinephrine, **[B]** norepinephrine and **[C]** dopamine. Results are their fractional excretion in response to the various secretagogues compared to control, and after incubation for the increasing period. The top three lines are the standard secretagogues: N = nicotine, K = potassium, and AT2 = angiotensin II. The bottom lines: Ed = edrophonium at the various concentrations.

thought to be sympathetic pre-ganglionic neuronal stimulation, with additional and less well understood influences by hormones such as angiotensin II, glucagon, vasoactive intestinal peptide, and PACAP [8,11,12].

Neuronal stimulation evokes catecholamine secretion from adrenomedullary cells by releasing acetylcholine, which binds to nicotinic cholinergic receptors on the chromaffin cell membrane. Considering that nests of cholinergic neurons are present in myocardial tissue [1–4], and that adrenomedullary tissue expresses

Table 1. Fractional excretion of catecholamines released from chromaffin cells in response to nicotine, potassium ion, and angiotensin II after pre-incubation with or without edrophonium for a period of 10 and 20 minutes

Incubation time	FE of CA without edrophonium	FE of CA with edrophonium	FE of CA without edrophonium	FE of CA with edrophonium
	10 minutes		20 minutes	
Norepinephrine				
Control	100	100	100	100
Nicotine	258 ± 28	264 ± 21	324 ± 33	301 ± 45
Potassium	185 ± 14	167 ± 22	248 ± 27	262 ± 35
Angiotensin II	198 ± 31	185 ± 27	285 ± 35	291 ± 40
Epinephrine				
Control	100	100	100	100
Nicotine	210 ± 17	241 ± 29	287 ± 22	259 ± 36
Potassium	242 ± 26	220 ± 16	310 ± 29	296 ± 31
Angiotensin II	183 ± 23	194 ± 26	264 ± 34	282 ± 39
Dopamine				
Control	100	100	100	100
Nicotine	295 ± 43	323 ± 31	457 ± 54	420 ± 47
Potassium	303 ± 47	288 ± 41	385 ± 61	364 ± 58
Angiotensin II	246 ± 55	223 ± 62	298 ± 56	273 ± 69

Numbers are percent increase (average ± SD) compared to control, i.e., catecholamines secretion after incubation with physiologic solution for the corresponding 10 or 20 minutes.
FA = fractional excretion, CA = catecholamines.

acetylcholinesterase, in this study we explored whether in primary cultures of adrenomedullary cells there is a population of cholinergic neurons that secrete acetylcholine tonically, resulting in occupation of nicotinic receptors and secretion of catecholamines even in the unstimulated condition.

The kinetics of the response of bovine adrenomedullary cells to acetylcholine and the effect of associated factors such as potassium depolarization, calcium concentration, and nicotinic and muscarinic agents were previously shown by Schneider et al. [5]. In part of their study the authors looked at the effect of eserine and neostigmine on catecholamine secretion in response to acetylcholine. The results were not entirely consistent as eserine showed no effect while neostigmine did, although little. A possible explanation is that the response was maximal in the presence of acetylcholine. Our study design was aimed at directly addressing the issue with the current superior assays for catecholamine measurements. The results failed to support the existence of a population of cholinergic neurons, because inhibition of acetylcholinesterase did not affect catecholamine secretion, at baseline or in response to the secretagogues nicotine, potassium ion, or angiotensin II.

Several studies have suggested different functions of adrenomedullary cholinesterase, beyond the classical enzymatic termination of actions of acetylcholine [13]. For instance, in PC12 cells, Huperzine A, a cholinesterase inhibitor, reduces hydrogen peroxide-induced cellular injury [14], consistent with a protective effect. Another potential role is in neuronal differentiation, since in PC12 cells the expression of cholinesterase activity is associated with neurite outgrowth [15,16]. Another study used an antisense oligonucleotide to inhibit acetylcholinesterase in PC12 cells and also indicated a role in neuronal differentiation [17].

Consistent with our findings in bovine adrenomedullary cells, in guinea pig adrenomedullary chromaffin cells, physostigmine fails to alter catecholamine secretion at baseline or in response to potassium ion [18]. The results of these studies were different from those that used the PC12 model for chromaffin cells. Therefore, in view of the current knowledge, there is room for revisiting this issue. It has to be remembered that the present study was an *in vitro* study performed in bovine adrenomedullary cells, and understanding the mechanism in this model may imply the possible mechanism in catecholamine secretion in humans.

In conclusion, in bovine adrenomedullary chromaffin cells, inhibition of acetylcholinesterase acutely does not augment catecholamine secretion, nor does it enhance the secretory responses to nicotine, angiotensin II, or potassium ion. The results cast doubt on the notion that a population of tonically active cholinergic cells exists within the adrenal medulla.

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