NICOTINE STIMULATES SECRETION OF BOTH CATECHOLAMINES AND ACETYLCHOLINESTERASE FROM CULTURED ADRENAL CHROMAFFIN CELLS¹

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Abstract

There is conflicting evidence from studies on sympathetic ganglia and the adrenal medulla concerning the morphological and biochemical localization and physiological role(s) of the enzyme acetylcholinesterase (AChE). Furthermore, the origin of the AChE released from the adrenal medulla (whether from chromaffin cells or splanchnic nerve, or both) has not been firmly established. We have examined the efficacy of cholinergic agonists to release endogenous AChE and catecholamines (CA) from monolayer cultures of purified bovine adrenal chromaffin cells. The nicotinic agonist (nicotine), but not the muscarinic agonist (methacholine), released both AChE and CA from the adrenal chromaffin cells. The concomitant release of CA and AChE evoked by nicotine was Ca^{++} dependent with a correlation coefficient $r = 0.82$ ($p < 0.001$). The results show that adrenal chromaffin cells *in vitro*, a system free of splanchnic nerve elements, can still release AChE. The finding that concomitant release of AChE and catecholamines occurs on exposure of the cells to nicotinic agonists suggests that released AChE may have a physiological role at neuroeffector junctions.

At the neuromuscular junction, acetylcholinesterase (AChE) is localized to the postsynaptic membrane and plays an important role in the control of normal neuromuscular activity by hydrolyzing acetylcholine (ACh) following its release from nerve terminals. By contrast, the function of AChE in ganglionic transmission has not been firmly established (for review see Silver, 1974). Anticholinesterases do not affect ganglionic transmission as dramatically as they affect neuromuscular transmission. It has been suggested that in the ganglion the termination of transmitter action is not primarily dependent on the hydrolysis of ACh by AChE but depends more on the diffusion of ACh from the synapse (Emmelin and McIntosh, 1956).

Morphological observations on cholinergically innervated sympathetic ganglia (Somogyi and Chubb, 1976;

Gisiger et al., 1978) and the adrenal medulla (Somogyi et al., 1975) show that relatively little AChE is associated with the plasma membrane adjacent to the presynaptic cholinergic terminals. Rather, AChE has been demonstrated by ultrastructural histochemistry to be sequestered in cisternae of the endoplasmic reticulum of the target cells. The function of the cisternal AChE is not known. Recent evidence suggests that spontaneous release of AChE occurs from neuronal cells *in vivo* (Kreutzberg and Toth, 1974) and *in vitro* (Skangiel-Kramska and Niemierko, 1975) and from cultured muscle (Wilson et al., 1973), spinal cord (Oh et al., 1977), sympathetic ganglia (Gisiger and Vigny, 1977), and neuroblastoma (Oh et al., 1977; Kimhi et al., 1980; Lazar and Vigny, 1980) cells. Enhanced AChE release has also been demonstrated from unidentified central neurons into cerebrospinal fluid following electrical and pharmacological (Chubb et al., 1976; Bareggi and Giacobini, 1978; Greenfield et al., 1979; Greenfield and Smith, 1979) stimulation, into the perfusates collected from cannulated cat substantia nigra and caudate nucleus (Greenfield et al., 1980) and retrogradely perfused bovine adrenal glands (Chubb and Smith, 1975), and into the incubation medium obtained from stimulated rat phrenic nerve-hemidiaphragm preparations (Skau and Brimijoin, 1978).

In an earlier study (Mizobe and Livett, 1980) we provided evidence for the production and spontaneous

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release of AChE from primary monolayer cultures of bovine adrenal chromaffin cells. Monolayer cultures of adult bovine chromaffin cells can be isolated and maintained under conditions free of non-neuronal cells and splanchnic nerve influence (Livett et al., 1978). These cultured cells are readily accessible to reagents added to the incubation medium which can be easily and rapidly changed. Moreover, release is not complicated by diffusion barriers present in intact tissue. Therefore, primary cultures of bovine adrenal chromaffin cells provide a useful experimental system for investigating the evoked release of AChE.

We now show that in adrenal chromaffin cells, an *in vitro* system free of splanchnic nerve elements, concomitant release of AChE and catecholamines occurs following nicotinic stimulation. The origin of the released AChE is not certain. It could conceivably come from exocytosis of chromaffin vesicles, or from the cisternae. In view of the histochemical evidence that most of the AChE is located in cisternae, we suggest that the release of AChE is parallel but separate from the release of catecholamines, the release of AChE most likely occurring from the cisternae into the extracellular space.

Materials and Methods

Cell culture and pharmacological stimulation

Primary cultures of adrenal chromaffin cells were established as monolayers on collagen-coated plastic dishes and maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco)—10% fetal calf serum, heat inactivated (FCS) (Livett et al., 1978) containing 5-fluorodeoxyuridine (10^{-5} M) and cytosine arabinoside (10^{-5} M), to prevent the proliferation of non-neuronal cells (see Livett et al., 1979; Mizobe et al., 1979). After 7 to 11 days in culture, the cells were washed thoroughly with DMEM and a HEPES-buffered Krebs-Ringer saline (KRH) (Mizobe et al., 1979). Afterward, the cells were incubated in KRH medium with or without (control) a secretagogue for 8 min at 37°C to study the release of endogenous catecholamines and AChE. Nicotine and methacholine were used as secretagogues.

Assay of catecholamines. Endogenous catecholamines (both within the cells and released into the media) were assayed by the two-wavelength differential spectrofluorimetric procedures of Renzini et al. (1970) and Miura et al. (1977) as described elsewhere (Livett et al., 1981).

Assay of AChE. AChE activity was measured by the radiometric method of Johnson and Russell (1975) with minor modification. The assay mixture contained 80 μ l of the supernatant of incubation media or cell homogenates in KRH medium (pH 7.2) and 20 μ l of acetylcholine substrate ($[^3\text{H}]\text{acetylcholine chloride}$, specific activity 3.9 Ci/mol, final 5.1×10^{-4} M). Reactions were started by addition of the substrate, and incubations were carried out at 37°C for period of 10 to 20 min. The reaction was stopped by adding 0.1 ml of stopping mixture (1 M chloroacetic acid, 0.5 M NaOH, and 2 M NaCl, pH 2.5), followed by adding 4.5 ml of scintillation mixture (100 ml of isoamyl alcohol, 4 gm of 2,5-diphenyloxazole (PPO), 50 mg of 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP) in 1 liter of toluene). Appropriate blanks were included

in which KRH medium was added instead of the enzyme samples. Radioactivity was measured in a scintillation counter with a counting efficiency of 49%. The enzyme reaction was linear with time for more than 25 min, was proportional to enzyme concentration, and was inhibited completely by 4×10^{-5} M eserine. Total cholinesterase activity, both within the cells and released into the incubation medium, was inhibited 92 to 96% by the specific AChE inhibitor, 1,5-bis-(4-allyldimethylammonium-phenyl)pentan-3-one dibromide (BW 284C51, 5×10^{-6} M), indicating that essentially all the activity was present as *true* acetylcholinesterase (Klingman et al., 1968; Silver, 1974).

Results

Release of catecholamines and AChE. Figure 1 shows the time course of the release of endogenous catecholamine and cellular AChE from bovine adrenal chromaffin cells stimulated with nicotine (10^{-5} M). The release of endogenous catecholamines evoked by nicotine was quite rapid and 90 to 100% of maximum release occurred within 5 min, consistent with our earlier observations on the time course of release of $[^3\text{H}]\text{noradrenaline}$ evoked by ACh (Mizobe et al., 1979). Nicotinic stimulation produced a similar rapid release of AChE which occurred in parallel with the release of catecholamines (Fig. 1).

Characterization of nicotinic response. The nicotinic receptor response of many cell types shows a characteristic activation at low levels of agonist (10^{-7} to 10^{-5} M nicotine) and an inhibition ("desensitization") (Katz and Thesleff, 1952) at higher levels ($>10^{-4}$ M). In accord with this general property, the nicotine-evoked release of endogenous catecholamines and AChE in the presence of Ca^{2+} (Fig. 2) showed similar dose response curves with inhibition at high concentrations of agonist. The release of both catecholamines and AChE evoked by nicotine was dependent on extracellular Ca^{++} as shown in Figure 2. When the cells were exposed to nicotine in the absence of extracellular Ca^{++} , the release of AChE and catecholamines (Mizobe et al., 1979) was at or below the basal level. Nicotine-induced release of cellular AChE was highly significant at 5×10^{-6} M and 10^{-5} M (the Student's *t* test, $p < 0.001$), even though the maximum release (at 10^{-5} M) was only 1% higher than basal release. In contrast, the muscarinic agonist, methacholine (10^{-6} to 10^{-3} M) did not stimulate the release of endogenous catecholamines and AChE (data not shown). Nicotine (10^{-5} M) and methacholine (10^{-4} M) by themselves did not have any effect on the enzyme assay for AChE activity.

Proportionality of AChE and catecholamine released. The net evoked release of the AChE was proportional to that of catecholamines over the range of nicotine concentrations used (5×10^{-7} to 10^{-5} M) (Fig. 3). The correlation coefficient ($r = 0.819$) between released AChE and catecholamines was significant at the $p < 0.001$ level.

Discussion

Our results show that nicotinic stimulation of chromaffin cells *in vitro* brings about the simultaneous release of AChE and endogenous catecholamines. The release evoked by ACh and nicotine was Ca^{++} -dependent, suggestive of exocytosis. There is good evidence that

Figure 1. Time course of release of endogenous catecholamines and AChE in the presence and absence of nicotine. Chromaffin cells (2.5×10^5 cells/well) were maintained in 16 mm, 24-well plastic cluster dishes (Falcon) containing 0.5 ml of DMEM-10% FCS. At day 8, cultures were washed with DMEM (serum free) and incubated in DMEM for 1 hr at 37°C . The cultures were then washed twice by incubation in KRH medium at 37°C for 15 min. After pre-incubation in 0.3 ml of KRH medium for an additional 8 min, the cultures were incubated in 0.3 ml of KRH medium with ($\circ$, $\bullet$) or without (∇ , $\blacktriangledown$) 10^{-5} M nicotine to evoke release. At the time indicated, the incubation media were removed from the cells, placed on ice, and centrifuged at $30,000 \times g$ for 30 min. The supernatant was recovered to measure catecholamines ($\circ$, ∇) and AChE activity ($\bullet$, $\blacktriangledown$). The cells were scraped off the plate and homogenized in 1.5 ml of KRH medium, and aliquots were used for assay of catecholamines and AChE activity (see "Materials and Methods"). The 8-day-old cultures contained 212 ± 5 (mean $\pm$ SEM, $n = 14$) nmol of catecholamines per 10^6 cells, and an AChE activity of 3.74 ± 0.11 ($n = 14$) nmol of ACh hydrolyzed min^{-1} per 10^6 cells. The release of endogenous catecholamines and AChE from the cells into the medium is expressed as a percentage of the total content in the cells. The results of a typical preparation are shown where each point represents the average of duplicate determinations.

release of catecholamines from the cells occurs by exocytosis of the chromaffin granules (Douglas, 1968; Fenwick et al., 1978); however, there is no convincing evidence that AChE is secreted by this mechanism. Biochemical studies have given conflicting information about the distribution of AChE in chromaffin cells. Whereas Wilson and Kirshner (1977) found no evidence for AChE being localized to chromaffin vesicles, Gratzl et al. (1981) reported both membrane-bound and soluble (latent) forms of the enzyme associated with purified chromaffin vesicles. Cytochemical studies on glutaraldehyde-fixed tissue by Somogyi et al. (1975) on the cow, and more recently by Millar and Unsicker (1981) on the rat, and by Carmichael (1982) on cow, pig, and hamster adrenals have localized AChE activity on the external plasma membrane, within the smooth endoplasmic reticulum, and on the nuclear envelope of chromaffin cells but *not* on or within the chromaffin vesicles. However, when Vibratome sections of fresh adrenal medullae were incubated with the enzyme substrates for up to 60 min prior to fixation, Carmichael (1982) demonstrated that low but detectable levels of AChE activity were associated with chromaffin vesicles. The cytochemical results,

when taken together with the biochemical data of Gratzl et al. (1981), suggest that AChE is normally associated with the membrane and soluble contents of chromaffin vesicles where it is present either in low amounts or in a latent form. Since in the present experiments, only a few percent of the total AChE content was released by nicotinic stimulation, a minority source such as the chromaffin vesicles cannot be excluded.

In the intact animal, activation of ACh receptors on adrenal chromaffin cells is brought about by release of ACh from splanchnic nerve terminals innervating the medulla, resulting in the release of catecholamines into the circulation. In a similar manner, our earlier studies on bovine adrenal chromaffin cells showed that [^3H]noradrenaline ([^3H]NA) taken up and stored in reserpine-sensitive sites was released from the cells by activation of nicotinic (but not muscarinic) ACh receptors (Mizobe et al., 1979). Earlier studies by Chubb and Smith (1975) on perfused bovine adrenal glands suggest that, after stimulation with nicotinic agonists and high K^+ , AChE is released into perfusates from the glands, i.e., primarily from the chromaffin cells and possibly from the splanchnic nerves as well. Our present observations with the

Figure 2. Dose response curves for release of endogenous catecholamines and AChE by nicotine in the presence and absence of Ca^{2+} . At day 7 to 11, adrenal chromaffin cells were processed for release experiments as described in the legend to Figure 1. The cells were pre-incubated for 8 min in KRH medium with no Ca^{++} added, or containing 2.2 mM Ca^{2+} , and then incubated for 8 min with various concentrations of nicotine in the absence (∇ , $\blacktriangledown$) or presence ($\circ$, $\bullet$) of Ca^{++} at 37°C . Endogenous catecholamines ($\circ$, ∇) and AChE ($\bullet$, $\blacktriangledown$) (both within the cells and released into the media) were assayed as described under "Materials and Methods." These cultures from six preparations contained 315 ± 64 nmol of catecholamines per 10^6 cells and AChE activity of 4.97 ± 1.14 nmol min^{-1} per 10^6 cells. Basal release, which was obtained from duplicate wells, has been subtracted from the release data. The basal release (i.e., the release in the absence of nicotine) from the cells plated on multiwells amounted to $8.8 \pm 1.0\%$ ($n = 12$) of endogenous catecholamines in the presence of Ca^{2+} (2.2 mM) and $9.3 \pm 1.7\%$ ($n = 6$) in the absence of Ca^{2+} ; $1.37 \pm 0.25\%$ ($n = 12$) of AChE in the presence of Ca^{2+} and $1.57 \pm 0.30\%$ ($n = 6$) in the absence of Ca^{2+} . The results are given as mean $\pm$ SEM ($n = 4$ to 12).

isolated chromaffin cells in culture which are free from any splanchnic nerve influence show that upon depolarization AChE activity is released from the chromaffin cells themselves. Since most of the AChE activity in the adrenal medulla has been localized to cisternae of endoplasmic reticulum, with some on the plasma membrane, it is most likely that the released AChE originates from these cisternae (see Chubb, 1977). However, the possibility is not excluded that some, or all, of the released AChE activity might originate from the plasma membrane or from chromaffin vesicles (Gatzl et al., 1981; Carmichael, 1982). Whatever the source of the released AChE, the mechanism of this release seems to share features of stimulus-secretion coupling (Douglas, 1968) responsible for the release of catecholamines from chromaffin cells.

Our earlier studies (Mizobe and Livett, 1980, 1982) showed that a specific anti-acetylcholinesterase, BW-

284C51, which does not readily penetrate the plasma membrane of cells (McIsaac and Koelle, 1959; Silver, 1974) restored the efficacy of ACh to stimulate the bovine adrenal chromaffin cells, indicating that AChE located on the external surface of the plasma membrane may hydrolyze exogenously applied ACh. The present results showing that nicotine stimulates the release of AChE from the chromaffin cells suggests that exogenously applied ACh may be hydrolyzed not only by AChE located on the plasma membranes, but also by soluble AChE secreted from the cells in response to the agonist.

The function of the released AChE is not known (Chubb, 1977). In the adrenal medulla the secreted AChE might function to remove excess ACh from any further pharmacological effects, particularly in those regions where membrane-bound AChE is sparse or not present (Somogyi et al., 1975), and to provide choline for re-

Figure 3. Correlation between AChE released and endogenous catecholamine released from bovine adrenal chromaffin cells by nicotine. Data are expressed as percentage of the total cellular content of AChE or endogenous catecholamines released by nicotine over the range 5×10^{-7} M to 10^{-5} M. Basal release has been subtracted from the release data (see legends to Figs. 1 and 2 for details). The solid line is drawn according to the equation $y = 8.87x + 0.78$, as determined by linear regression analysis (correlation coefficient $r = 0.819$, $p < 0.001$, $n = 27$).

uptake or resynthesis of ACh in splanchnic nerve terminals as proposed for sympathetic ganglia (Gisiger and Vigny, 1977). Recently, it has been reported that highly purified AChE from both fetal calf serum and electric eel has peptidase activity and can hydrolyze both substance P (Chubb et al., 1980) and Leu- and Met-enkephalin (Chubb et al., 1982). AChE seems to show some selectivity in its action because it does not hydrolyze two other neuropeptides (somatostatin and neurotensin) endogenous to the adrenal medulla or a number of other neuropeptides, including β -endorphin, oxytocin, vasopressin, and angiotensin II (Chubb et al., 1982). Inasmuch as substance P and the enkephalins are endogenous components of the splanchnic nerve that innervates the adrenal medulla (Schultzberg et al., 1978; Linnoila et al., 1980) and act as inhibitory modulators of the nicotinic response of chromaffin cells to release catecholamines (Livett et al., 1979; Mizobe et al., 1979; Role et al., 1981), it suggests that another function of the released AChE might be to hydrolyze these peptides, so terminating their biological activity. Whatever the precise function(s) of the released AChE, the present demonstration that AChE and catecholamines are released concomitantly upon nicotinic stimulation provides a mechanism by

which the chromaffin cell could ensure prompt termination of the effects of splanchnic nerve stimulation.

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