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Secretion of acetylcholinesterase and butyrylcholinesterase from the guinea-pig isolated ileum

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1 Strips of longitudinal muscle from guinea-pig ileum, retaining Auerbach's plexus, were superfused with oxygenated Krebs solution. Addition of 50 mM KCl led to a pronounced Ca^{2+}-dependent increase in the activities of both acetylcholinesterase and non-specific cholinesterase (butyrylcholinesterase) in the perfusate but with no change in lactate dehydrogenase activity.

2 No release of acetylcholinesterase, either spontaneous or K^+-evoked was observed in tissue freed of the nerve plexus, although release of butyrylcholinesterase still occurred.

3 Carbachol induced a marked Ca^{2+}-dependent increase in the release of acetylcholinesterase but had no effect on the release of butyrylcholinesterase or lactate dehydrogenase. This carbachol-evoked increase in acetylcholinesterase release was blocked by hexamethonium but not by atropine.

4 Four readily soluble molecular forms of acetylcholinesterase and three soluble molecular forms of butyrylcholinesterase were present in innervated longitudinal muscle strips, but insignificant amounts of acetylcholinesterase were detected in denervated strips of muscle. Only one of the four molecular forms of acetylcholinesterase was recovered in the perfusates.

5 It is concluded that acetylcholinesterase is secreted from the nerves of Auerbach's plexus in response to depolarizing stimuli or to nicotinic cholinergic stimulation, while butyrylcholinesterase is secreted from non-neural elements, possibly the longitudinal muscle cells, of guinea-pig ileum in response to a depolarizing stimulus.

Introduction

Neurones within the central nervous system can release acetylcholinesterase (EC 3.1.1.7; AChE) upon stimulation (Chubb *et al.*, 1976; Greenfield & Smith 1979; Greenfield, 1984; Vogt *et al.*, 1984; Appleyard *et al.*, 1988; Appleyard & Smith, 1987a). The release of AChE has also been demonstrated in the periphery, e.g. from the adrenal medulla (Chubb & Smith, 1975b), the phrenic nerve-diaphragm preparation (Skau & Brimijoin, 1978), sympathetic ganglia (Gisiger & Vigny, 1977), fibroblast cells (Bartos & Glinos, 1976) and platelets (Chuang *et al.*, 1976).

The gastro-intestinal tract is very rich in AChE (Koelle, 1953) and certain clinical observations have raised the possibility that both AChE and non-specific cholinesterase (EC 3.1.1.8, butyrylcholinesterase, BuChE) might be released from the gastro-intestinal tract. Thus several groups who have examined the cholinesterase content of amniotic fluid have detected the presence of AChE (Wald *et al.*, 1980; Dale, 1980; Voigtlander *et al.*, 1981; Garry *et al.*, 1981; Crandall *et al.*, 1982; Read *et al.*, 1982) in cases of foetal exomphalos. It has been suggested by Wald *et al.* (1980) that the AChE is derived from peripheral nervous tissue such as the intestinal nerve plexi which are exposed to the amniotic fluid in this condition. Indeed soluble AChE has been found in rectal biopsy material from children with Hirschsprung's disease (Dale *et al.*, 1979). Secretion of BuChE from intestinal tissue in cases of exomphalos is also suggested, both by the occasional presence of additional electrophoretic bands of BuChE (Wald *et al.*, 1980) and by the higher ratio of BuChE to AChE activities in amniotic fluid in cases of exomphalos compared to cases with neural tube defects, in the absence of any visible blood contamination (Goldfine *et al.*, 1983; Wald *et al.*, 1984; Burton, 1986).

In the present study we have examined whether AChE and BuChE can be secreted from an intestinal tissue, namely the longitudinal muscle layer of the

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guinea-pig small intestine, that can be isolated either with, or without, its associated myenteric nerve plexus (Ambache & Freeman 1968; Paton & Abou Zar, 1968). In this tissue the bulk of AChE present is confined to the nerve plexus, whereas the BuChE is confined to the muscle (Ambache *et al.*, 1971). A preliminary account of these results has been given (Appleyard & Smith, 1987b).

Methods

Tissue preparation

Albino guinea-pigs (200–300 g) of either sex were stunned by a blow on the head, then decapitated. The small intestine was removed, about 8–12 cm of the terminal ileum being discarded, and placed in warmed oxygenated Krebs solution containing (mM): NaCl 118, KCl 4.7, $MgSO_4$ 1.14, KH_2PO_4 1.18, $NaHCO_3$ 25, $CaCl_2$ 2.52 and glucose 11.1; gassed with 95% O_2, 5% CO_2. Longitudinal muscle strips were prepared by the method of Rang (1964), and Paton & Aboo Zar (1968), with slight modifications to minimize damage to the plexus. A piece of ileum, approximately 10 cm long, was stretched over a glass pipette of 7 mm diameter and the mesentery was cut away. The longitudinal muscle layer was separated from the underlying circular muscle by stroking tangentially away, in both directions, from the mesenteric attachment at one end of the strip with a small piece of cotton wool, followed by gentle traction to strip it from the whole length. The whole procedure was carried out under warmed oxygenated Krebs solution to minimize the damage to the plexus which occurred if the preparation were not kept moist. This method of preparation yielded strips retaining Auerbach's plexus.

To obtain plexus-free strips, a longer strip of longitudinal muscle was pulled off, the ileum being pulled upwards on the supporting glass pipette by applying gentle traction at the proximal end. The plexus-retaining proximal portions could be identified by their striated appearance under oblique light, while the distal plexus-free strips had a smooth appearance.

In vitro *superfusion*

Sections of longitudinal muscle, weighing approximately 150 mg were superfused with oxygenated Krebs solution at 37°C in 0.4 ml chamber at a rate of 0.25 ml min^{-1}. After an initial washout period of 10 min, fractions of 0.5 ml were collected every two minutes into vials containing 25 μg bovine serum albumin to preserve the enzymes.

K^+-evoked release was studied by replacing an equivalent amount of NaCl by 50 mM KCl in the perfusing medium for 4 min, 10 min after starting fraction collection. The effects of K^+ stimulation upon plexus-free strips were also studied.

In a further series of experiments carbachol (0.01 mM) was added to the perfusion fluid for 4 min, 10 min after starting the collection of fractions. The effects of the cholinoceptor antagonists hexamethonium bromide (1×10^{-3} M) and atropine sulphate (0.5×10^{-6} M) upon carbachol stimulation were also studied, these drugs being present in the perfusion fluid from the start of the superfusion.

All experiments were repeated in Ca^{2+}-free medium containing 1.0 mM EDTA.

Biochemical analysis

All perfusates were assayed for AChE, BuChE and lactate dehydrogenase (LDH) activities. Acetylcholinesterase and BuChE were assayed by a modified version (Chubb & Smith, 1975a) of the Ellman method (Ellman *et al.*, 1961) using 1 mM acetylthiocholine as substrate at pH 7.0 (30°C); the two activities were distinguished (Silver, 1974) by use of the specific AChE inhibitor BW284c51 (1,5-*bis*-(4 allyldimethylammonium phenyl)-pentone-3-one dibromide 1.5×10^{-6} M) and the butyrylcholinesterase inhibitor ethopropazine (10^{-5} M). It should be noted that the activities given for butyrylcholinesterase are submaximal since the substrate was acetylthiocholine, not butyrylthiocholine, and the substrate concentration was below the value that gives V_{max}; however, the substrate concentration was optimal for AChE. Lactate dehydrogenase (LDH) was assayed fluorometrically (Brooks & Olken, 1965).

At the end of the perfusion the tissue samples and the contents of the chamber were homogenized in 1.0 ml of 50 mM phosphate buffer pH 7.0 at 0°C. Samples were centrifuged (1,000 *g* for 10 min) to remove particulate matter and then assayed for choline acetyltransferase (Fonnum, 1975) and AChE activity. Aliquots of homogenate were further centrifuged (at 100,000 *g* for 1 h) to sediment the membranes; the supernatant was then subjected to polyacrylamide gel electrophoresis (Chubb & Smith, 1975a). The perfusates from a number of identical experiments, for both the pre-stimulation and the post-stimulation fractions, were combined and concentrated by vacuum dialysis against 0.39 M Tris/glycine buffer (pH 8.1) and were then analysed by polyacrylamide gel electrophoresis. The gels were incubated with acetylthiocholine to reveal bands of cholinesterase activity (Chubb & Smith, 1975a) using BW 284c51 to distinguish between AChE and BuChE.

Analysis of results

Graphical representation of results To minimize the effect of experimental variation in the resting levels of enzymes in the perfusates, the results of each experiment were standardised for graphical presentation. Basal levels of release for each experiment were determined by calculating the mean value of the activities of the pre-stimulation fractions for the time period during which the release had reached a constant level. The enzyme activity in each sample for a particular experiment was expressed as a percentage of this mean basal release, which was taken as 100%. To obtain combined results for each group of experimental animals the mean ± s.e.mean of these percentages was then calculated for each sample.

Statistical procedures Statistical analysis of the raw data was performed by the use of a one-tailed permutation test (Lindgren, 1976), which has the effect of comparing the mean of the enzyme activity in the last three pre-stimulation fractions with the mean of that in the first three post-stimulation fractions.

For each piece of tissue, the difference (mean fraction after minus mean fraction before) was calculated for the 20 different ways of choosing three fractions from the six as 'before' and three fractions from the six as 'after'. These twenty values were then ranked and thus a rank was assigned to the actual experimental result. A statistic could then be formed by summing the ranks for each piece of tissue. The distribution of this statistic could then be calculated under the null hypothesis that the six fractions are all the same value against the alternative hypothesis that the values for the three fractions after stimulation are larger than the values for the three fractions before stimulation. The result was considered to be significant if it fell within the lower 5% of the distribution, since lower values are more likely under the alternative hypothesis.

Correlations between the activities of AChE and BuChE in perfusates and the tissue contents of these enzymes and choline acetyltransferase were analysed using the Spearman-Rank correlation test.

Results

Basal release of AChE, BuChE and LDH and the effect of depolarizing concentrations of K^+

There was a rapid washout of all three enzymes over the first six minutes of the perfusion; ten minutes after the fraction collection began all enzyme levels were low and relatively constant (Figure 1)

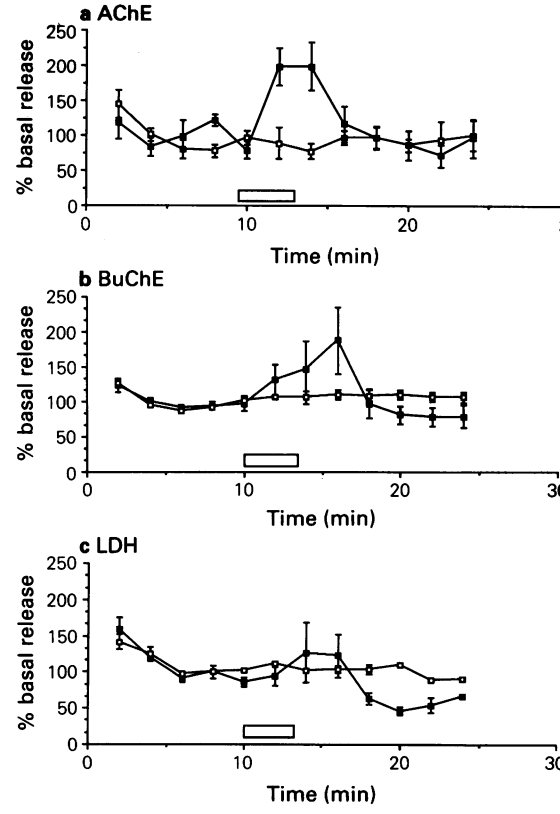

Figure 1 Activities of (a) acetylcholinesterase (AChE), (b) butyrylcholinesterase (BuChE) and (c) lactate dehydrogenase (LDH) in superfusates of Auerbach's plexus showing both the spontaneous release and the secretion of the cholinesterases evoked by depolarizing K^+ stimulation. (□) Unstimulated controls, $n = 4$. (■) Stimulated with 50 mM K^+ during period denoted by the bar, $n = 7$. Activities are expressed as a percentage of the mean basal release in each case. Each point represents the mean and vertical lines show s.e.mean.

with the following basal activities ($n = 11$): AChE = 1.99 ± 0.88, BuChE = 14.1 ± 4.7, LDH = 59.1 ± 3.5 mu mg^{-1} tissue ml^{-1} perfusion fluid. The hydrolysis of acetylthiocholine that was insensitive to BW284c51 was inhibited by ethopropazine (10^{-5} M), thus classifying the enzyme as butyrylcholinesterase (Silver, 1974). The basal levels of AChE and BuChE in perfusates were both significantly correlated with the tissue contents of AChE ($P < 0.013$) and BuChE ($P < 0.007$), respectively, but there was no correlation between basal LDH and the tissue content of LDH.

Introduction of 50 mM KCl into the perfusion fluid, ten minutes after starting superfusion, led to a

Table 1 Effect of depolarising concentration (50 mM) of K^+ upon the release of acetylcholinesterase (AChE), butyrylcholinesterase (BuChE) and lactate dehydrogenase (LDH) from Auerbach's plexus when Ca^{2+} was omitted from the perfusion medium

Enzyme	*Pre-stimulation*	*Post-stimulation*
AChE	95.9 ± 19.0	95.8 ± 13.2
BuChE	102.4 ± 6.6	140.9 ± 18.5
LDH	89.4 ± 5.2	128.2 ± 21.5

All activities are expressed as a percentage of the mean basal release. Pre-stimulation values are the mean ± s.e.mean of the three fractions immediately preceding stimulation, post-stimulation values are the mean ± s.e.mean of the three fractions immediately following stimulation, $n = 4$. None of the post-stimulation values was significantly different from the corresponding pre-stimulation value.

rapid and statistically significant ($P = 0.001$) increase in the AChE activity in the perfusate, the levels returning to normal upon restoration of normal K^+ concentrations (Figure 1). A less marked (but statistically significant with $P < 0.005$) rise in BuChE activity of slower onset also occurred in response to depolarising concentrations of K^+, but there was no change in the LDH activity of the perfusates (Figure 1). These K^+-evoked increases in AChE and BuChE activities were Ca^{2+}-dependent since there were no significant increases of AChE and BuChE activities in response to 50 mM K^+ in calcium-free perfusates (Table 1).

When plexus-free tissue was used no release of AChE, either basal or K^+-evoked, could be detected. However, BuChE activities in the perfusates, both basal and K^+-evoked, were comparable to those of plexus-retaining tissue, as were levels of LDH (data not shown). Classification of the tissue as denervated was shown to be correct by the absence of significant choline acetyltransferase activity in the tissue homogenates. AChE activity was also negligible in the same tissue homogenates compared to the high activities detected in plexus-retaining tissue.

Effect of carbachol on the release of AChE, BuChE and LDH

Introduction of 0.01 mM carbachol into the perfusion medium led to a significant ($P = 0.002$) increase in AChE release that was rapid in onset. However, there was no significant accompanying change in BuChE activity or LDH activity in the perfusates (Figure 2). No increase in the release of AChE was observed in response to carbachol stimulation when

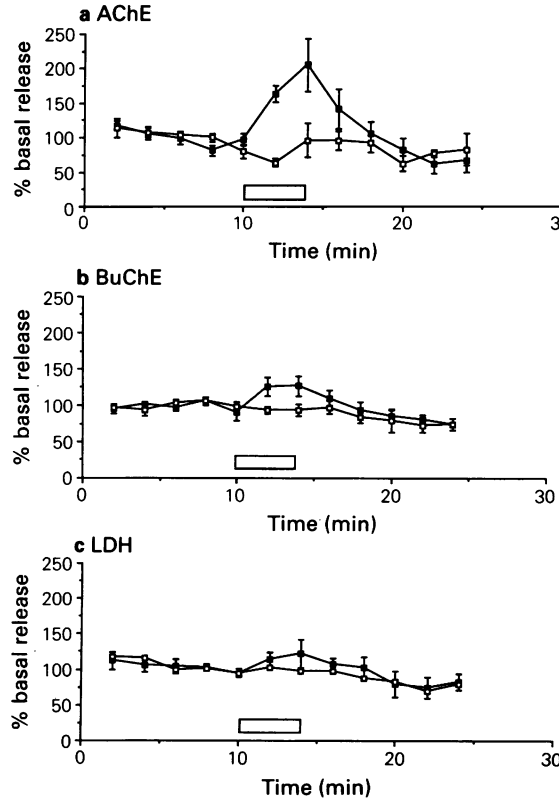

Figure 2 Effect of carbachol (0.01 mM) stimulation (bar) upon the release of (a) acetylcholinesterase (AChE), (b) butyrylcholinesterase (BuChE) and (c) lactate dehydrogenase (LDH) into superfusates of Auerbach's plexus in medium containing 2.52 mM Ca^{2+} (■) ($n = 8$) and with no added calcium salts (□) ($n = 5$). Activities are expressed as a percentage of the mean basal release in each case. Each point represents the mean and vertical lines show s.e.mean.

Ca^{2+} was omitted from the perfusion fluid (Figure 2). The carbachol-evoked release of AChE was not blocked by the addition of 0.005 mM atropine to the perfusion fluid (Figure 3); again release was found to be Ca^{2+}-dependent, and there was no accompanying change in BuChE and LDH activities of the perfusates (Table 2). The addition of hexamethonium (1 mM) blocked the carbachol-evoked release of AChE (Figure 3).

The tissue samples were analysed for their choline acetyltransferase activity, as a measure of the extent of the cholinergic innervation. There was significant correlation (Spearman-Rank, $P < 0.05$) between the choline acetyltransferase activity of the tissue and the maximum amount of AChE released by the tissues in response to stimulation with either K^+ or carbachol (Figure 4).

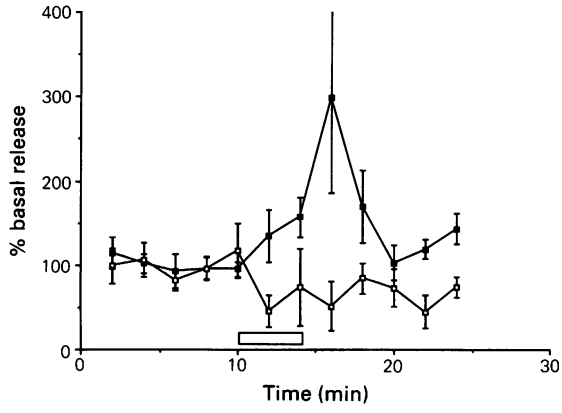

Figure 3 Effect of the cholinoceptor antagonists atropine (■) ($n = 4$) and hexamethonium (□) ($n = 4$) on the carbachol-evoked (bar) release of acetylcholinesterase (AChE) into superfusates of Auerbach's plexus. Activities are expressed as a percentage of the mean basal release. Each point represents the mean and vertical lines show s.e.mean.

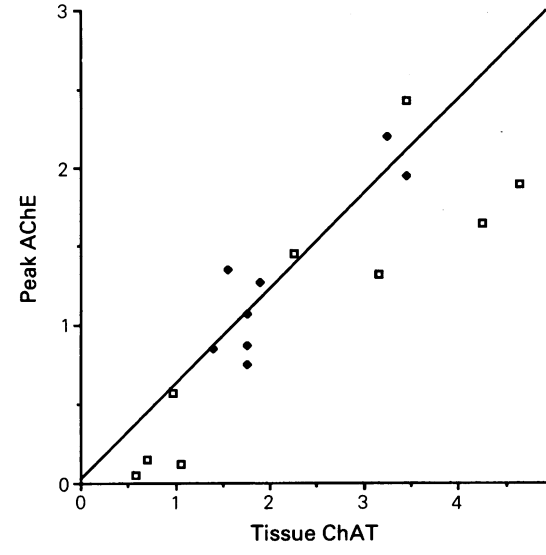

Figure 4 Correlation between peak levels of acetylcholinesterase (AChE) in the perfusate and the tissue content of choline acetyltransferase (ChAT) in superfused strips of Auerbach's plexus stimulated with a depolarizing concentration of K^+ (□) or 0.01 mM carbachol (◆).

Isoenzymes of AChE in tissue and perfusates

Electrophoretic analysis of the soluble fraction of homogenates of plexus-retaining longitudinal muscle strips revealed five bands of cholinesterase activity. Two of these bands disappeared when the gels were

Table 2 Effect of various stimulating agents upon the release of butyrylcholinesterase (BuChE) and lactate dehydrogenase (LDH) from Auerbach's plexus

	BuChE	
Stimulation	*Pre-stimulation*	*Post-stimulation*
Carbachol 0.01 mM + Hexamethonium 1 mM ($n = 4$)	94.0 ± 6.4	118.9 ± 21.6
Carbachol 0.01 mM + Atropine 500 nM ($n = 4$)	102.0 ± 4.1	95.6 ± 4.0
	LDH	
Stimulation	*Pre-stimulation*	*Post-stimulation*
Carbachol 0.01 mM + Hexamethonium 1 mM ($n = 4$)	96.9 ± 6.2	124.3 ± 17.1
Carbachol 0.01 mM + Atropine 500 nM ($n = 4$)	97.1 ± 1.3	92.6 ± 11.7

All activities are expressed as a percentage of the mean basal release. Pre-stimulation values are the mean ± s.e.mean of two fractions immediately preceding stimulation, post-stimulation values are the mean ± s.e.mean of the three fractions immediately following stimulation.
None of the post-stimulation values was significantly different from the corresponding pre-stimulation values.

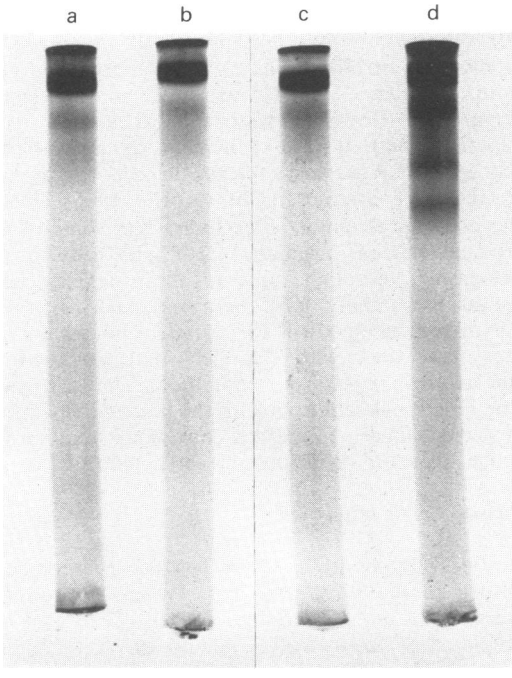

Figure 5 Polyacrylamide gels of high-speed supernatants of tissue homogenates of innervated and denervated strips of guinea-pig longitudinal muscle stained to reveal cholinesterase activity. The samples were applied at the top and the direction of migration was towards the anode. Gels (a) and (b) are from the homogenates of longitudinal muscle with nerve plexus removed whilst gels (c) and (d) are from homogenates of longitudinal muscle with the plexus intact. Gels (a) and (c) were stained in the presence of the acetylcholinesterase inhibitor BW284c51.

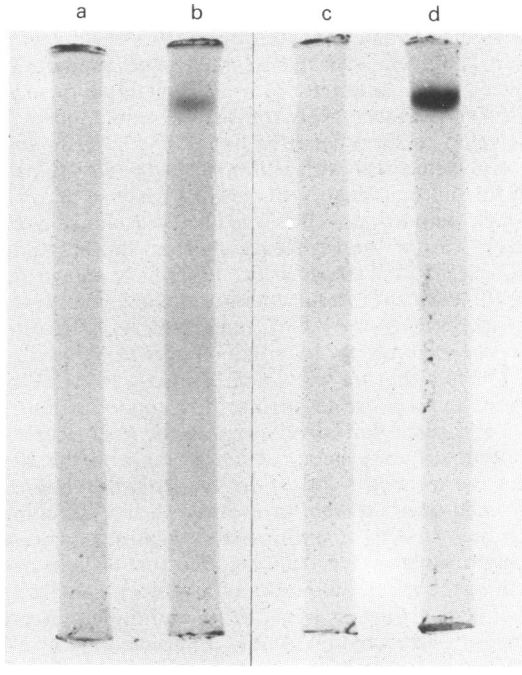

Figure 6 Polyacrylamide gels of superfusates of Auerbach's plexus before (a, b) and after (c, d) carbachol stimulation, stained to reveal cholinesterase activity. The samples were applied at the top and the direction of migration was towards the anode. Gels (a) and (c) were incubated in the presence of the acetylcholinesterase inhibitor BW284c51, whilst gels (b) and (d) were incubated in the absence of inhibitor.

incubated in the presence of the acetylcholinesterase inhibitor BW284c51 and two were reduced in intensity of staining, suggesting that the tissue contains up to four molecular forms of soluble AChE and three of BuChE (Figure 5). No isoenzymes of AChE could be detected in homogenates of denervated tissue. BuChE was present in both preparations: since there was no noticeable difference in the intensity of staining in the presence of BW284c51 for both innervated and denervated tissue, BuChE appears to be confined to the non-nervous elements of the tissue.

Electrophoretic analysis of the perfusates for AChE revealed that the AChE activity, both basal and carbachol-evoked, was attributable to a single molecular form (Figure 6).

Discussion

The results indicate that both AChE and BuChE are secreted from the guinea-pig ileum. Secretion of AChE could be evoked in a Ca^{2+}-dependent manner by both depolarizing concentrations of K^+ and the cholinoceptor agonist carbachol. In neither case was the increase in activity due to non-specific tissue damage since levels of the soluble cytoplasmic marker LDH remained unchanged. Furthermore, only one of the four soluble isoenzymes of AChE present in Auerbach's plexus was present in the perfusate. This is analogous to the situation in the adrenal medulla (Chubb & Smith, 1975b) and brain (Chubb *et al.*, 1976; Greenfield & Shaw, 1982) where only one of several soluble isoenzymes present in the tissue is secreted.

Origin of the secreted cholinesterases

The secreted AChE would appear to be of neuronal origin, rather than deriving from the longitudinal muscle, since no AChE release, either spontaneous or evoked, could be demonstrated from tissue from which the nerve plexus had been removed. Indeed, denervated tissue was found to contain negligible

AChE activity; the small amount present can be attributed to the presence of nervous elements which were not removed during the tissue preparation, since small amounts of the cholinergic neurone marker choline acetyltransferase could also be detected in the denervated tissue. The latter findings are in agreement with earlier histochemical (Koelle, 1955) and manometric studies (Ambache *et al.*, 1971) which found that AChE was localised almost exclusively in the nerve plexus. Histochemical studies (Koelle, 1955; Garrett *et al.*, 1972) have shown that neurones of the intermyenteric ganglion stain weakly to moderately for AChE, and that AChE-positive nerves innervate the longitudinal muscle.

The fact that the maximum amounts of AChE released in response to depolarizing concentrations of K^+ and carbachol correlated with the tissue levels of choline acetyltransferase is consistent with the view that the secreted AChE is derived from the cholinergic neurones of the myenteric plexus. Immunostaining with antibodies against choline acetyltransferase has demonstrated that at least one-fifth of the nerve cell bodies of myenteric ganglia are cholinergic (Furness *et al.*, 1983), and these cells presumably also contain AChE. Pharmacological evidence demonstrates two types of intrinsic cholinergic neurone within the enteric nervous system (Furness & Costa, 1980): those which act upon the smooth muscle (Bennett & Burnstock, 1968) and cholinergic interneurones with inputs onto neurones of the myenteric plexus (Hirst *et al.*, 1974). In addition, there are extrinsic cholinergic inputs to the enteric nervous system, arising from the bulbar and sacral outflow of the central nervous system (Furness & Costa, 1980). The secreted AChE could derive from the processes and axons of any of these cholinergic nerves. In addition AChE is present in non-cholinergic peripheral neurones (Silver, 1974) and these could contribute to the secreted AChE.

The carbachol-evoked secretion of AChE would appear to involve nicotinic receptors since it is blocked by hexamethonium but not by atropine. Extracellular and intracellular recording studies have demonstrated a population of neurones in the myenteric plexus which have an excitatory nicotinic synaptic input (North, 1982). It is possible that these include AChE-staining cells; AChE could therefore be secreted from these cells in response to stimulation of the cholinergic input.

Most, if not all, of the secreted butyrylcholinesterase seems to derive from non-nervous tissue, such as the longitudinal muscle, rather than from the nerve plexus, since removal of the nerve plexus did not greatly affect either the spontaneous or the K^+-evoked secretion of BuChE. Biochemical analysis has demonstrated that the bulk of BuChE in the guinea-pig remains after denervation of the longitudinal muscle (Ambache *et al.*, 1971); this agrees with our observation that no appreciable difference in intensity of BuChE stains could be detected in gels from innervated and denervated muscle strips (Figure 5). However, histochemical studies have demonstrated that most of the neurones and interstitial cells of Auerbach's plexus in cat, rabbit and monkey also contain BuChE (Koelle, 1955). Hence, the possibility cannot be excluded that some of the secreted BuChE originates from glial cells in the guinea-pig, although most of these satellite cells would presumably have been removed when the plexus was stripped off the muscle. Our results are most consistent with the view that the secreted BuChE originates from longitudinal muscle cells. The functional significance of the secreted BuChE is at present unclear; indeed a convincing function for BuChE is yet to be proposed (Kutty, 1980).

Possible implications

Acetylcholinesterase secretion has also been demonstrated in the substantia nigra, where it produces behavioural and electrophysiological effects that are unrelated to its hydrolytic action on acetylcholine (Greenfield, 1984). For example, application of AChE decreases the firing rate of pars compacta neurones (Greenfield *et al.*, 1981). Therefore secreted AChE might possibly have similar non-cholinergic actions within the gut. Consistent with such a notion is the finding by Ambache *et al.* (1986) of a proteinaceous component derived from Auerbach's plexus that will contract longitudinal muscle from the guinea-pig ileum.

A function of secreted AChE within the gut is also suggested by the fact that parasitic intestinal nematodes secrete large amounts of AChE but only during their gut-dwelling life-stages (Philip, 1984). It has been proposed that AChE secretion has survival value for the parasites; AChE may act as a biochemical 'holdfast' by inhibiting locally the peristaltic movements of the gut during the expulsion process (Ogilvie & Jones, 1971).

The secretion of acetylcholinesterase from the gut could have implications in disease states such as Hirschsprung's disease. Many studies have shown markedly elevated levels of soluble AChE within aganglionic rectal mucosal tissue of patients with this disease (Dale *et al.*, 1979; Bonham *et al.*, 1985); secretion of AChE could therefore be affected and contribute to the spasticity of the affected region. There have also been accounts of abnormal forms of AChE in rectal biopsies from patients with Hirschsprung's disease (Bajgar & Hak, 1979).

Finally our observations may account for certain findings on human amniotic fluid in cases where the foetus is abnormal. Secretion of AChE from the

exposed gastrointestinal tissue could account for its presence in the amniotic fluid in cases of exomphalos (Wald *et al.*, 1980; Dale, 1980; Voigtlander *et al.*, 1981; Garry *et al.*, 1981; Crandall *et al.*, 1982; Read *et al.*, 1982). Secretion of BuChE, in addition, would explain why the ratio of BuChE to AChE in amniotic fluid is higher in cases of foetal exomphalos (Goldfine *et al.*, 1983; Wald *et al.*, 1984), compared to cases of foetal neural tube defects where the main change is a greatly increased amount of AChE derived from cerebrospinal fluid (Smith *et al.*, 1979).

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