VASOACTIVE INTESTINAL POLYPEPTIDE (VIP) AS A CHOLINERGIC CO-TRANSMITTER: SOME RECENT RESULTS

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The neuropeptide vasoactive intestinal polypeptide (VIP) copurifies with mammalian brain cholinergic synaptosomes when these are separated from the total brain synaptosome fraction by an immunoadsorption procedure based on an antiserum to a cholinergic-specific antigen. VIP must therefore be reckoned to be a cholinergic co-transmitter in brain. In electromotor and myenteric neurones the two transmitters are differently packaged. The frequency-dependence and pharmacology of release show that the intracellular dynamics of the storage and release processes are quite different for the two neurotransmitters. However in the ileum a portion of the vesicle-bound acetylcholine is recovered in the VIP-storing particles and this might indicate a precursor-product relationship for the two types of vesicle in this system analogous to that which has been proposed for electron-dense and electron-translucent vesicles in noradrenergic nerves.

Introduction

The phenomenon of cotransmission

In recent years it has become apparent - largely through the application of immunocytochemical techniques - that nerve terminals containing classical small-molecular-mass transmitters may often also contain neuroactive neuropeptides. This has led to the concept of co-transmission, in which a terminal may release, in addition to a classical transmitter, one or more neuropeptides. This raises a number of new problems for our understanding of synaptic transmission. We may ask, for example
- what is the function of the neuropeptide?
- how is it stored in the terminal: together with the classical transmitter in synaptic vesicles or in a separate class of storage granules?
- what is the frequency-dependence of its release and how does this compare with that of the classical transmitter?

Answers to the second and third questions may help to provide an answer to the first.

Vasoactive intestinal polypeptide - a cholinergic co-transmitter

For those of us particularly interested in cholinergic function the discovery, in recent years, that vasoactive intestinal polypeptide

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0309-1651/89/121039-13/$03.00/0

Table I Acetylcholine and VIP contents of some tissues rich in cholinergic endings

Species and Tissue	Acetylcholine ($nmol.g^{-1}$)	VIP ($fmol.g^{-1}$)
Guinea pig		
Myenteric plexus	130 ± 20	4800 ± 600
Brain	20 ± 5	1400 ± 100
Muscle	2 ± 1	-
Torpedo		
Electric organ	920 ± 90	130 ± 16
Electric lobes	25 ± 2	820 ± 50

Values are means ± SEM of 3 or more determinations; for *Torpedo* they are porcine VIP equivalents

(VIP) is often associated with both central and peripheral cholinergic synapses is of considerable importance (reviewed by Agoston, 1988). Table I gives the acetylcholine and VIP content in several tissues of guinea pig and in the electromotor system of *Torpedo*. The myenteric plexus-longitudinal muscle preparation of guinea-pig ileum is one of the richest sources, among mammalian tissues, of cholinergic terminals; it is also a rich source of VIP; brain tissue is much less rich in both neurotransmitters, only about 5% of nerve terminals being cholinergic. Electric organ is a rich source of cholinergic nerve terminals, between 500 and 1000 times richer than muscle, from which the electrocytes are embryonically derived, while the electric lobes, prominent paired nuclei on the brain stem just caudal to the cerebellum, contain the cell bodies of the cholinergic electromotor neurones. Interestingly these structures also contain VIP-like immunoreactivity (VIPLI) (Agoston and Conlon, 1986). The values in Table I were obtained by radioimmune assay using porcine VIP as a standard, but since the displacement curves of *Torpedo* VIPLI and porcine VIP in the assay are not identical, the true values could be much higher.

Copurification of VIP with cholinergic synaptosomes

In recent years our group has identified two minor gangliosides, designated Chol-1 α and β, which are specific for cholinergic nerve terminals both central and peripheral and are conserved from *Torpedo* to mammals (Ferretti and Borroni, 1986/refs). Antisera to these gangliosides raised in sheep allow the cholinergic subpopulation of mammalian brain synaptosomes to be separated out by immunoadsorption (Agoston et al, 1988a/refs). Briefly, after addition of the antiserum in saturating amounts to the synaptosome preparation, those with attached anti-Chol-1 antibodies (i.e. the cholinergic subpopulation) selectively adhere to Sepharose beads to which a high-titre anti-sheep IgG monoclonal antibody has been attached and can be subsequently harvested. Such synaptosomes show up to an 18- to 20-fold enrichment of classical cholinergic markers, consistent

with a 5-6% representation of cholinergic synaptosomes in the total synaptosome population. VIP copurifies with the cholinergic markers but with a lower enrichment factor (Table II). Lactate dehydrogenase, a general cytoplasmic marker, shows, as expected, no enrichment. It has been concluded from the enrichment factors and yields obtained that about 50% of all cholinergic synaptosomes contain VIP and 75% of all VIP-containing synaptosomes are Chol-1-positive and thus cholinergic. Such results are consistent with the conclusions of earlier immunocytochemical studies (Eckenstein and Baughman, 1984).

Table II Copurification of VIP with cholinergic markers in affinity-purified synaptosomes

Component	Purification factor	Yield %
Acetylcholine	19	-
Choline acetyltransferase	15	3.3
VIP	8	4.0
Lactate dehydrogenase	1	-

(Results are those of Agoston et al, 1988a)

The guinea-pig myenteric plexus

Isolation of two classes of storage vesicle

When cytoplasmic extracts of myenteric plexus-longitudinal muscle preparations are submitted to high-resolution centrifugal density-gradient fractionation in a zonal rotor and the various density-gradient fractions assayed after the run, acetylcholine, substance P, somatostatin and VIP are each found (Agoston et al, 1985b) to be associated with distinct populations of particles differing is size and density (Fig 1a and Table III).

The lightest and smallest particles (designated peak I) contain acetylcholine and have the typical morphology of synaptic vesicles. The particles associated with VIP (designated peak VI) were by contrast larger and denser than synaptic vesicles and a considerable proportion of them contained dense cores (Fig 1c). Interestingly, acetylcholine was bimodally distributed in the gradient; while most of it was recovered in peak I, a second peak (II) coincided with that of VIP. Attempts to dissociate peaks II and VI by exploiting possible differences in density (Fig 2a), size (Fig 2c) or osmotic fragility (Fig 1d) failed (Agoston and Whittaker, 1989). It has therefore been concluded that VIP-rich vesicles in myenteric neurones also store some acetylcholine. Putative synaptic vesicle markers, Mg-ATPase, synaptophysin and vesicle-specific proteoglycan, were also bimodally distributed in the gradient (Figs 2b and 2d) with a less dense peak coinciding with the acetylcholine-rich

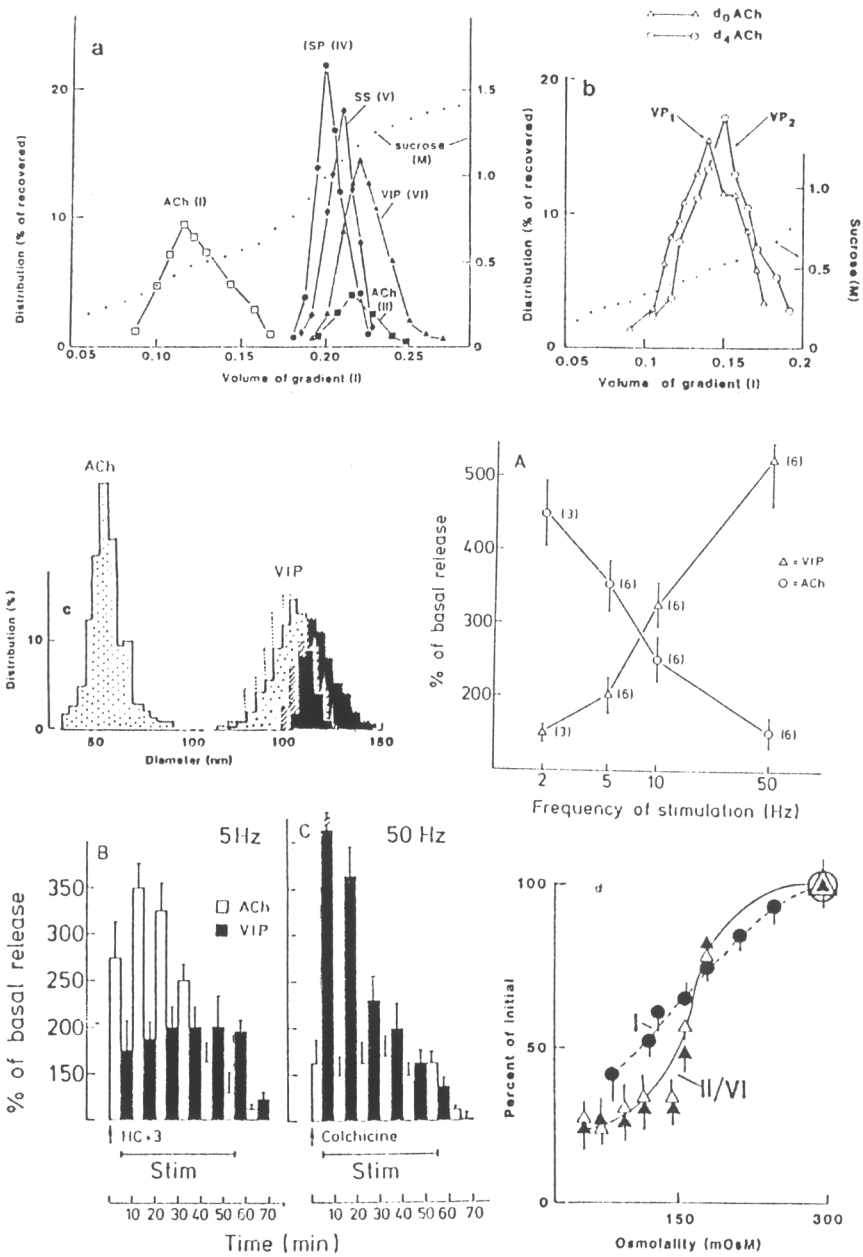
synaptic vesicles and a denser peak embracing the three peaks of neuropeptides (Fig 2b). This indicates that the denser neuropeptide-storing vesicles resemble synaptic vesicles in their chemical

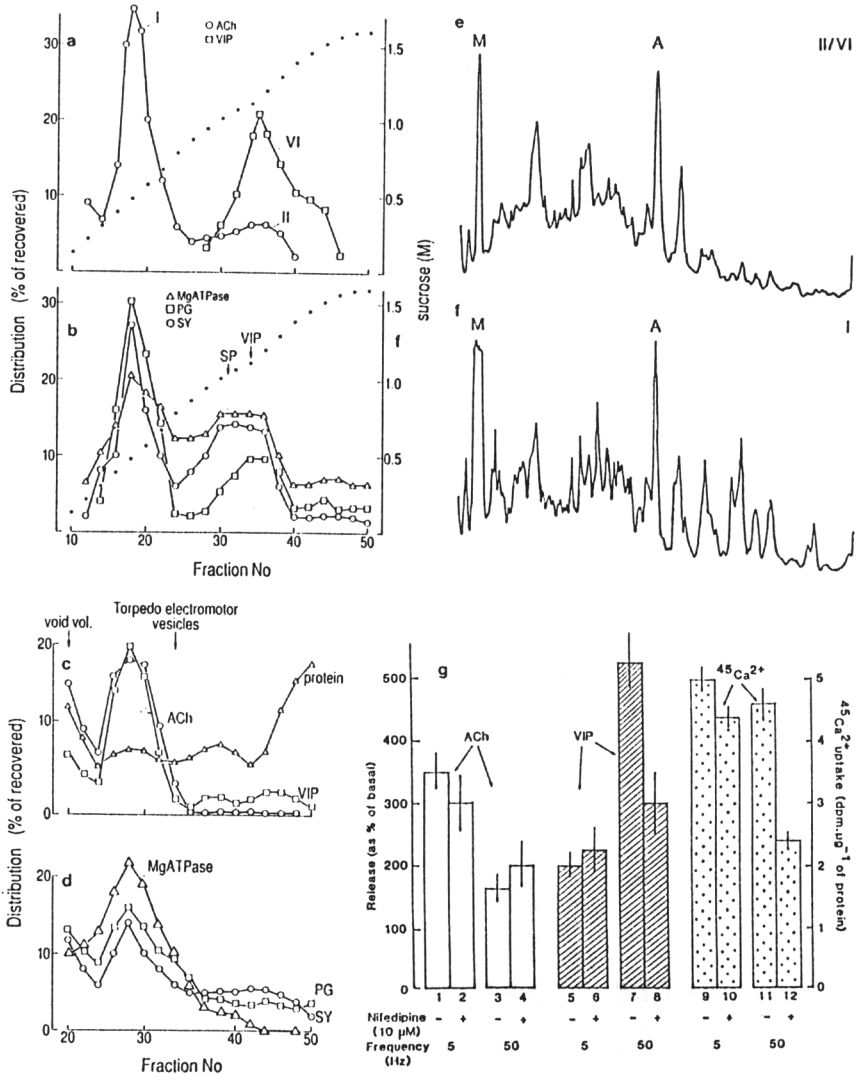
Table III Mean diameters, densitites and neuropeptide contents of storage vesicles from the guinea-pig myenteric plexus

Peak	Substance	Diameter[a]	Density[b]	Vesicular concentration	
		(nm)	($g.ml^{-1}$)	units	units.(mg of protein)$^{-1}$
I	Acetylcholine	61 ± 6	1.066	nmol	34 ± 4
IV	Substance P	65 ± 3	1.123	pmol	157 ± 14
V	Somatostatin	87 ± 3	1.138	pmol	70 ± 14
II/VI	VIP+acetylcholine	110 ± 6[c]	-	-	-
	VIP	-	1.148	pmol	25 ± 4
	Acetylcholine	-	1.144	nmol	11 ± 3

[a] Mean equitorial diameter ± SD for 1120 to 1860 profiles
[b] Means of peak fractions; SDs were approximately 1% of the mean
[c] For empty and dense-cored vesicles combined (see Fig 1c)
Results are those of Dowe et al (1980) and Agoston et al (1985b)

***Fig 1** (see facing page) (**a**) Distribution of acetylcholine (ACh, peak I), substance P (SP, peak IV), somatostatin (SS, peak V) and VIP (peak VI) in a zonal density gradient after centrifugal separation of a cytoplasmic extract of guinea-pig myenteric plexus. Note the presence of a small second peak of ACh (peak II) coinciding with VIP. Results of Agoston et al (1985b). (**b**) Incorporation of newly formed d_4 ACh into the synaptic vesicle fraction after superfusion with d_4 choline and stimulation. The denser vesicles corresponding to the shoulder in the distribution of d_0 (endogenous) ACh are preferentially labelled and comprise the recycling population. Results of Agoston et al (1985a). (c) Size distribution of vesicles present in peaks I and VI. In the latter, the dotted lines are the observed distribution of profile diameters, the continuous lines the calculated particle size distribution. Hatched and solid blocks show the observed and calculated subpopulation of dense-cored vesicles. The electron-lucent populations probably belong to the incompletely separated SS-containing particles. For means and SDs see Table 3. (**d**) Osmotic fragility of peaks I and II/VI. ACh, filled circles and open triangles; VIP, filled triangles. Results of Agoston and Whittaker (1989). (**A**) Frequency dependence of VIP and ACh release from superfused myenteric plexus-longitudinal muscle strips. At 8 Hz, the release of both transmitters as a percentage of basal release is the same. (**B,C**) Effect of (B) hemicholinium-(HC-)3, (C) colchicine on the release of ACh and VIP from superfused myenteric plexus-longitudinal muscle strips stimulated at (B) low, (C) high frequency. Results of Agoston et al (1988b).*


a
(SP (IV)
SS (V)
sucrose (M)
VIP (VI)
ACh (II)
ACh (I)
Distribution (% of recovered)
Volume of gradient (l)
b
d_0 ACh
d_4 ACh
VP_1
VP_2
Sucrose (M)
c
ACh
VIP
Distribution (%)
Diameter (nm)
A
% of basal release
Δ = VIP
○ = ACh
Frequency of stimulation (Hz)
B
5Hz
C
50 Hz
ACh
VIP
% of basal release
IIC+3
Colchicine
Stim
Time (min)
d
Percent of initial
II/VI
Osmolality (mOsM)


a
ACh
VIP
I
VI
II
b
MgATPase
PG
SY
VIP
SP
Distribution (% of recovered)
Fraction No
sucrose (M)
e
M
A
II/VI
f
M
A
I
void vol.
Torpedo electromotor vesicles
c
protein
ACh
VIP
d
MgATPase
PG
SY
g
ACh
VIP
45Ca2+
Release (as % of basal)
45Ca2+ uptake (dpm.µg-1 of protein)
Nifedipine (10 µM)
Frequency (Hz)

composition and this is also borne out by similarities in their protein composition (Figs 2e and f).

Differences in intracellular dynamics

Frequency dependence of release. When strips of myenteric plexus-longitudinal muscle preparations are stimulated at different frequencies in a superfusion chamber and the release of acetylcholine and VIP monitored, a striking difference emerges between the frequency dependence of their release (Agoston et al, 1988b) (Fig 1A). Whereas the release of acetylcholine is favoured by a low (2 Hz) frequency of stimulation, VIP release is maximal at high (50 Hz) frequency. At 8 Hz, the releases (as a % of basal release) are equal. These results are similar to those previously obtained with another VIP-rich cholinergic innervation, that to the salivary gland. In such nerve terminals acetylcholine and VIP are also packaged in synaptic vesicles and dense-cored vesicles (Lundberg, 1981; Lundberg and Hökfelt, 1983).

Pharmacology of release. Figs 1B and C show the results of stimulation experiments carried out in the presence of hemicholinium-3, which, by inhibiting the uptake of choline and the resynthesis of acetylcholine, blocks vesicle recycling, and colchicine, which blocks axonal transport (Agoston et al, 1988b). In the hemicholinium experiment (Fig 1B) stimulation at low (5 Hz) frequency was selected to favour the release of acetylcholine (white blocks), though some evoked release of VIP (black blocks) does occur. Hemicholinium causes a marked diminution in acetylcholine output, leaving VIP release unaffected. In Fig 1C, the conditions (50 Hz) favoured VIP release (compare height of first black blocks with corresponding blocks in panel B), but not that of acetylcholine (compare first white blocks in the two panels; those of panel C are

***Fig 2** (see facing page) **(a,b)** Attempted separation by (a) density, (b) size of peaks II and VI. In (a) the zonal density gradient was designed to give maximum resolution in the region of peak II/VI. In (b) a single peak of acetylcholine (ACh) and VIP activity emerged with a retention volume between that of chromaffin granules ('void volume') and Torpedo electromotor synaptic vesicles. **(c,d)** The three synaptic vesicle markers, proteoglycan (PG), synaptophysin (SY) and Mg^{2+}-ATPase are also inseparably associated with peak II/VI. **(e,f)** Densitometriy traces of silver-stained electropherograms of the proteins of (e) synaptic vesicles (peak I) and (f) peak II/VI. Both preparations were contaminated with myosin (M) and actin (A), probably derived from the longitudinal muscle and had numerous common components. **(g)** The effect of 10 μM nifedipine on the evoked release of acetylcholine (ACh, white blocks), that of VIP (hatched blocks) and the uptake of $^{45}Ca^{2+}$. It will be seen that only the voltage-sensitive calcium channel activated at high (50 Hz) frequency and the high-frequency release of VIP are nifedipine-sensitive. Results are those of Agoston and Whittaker (1989) and Agoston and Lisziewicz (1989).*

much lower than the corresponding ones of panel B). The presence of colchicine, however, rapidly blocks the release of VIP while leaving that of acetylcholine unaffected. This shows that VIP release is dependent on a steady supply of VIP-containing vesicles from the cell bodies of the neurones via axonal transport whereas acetylcholine release can be sustained by local recycling without replenishment of synaptic vesicles by axonal transport, at least within this time scale. The ability of myenteric synaptic vesicles to recycle has been shown by the rapid incorporation into them of labelled (i.e. newly synthesized) acetylcholine and its subsequent release (Fig 1B) (Agoston et al, 1985a). Such highly labelled vesicles differ in density from reserve vesicles and separate from them apparently as a result of osmotic dehydration following partial loading, as has also been observed with electromotor synaptic vesicles.

The clear difference in intracellular dynamics of the acetylcholine-rich synaptic vesicles and the VIP-rich vesicles seen in these experiments is also demonstrated by differences in their response to the L-type voltage sensitive calcium-channel blocker nifedipine (Fig 2g) (Agoston and Lisziewicz, 1989). This drug has little effect on either acetylcholine (white blocks) or VIP (hatched blocks) release at low frequency (Fig 2g, blocks 1-4) but at high frequency (blocks 5-8) it markedly inhibits VIP while having little effect on acetylcholine release. Note that the increase in frequency again depressed acetylcholine release (compare blocks 1 and 5) but enhanced that of VIP (compare blocks 3 and 7). The stippled blocks (9-12) show the effect of the drug on $^{45}Ca^{2+}$ uptake at low (9 and 10) and high (11 and 12) frequencies. Ca^{2+} uptake is largely frequency independent but is much more sensitive to nifedipine at high frequency than at low frequency. We interpret this to mean that the preferred mode of Ca^{2+} entry at low frequency is via the nifedipine-insensitive N-channels but at high frequency by the nifedipine-sensitve L-channels. It would also seem that VIP release is preferentially dependent on Ca^{2+} entry through L-type channels whereas acetylcholine release is preferentially dependent on Ca^{2+} entry through N-type channels. A neat explanation of the inverse frequency dependence of acetylcholine and VIP would be to suppose that the exocytosis of the VIP-rich vesicles occurs at a different site in the terminal (where L-type Ca^{2+}-channels are concentrated) from that at which acetylcholine-rich synaptic vesicles undergo exocytosis and where N-type channels may be concentrated (Fig 3).

It has been suggested (Geffen and Livett, 1971; Lowe et al, 1988) that in the adrenergic system the membranes of dense-cored vesicles, which contain the small-molecular-mass transmitter noradrenaline as well as neuropeptides are retrieved to form small, electron-lucent vesicles which are competent to take up and release noradrenaline and recycle in the terminal. It may be that this precursor-product relationship also exists, at least in cholinergic neurones of the guinea-pig myenteric plexus, between acetylcholine-containing, VIP-rich storage particles and acetylcholine-rich synaptic vesicles, which, as we have seen, are able to recycle and thus to sustain acetylcholine release independently of axonal transport.

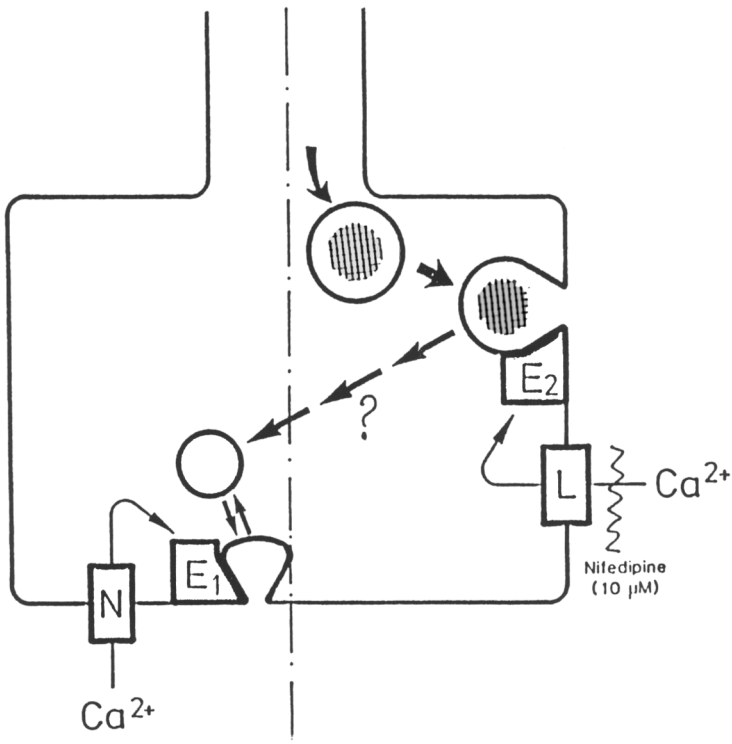


***Fig 3** Scheme showing dense-cored and recycling synaptic vesicles and their possible relationship in the cholinergic terminals of the myenteric plexus.*

The electromotor system of *Torpedo*

As already mentioned, VIPLI is present throughout the electromotor neurone. The absolute amounts have not yet been determined and the quantitative results in Figures and Tables are expressed as 'porcine VIP equivalents'.

VIPLI-containing particles separate completely from the acetylcholine-rich synaptic vesicles when a cytoplasmic extract of electric organ is separated on a zonal density gradient (Agoston et al, 1989a; Fig 4a). Both types of particle are associated with the putative stable vesicle markers synaptophysin and vesicle-specific proteoglycan (Fig 4b). Ligation of the electromotor nerve trunks causes a rise in the VIPLI content of the electromotor cell body and the axon proximal to the ligature but a fall in the distal axon and the terminals in the electric organ (Agoston and Conlon, 1986 and Table IV). This is sufficiently great after 10 days to be apparent immunohistochemically; it parallels the accumulation of proteoglycan above the ligature.

Synaptic vesicles and VIPLI-containing particles can also be separated out from axons (Agoston et al, 1989a). There is an interesting difference between them. In confirmation of Kiene and Stadler (1987), synaptic vesicles from electromotor axons are less dense than those isolated from their terminals in the electric organ; they also contain very little acetylcholine - about one-thousandth of that present in terminal synaptic vesicles (Table V). These two parameters are probably related, since in the axon, a relatively light inorganic monovalent cation may replace the denser organic cation acetylcholine. By contrast, axonal VIPLI-containing particles are essentially identical to those isolated from the terminal. Interesting also (Table V col 6, compare lines 1 and 2)

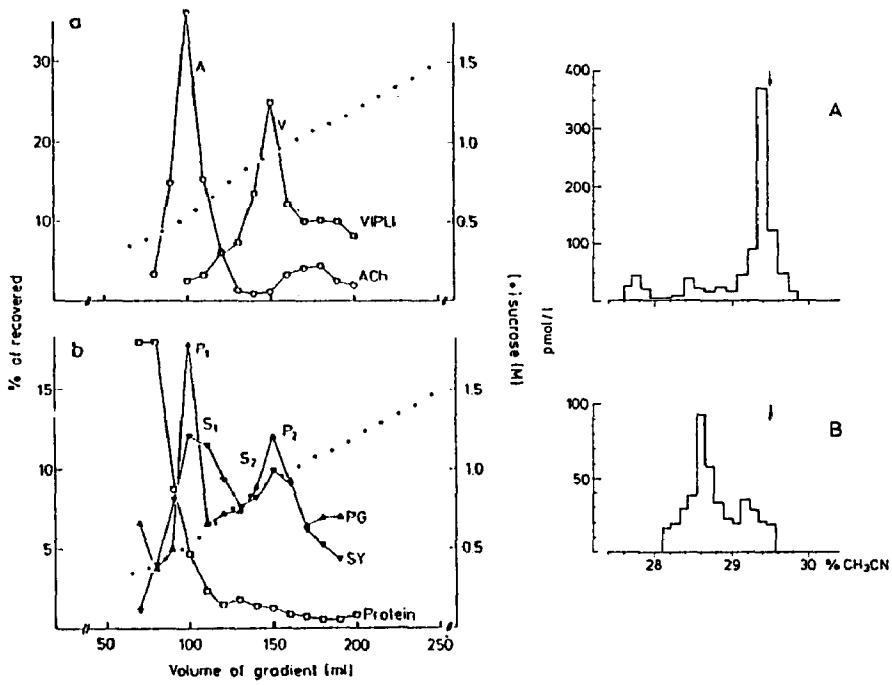


***Fig 4** (**a**) Separation of particles in cytoplasmic extracts of* Torpedo *electric organ rich in ACh and VIPLI by zonal density gradient separation. (**b**) Distribution of two putative stable synaptic vesicle markers in the same gradient, synaptophysin (SY) and vesicle-specific proteoglycan (PG). Peaks S_1 and P_1 of the two markers coincide with the ACh peak A and peaks S_2 and P_2 with the VIPLI peak V. (**A,B**) Reverse-phase analytical HPLC on Supercosil-18-DB under near isocratic conditions of the VIPLI in extracts of (A) electric lobe, (B) electric organ. The column was eluted with increasing concentrations of acetonitrile (abscissae). The arrow shows the concentration at which porcine VIP emerges from the column. Results are those of Agoston et al (1989a).*

is the lower Mg^{2+}-ATPase in axonal synaptic vesicles compared with those from the terminal. Does the ATPase get switched on in the terminal thus generating an enhanced proton gradient needed for uptake of acetylcholine there? By contrast, the Mg^{2+}-ATPase levels of axonal and terminal VIPLI-containing vesicles are essentially the same (Table V col 6, compare lines 3 and 4).

Table IV VIP-like immunoreactivity in the electromotor system of *Torpedo* and changes induced by ligation of nerve

Tissue	VIP-like immunoreactivity[a]	
	$fmol.g^{-1}$	% change on ligation
Electric lobe	740 ± 70	+ 84 ± 19
Electromotor nerve trunks	180 ± 30	+ 34 ± 11[b]
Electric organ	130 ± 20	- 47 ± 5
Rest of cns	550 ± 50	+ 0.5 ± 14

[a] Means of 3 expts ± SD, porcine VIP equivalents; increases are positive, decreases negative

[b] Proximal to ligature

Results are those of Agoston and Conlon (1986)

Table V Comparison of axonal and terminal synaptic and dense vesicles from *Torpedo* electromotor neurones

Site	Density ($g.ml^{-1}$)	Content (units per 100 units of synaptophysin: Acetylcholine (nmol)	VIPLI (fmol-eq)	Proteoglycan (SV-eq)	Mg^{2+}-ATPase (nmol P_i.h-1)
Synaptic vesicles					
Terminals	1.070	328	-	100	688
Axons	1.057	0.23	-	93	236
Dense vesicles					
Terminals	1.122	-	2432	84	876
Axons	1.100	-	2414	75	733

Results are those of Agoston et al (1989a)

These figures clearly indicate that the two transmitters again differ in their intracellular dynamics: they are packaged in distinct particles and at the level of axonal transport VIPLI is contained within a fully charged particle whereas synaptic vesicles only become fully charged when they reach the terminal.

In future work it will be interesting to see whether other differences in optimal frequency of release and sensitivity to drugs are similar to those found with salivary gland and myenteric plexus.

Acetylcholine in this system has not been detected in VIPLI-containing vesicles and synaptic vesicles appear, on the basis of morphological evidence, to be formed in the Golgi region of the electromotor perikarya. Another possible difference from mammalian systems is the finding that in electromotor neurones, VIPLI is subject to intravesicular modification. A comparison of the retention volumes of somatic and terminal VIPLI in reverse phase high performance liquid chromatography (HPLC) shows that a small hydrophobic sequence is cleaved off during axonal transport; whereas VIPLI isolated from electromotor cell bodies consists of a single component with a retention time close to that of porcine VIP (Fig 4A, peak and arrow), this component has all but disappeared when the storage particle reaches the terminal and has been replaced by a more hydrophilic one (Fig 4B).

Codistribution of PHI and VIP

It is now known that the 28-amino-acid peptide VIP is synthesized in the form of a propeptide precursor which is posttranslationally modified by proteolytic cleavage to generate VIP and various other fragments, the main of which is a 27-amino-acid peptide known as PHI, standing for 'the peptide with N-terminal histidine and C-terminal isoleucine amide'. VIP and PHI and the other propeptide cleavage products have been shown to coexist with VIP in a number of VIP immunopositive cells, but often not in equimolar amounts, suggesting different rates of further cleavage by tissue proteases.

We have studied the distribution of VIP- and PHI-containing particles by density and size, by applying both zonal density gradient separation and particle exclusion chromatography to cytoplasmic extracts of myenteric plexus. In all cases, the two peptides co-localized in exactly equimolar amounts (Agoston et al, 1989b). This suggests that the posttranslational modification occurs after packaging the propeptide and that any subsequent deviation from equimolarity is due to differential breakdown after release.

Conclusion

The work described here illustrates the value of a cell-biological approach to the problem of cotransmission. The results confirm the prevailing view that the classical transmitters and their accompanying neuropeptides are separately packaged in nerve terminals, within synaptic vesicles and larger, denser vesicles (which may or may not have a dense core) respectively. However, the composition of the membranes of the two particle types are similar

and it is possible that at least in some terminals the membranes of dense-cored vesicles are reutilized to form synaptic vesicles.

Within the neuropeptide-storing particles, posttranslational modification occurs: in the case of VIP, equimolecular amounts of another peptide, PHI, are generated and, in the electromotor system, the VIPLI present in the particles before they leave the cell soma is further modified during axonal transport. The nature of the peptidase(s) involved is not known but it is likely that a cathepsin is involved.

The acid pH required to activate the cathepsin might well be brought about by the same ATPase as is responsible for acidifying the interior of synaptic vesicles and thus providing the driving force for acetylcholine uptake. The peptide-containing and synaptic vesicle membranes may thus use a component common to both membranes for different purposes: a good example of evolutionary thriftiness!

Acknowledgement

This work was supported by grants Co 130/1-1 and Wh 1/4-2 to V.P.W. from the Deutsche Forschungsgemeinschaft.

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