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Characterization of Gastrin-Releasing Peptide Immunoreactivity in Distinct Storage Particles in Guinea Pig Myenteric and *Torpedo* Electromotor Neurones

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SHAW, C , V P WHITTAKER AND D V AGOSTON *Characterization of gastrin-releasing peptide immunoreactivity in distinct storage particles in guinea pig myenteric and* Torpedo *electromotor neurones* PEPTIDES **11**(1) 69–74, 1990 —Using high resolution centrifugal density-gradient separation of cytoplasmic extracts of guinea pig myenteric plexus and *Torpedo* electric tissue, we have succeeded in isolating fractions of storage particles rich in gastrin-releasing peptide (GRP) In extracts of myenteric plexus and gradients derived therefrom, the 10-amino acid GRP peptide (GRP-10) was the sole form present, this was bimodally distributed in the gradients, one peak copurifying with Golgi membranes and apparently consisting of immature storage particles, the other with other synaptophysin-rich neuropeptide-containing particles In extracts of electric organ, a tissue rich in cholinergic electromotor nerve terminals, and gradients derived therefrom, GRP-like immunoreactivity behaved in gel permeation and reversed phase high performance liquid chromatography like the 27-amino acid peptide (GRP-27) About half of the immunoreactivity sedimented in the centrifugal gradient to a region rich in particles containing vasoactive intestinal polypeptide-like immunoreactivity, the remainder was recovered in a very dense region of the gradient containing larger membrane fragments, including synaptosomes The electromotor nerves and cell bodies also contained GRP-27-like immunoreactivity in relatively high concentration as did the *Torpedo* gut It is concluded that this GRP-like peptide is packaged in dense storage particles in the electromotor neurones

Myenteric neurones Electromotor neurones GRP VIP Storage particles

GASTRIN-releasing peptide (GRP) is a neuropeptide found inter alia in mammalian intestine, lungs and brain which exhibits active site sequence homology with the amphibian peptide, bombesin (7). GRP is known to exist in mammalian tissue in two major molecular forms, GRP-27 and GRP-10, the smaller peptide corresponding to the C-terminal decapeptide amide of the larger. GRP and bombesin are now known to belong to a larger family of related peptides [for reviews see (16,17)]

In the Gottingen laboratory, much use has been made of two model systems, the myenteric plexus-longitudinal muscle (MPLM) preparation of guinea pig ileum (12) and the electromotor system of the elasmobranch fish *Torpedo marmorata* (an electric ray) (14), for cell-biological studies of neuropeptides, especially one, vasoactive intestinal polypeptide (VIP), which is often found to be colocalized with acetylcholine (ACh) in cholinergic terminals. Methods have been devised, using centrifugal density gradient fractionation of cytoplasmic extracts of guinea pig MPLM (8) and *Torpedo* electric organ (12,18) in zonal rotors, whereby VIP-containing storage granules may be separated from ACh-rich synaptic vesicles and storage vesicles containing other neuropeptides (1, 2, 4, 5). From extracts of MPLM, distinct fractions of vesicles storing substance P, somatostatin and VIP have been separated (2), but in the purely cholinergic *Torpedo* electromotor system, storage granules containing VIP-like immunoreactivity have been up to now the only peptide-containing particles to be isolated (5).

In the study reported here, we have identified and chromatographically characterized GRP immunoreactive peptides in extracts of guinea pig MPLM and *Torpedo* electric organs and have subsequently isolated and partially characterized their storage particles.

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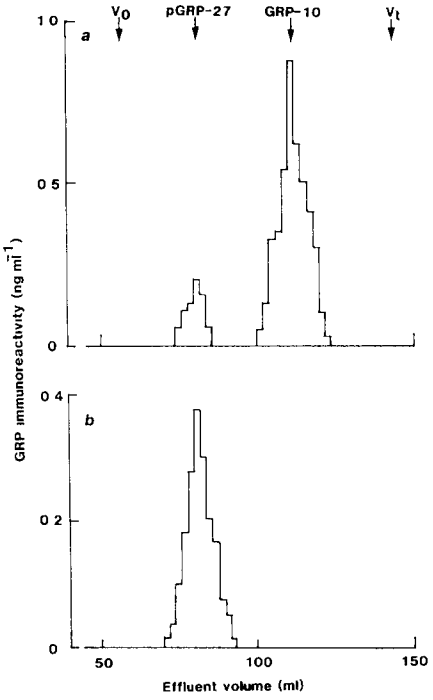

FIG 1 Gel permeation chromatography of acidic extracts of (a) guinea pig MPLM and (b) *Torpedo* electric organ. The columns were calibrated with blue dextran (V_o), porcine GRP-27 (pGRP-27) and GRP-10, and potassium dichromate (V_t) as indicated by the arrows Volumes of extract corresponding to about 100 mg of tissue were placed on the columns

METHOD

Preparation of Cytoplasmic Extracts and Storage of Tissues

From MPLM preparations. MPLM preparations were made as modified (1) from the original method (2). Briefly, tissue (8–10 g) from the ilea of 6 guinea pigs of 300–350 g body weight was homogenized at 4°C in 4 volumes of 0.16 M NaCl containing 10 mM 2-(*N*-2-hydroxyethyl-piperazine-*N'*-yl) ethane sulphonate (Hepes) buffer, pH 6.8, 1 mM EGTA, 0.1 mM EDTA and adequate concentrations of protease inhibitors as described (1). The tissue was first minced with scissors, further comminuted in a high speed blender (45,000 rev·min^{-1} for two 10-sec periods) and then homogenized in a Teflon glass homogenizer (ten passes at 800 rev·min^{-1} during two 2-min periods, all-round clearance 2%). The homogenate was centrifuged at 1000 × g for 20 min and the particulate material rehomogenized and recentrifuged as before. The combined supernatants (here referred to as cytoplasmic extracts) were subjected to centrifugal density gradient separation in a zonal rotor (see below).

From electric organ. The collagenous nature of the tissue required a more vigorous technique and utilized a modification (5,12) of our original method (18). Briefly, the tissue (70–80 g) was frozen in liquid nitrogen, crushed to a coarse powder, and after warming to −10°C, suspended in 0.935 M NaCl in a tissue to saline ratio of 1 8.1 The slurry was squeezed through cheesecloth to remove large fragments of tissue and then centrifuged at 10,000 rev·min^{-1} for 30 min to remove smaller fragments. The supernatant cytoplasmic extract was further fractionated by density gradient centrifuging (see below)

TABLE 1

GRP IMMUNOREACTIVITY IN *TORPEDO* AND GUINEA PIG TISSUES

Tissue	GRP Immunoreactivity* (ng·g^{-1} of tissue)
Whole intestine	98 6 ± 12 8
Electric lobe	40 4 ± 6 3
Electromotor nerves	0 60 ± 0 22
Electric organ	1 86 ± 0 36
Guinea pig MPLM	24 8 ± 4 0

*Values are means of four experiments ±SD and are expressed as porcine GRP equivalents

Other tissues. Torpedo gut, electric lobes and electromotor nerves were dissected from anaesthetized fish and immediately frozen in liquid N_2. Frozen tissues were stored at −80°C until crushing and extraction.

Density Gradient Centrifuging

The basic procedure was a modification (1, 5, 12) of our original method (18). Briefly, gradients are formed in a Beckman gradient maker from two solutions of sucrose-NaCl in proportions determined by the profile of a control cam. At a suitable point the denser of these two solutions is replaced by an even denser one, thereby generating a smooth density gradient with two inflexions in it. The gradient is formed horizontally in a 300-ml Beckman Ti-60 zonal rotor while the rotor is spinning and is held back from the outer wall of the rotor by a cushion of dense (1.6 M) sucrose

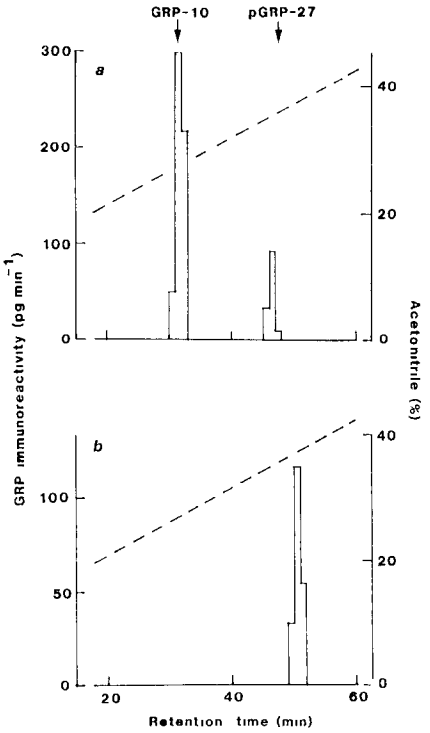

FIG 2 Reversed phase HPLC of acid extracts of (a) guinea pig MPLM and (b) *Torpedo* electric organ The arrows indicate the retention times of pGRP-10 and GRP-27 The volumes of extract chromatographed corresponded to about 40 mg of tissue

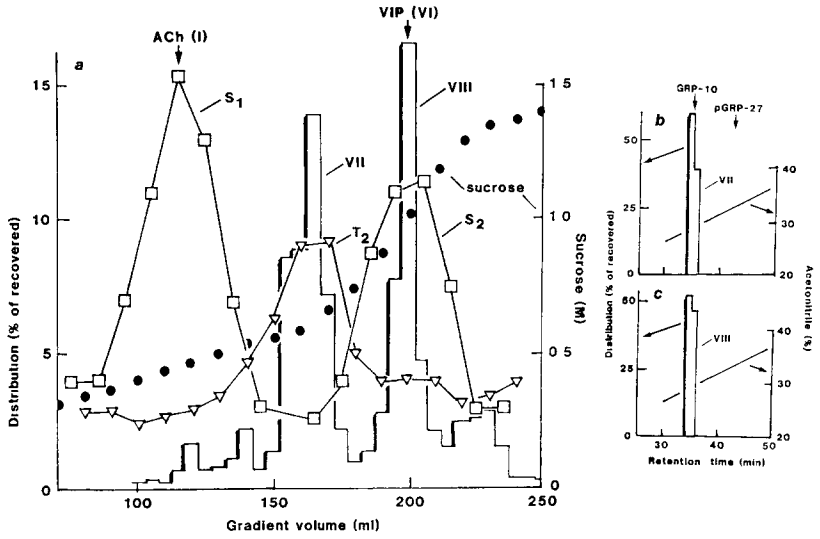

FIG 3 (a) Distribution of GRP immunoreactivity (blocks), the vesicle marker synaptophysin (squares) and the Golgi marker thiamine triphosphatase (inverted triangles) in a sucrose density gradient after centrifugal separation of a MPLM extract in a zonal rotor GRP immunoreactivity is found mainly in two peaks, designated, in extension of our previous terminology (2), VII and VIII Peak VII coincides with a peak (T_2) of the Golgi marker and peak VIII with a second peak (S_2) of the vesicle marker synaptophysin and peak VI of VIP (arrow). Negligible GRP immunoreactivity is found in a region of the gradient where acetylcholine (ACh)-rich synaptic vesicles congregate (arrow), here marked by the first peak (S_1) of synaptophysin (b,c) Behavior, on HPLC, of GRP immunoreactivity from (b) peak VII, (c) peak VIII, identifying these as being due to GRP-10

The addition of the sample of tissue extract and an overlay of the homogenization medium completes the gradient. Centrifuging is for 3 hr at 60,000 rev·min^{-1} after which the gradient is pumped out and collected in 5-ml fractions.

The gradient for work with extracts of MPLM utilized 0 32 M sucrose as the light solution, and 0.6 M sucrose as the dense solution; this was replaced, after 200 ml of gradient had been delivered, by 1.4 M sucrose. The volume of sample was 40 ml derived from 8–10 g of tissue.

For extracts of electric organ a more sharply inflected gradient was used. The light solution was 0.2 M sucrose-0.311 M NaCl, the first dense solution was 1.2 M and the second 1.6 M sucrose, the switch was after 100 ml of gradient had been delivered. The sample volume was 30 ml corresponding to 54 g of electric organ. The higher osmolarity of the light solution compared to that used with MPLM preparations is dictated by the high (ca. 800 mOsM) osmolarity of the *Torpedo*'s body fluids.

Analytical Techniques

GRP immunoreactivity. GRP was measured in crushed frozen tissues, tissue extracts, zonal fractions and column effluents by a C-terminal-specific radioimmunoassay as previously described (14) using synthetic porcine GRP as a standard. The antiserum used (14) showed equimolar cross-reactivity with porcine GRP-10 and -27, neuromedin C and amphibian bombesin but none at 1000-fold molar excess with other peptides including VIP, PHI, NMU, SP, NKA, NPK or NKB. Cross-reactivity with neuromedin B was 1%. To extract the neuropeptide from tissues, they were stirred at 4°C for 12–15 hr with 10 volumes of an ethanol-0.7 M HCl mixture, 3:1 v/v; particulate material was removed by centrifuging at 1600 × g for 30 min and the supernatants lyophilized. The advantage of adding ethanol to the acidic extractant

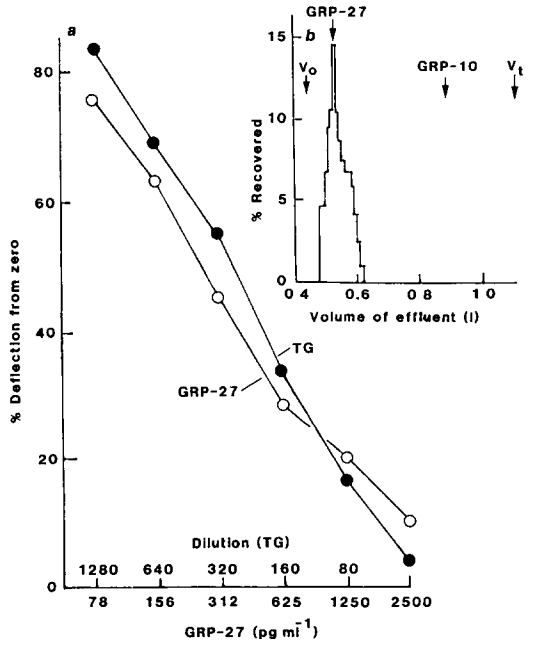

FIG 4 (a) Displacement curves of *Torpedo* gut GRP immunoreactivity (filled circles, TG) and authentic porcine GRP-27 (open circles) (b) Gel permeation chromatography of gut GRP immunoreactivity, which is seen to have the same retention volume as authentic porcine GRP-27 Arrows (V_o) void volume of column, (V_t) occluded volume, (GRP-27, GRP-10) retention volumes of GRP-27 and GRP-10, respectively

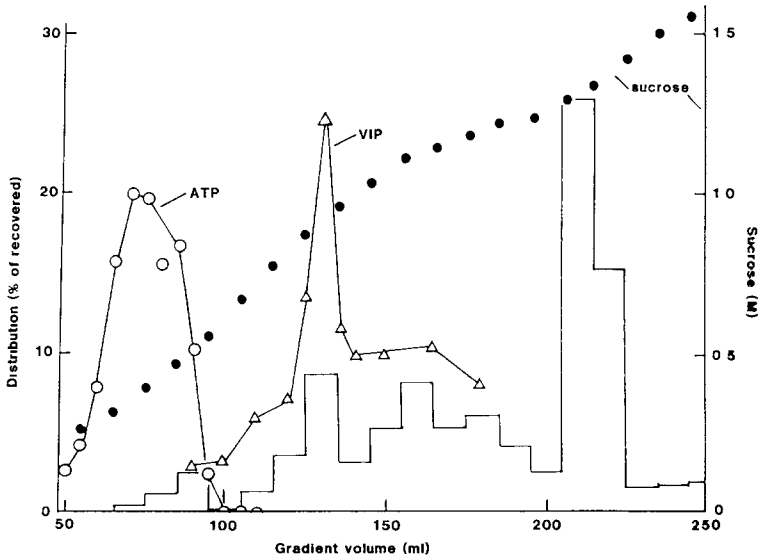

FIG 5 Distribution of GRP immunoreactivity (blocks) and synaptic vesicle-bound ATP (open circles) in a sucrose density gradient after centrifugal separation of a cytoplasmic extract of *Torpedo* electric organ in a zonal rotor The distribution of VIP-like immunoreactivity (triangles, VIP) is also shown

is that many high molecular mass proteins which interfere with the radioimmunoassay are removed (C. Shaw, unpublished observations) Samples of density gradient fractions were brought to 95°C by the addition of 10 volumes of boiling distilled water, maintained at that temperature for 5 min in a boiling water bath, chilled on ice and treated with acetic acid to a final concentration of 0 1 M. They were then lyophilized and stored at −20°C till assayed

Chromatography. Gel permeation chromatography of extracts (1 ml) was performed using a 90 × 1.6 cm column of Sephadex G-50 (superfine; Pharmacia, Uppsala, Sweden) equilibrated in 1 M acetic acid at 4°C and eluted at a flow rate of 10 ml·hr^{-1} Fractions (2 ml) were collected and the column was calibrated with blue dextran (V_o), porcine GRP-27 and GRP-10, and potassium dichromate (V_t). Immunoreactive fractions were subjected to reverse phase high performance liquid chromatography (HPLC) analysis using a Supelcosil LC-18-DB column (25 × 0 46 cm) (Supelco, Bellafonte, PA) equilibrated in 0 1% aqueous trifluoroacetic acid and eluted with a gradient from 10 to 42 5% acetonitrile in 0 1% aqueous trifluoroacetic acid in 60 min. The flow rate was 1.5 ml·min^{-1} and the fraction volume 1.5 ml Recoveries of GRP immunoreactivity after chromatography were over 90%

Synaptophysin. This putative synaptic vesicle marker (19), also known as p38 (10), has recently been shown to also be present in neuropeptide-containing particles from MPLM (1) and *Torpedo* electric organ (5) It was assayed by a dot-blot technique as described (1, 3, 5).

Thiamine pyrophosphatase. This Golgi membrane marker was measured by an adaptation (5) of a histochemical method (11).

RESULTS

MPLM Extracts

Chromatographic characterization of GRP immunoreactivity. Acid-ethanol extracts of guinea pig MPLM contained about 25 ng porcine GRP equivalents (Table 1). When extracts were submitted to gel permeation chromatography, the immunoreactivity was resolved into two immunoreactive species coeluting with porcine GRP-27 and GRP-10, respectively (Fig 1a). The latter form was the most abundant (about 5·1). Reverse phase HPLC (Fig 2a) also separated two immunoreactive peptides coeluting with porcine GRP-27 and GRP-10 in approximately the same ratio.

Characterization of storage particles containing GRP immunoreactivity Figure 3 shows the effect of separating a cytoplasmic extract of MPLM by density gradient centrifuging in a zonal rotor GRP immunoreactivity is bimodally distributed (Fig. 3a) The lightest peak, designated peak VII in extension of our previous terminology (2), coincides with a peak of the Golgi marker, thiamine pyrophosphatase. A denser peak (VIII) coincides with a peak of the storage vesicle marker, synaptophysin; another lighter peak of this marker coincides with a peak of ACh-rich synaptic vesicles (peak I, arrow). Peak VIII occurs in the same density range of the gradient known from earlier work (2) to contain peaks of substance P, somatostatin and VIP (in order of increasing density). A small peak or shoulder in the densest region of the gradient where synaptosomes and other larger tissue fractions accumulate is also seen

The immunoreactive material in peaks VII and VIII was submitted to HPLC Radioimmune assay of the effluents showed that the form of GRP present was exclusively GRP-10 (Fig 3b,c).

The Electromotor System of Torpedo

Chromatographic characterization of GRP immunoreactivity Of the various *Torpedo* tissues assayed, the gut had the highest level of GRP immunoreactivity, about 100 ng g^{-1} (Table 1). Gel permeation chromatography of gut extracts revealed only one GRP immunoreactive peak with a retention volume identical to that of porcine GRP-27 (Fig. 4b). The displacement curve of the *Torpedo* gut immunoreactivity is also almost identical to that of porcine GRP-27 (Fig 4a).

Relatively high levels of GRP immunoreactivity were also present (Table 1) in electric lobes, the portion of the *Torpedo* brain containing the cell bodies of the electromotor neurones innervating the electric organ. These comprise about half the mass of the lobes Lesser amounts of GRP immunoreactivity are present in the

electromotor nerves and in electric organ where the immunoreactivity is probably accounted for by the nerve terminals.

Gel permeation chromatography of electric organ extracts again revealed, as in *Torpedo* gut, only one GRP immunoreactive species with a retention volume identical to that of porcine GRP-27 (Fig. 4b). In reverse phase HPLC too, only one form was detected, this time with a retention time a little longer than porcine GRP-27. Thus, there may be a slight difference between *Torpedo* electric organ and porcine GRP-27

Partial characterization of storage particles. The distribution of GRP immunoreactivity in density gradients following centrifugal separation in a zonal rotor (Fig. 5) was more diffuse than previously observed for ACh or VIP-like immunoreactivity; a broad peak overlapping the much sharper peak of VIP-like immunoreactivity and a sharper peak considerably more dense than that of VIP-like immunoreactivity.

DISCUSSION

Extraction Procedures

The extraction procedure for GRP differed from the conventional one in that ethanol acidified with HCl was substituted for aqueous acetic acid. This has the advantage of removing proteins of large molecular masses that might otherwise interfere with the subsequent radioimmunoassay and has been thoroughly tested out (C Shaw, unpublished) The GRP content of the myenteric plexus, when extraction was performed in this way (Table 1, line 5), is, however, very close to that found by Hutchison *et al.* (9) using the acetic acid extraction procedure

Storage in MPLM

Studies during the past few years have shown that neuropeptides, like other physiologically active cell components (e.g , adrenaline, insulin), are packaged within the cells which secrete them in storage granules of varying size and density. A knowledge of such storage particles is essential for an understanding of the release process and of the posttranslational modification of the neuropeptide secreted.

The separation and characterization of peptide storage particles has been facilitated by the use of high resolution centrifugal density gradient separation in a zonal rotor (2, 8, 18) and also by the discovery of stable "marker" substances present in the limiting membrane of the storage granule (1,5). One of these is synaptophysin, a 38,000-Da intrinsic membrane protein (10,19) present in synaptic vesicles, but also other, neuropeptide-storing granules (1,5). Use has been made of this and also of the Golgi membrane marker thiamine pyrophosphatase to localize mature and immature, Golgi-associated storage particles in sucrose density gradients after centrifugal separation.

Fractionation of cytoplasmic extracts of MPLM in this way led to the identification of two peaks (VII and VIII) of GRP immunoreactivity in the density gradient, one of which (VII) was thiamine pyrophosphatase positive and the other of which (VIII) was synaptophysin positive. We tentatively conclude that peak VII contains immature storage particles associated with Golgi membranes which have not acquired synaptophysin, whereas peak VIII consists of mature storage particles with a normal content of storage particle membrane synaptophysin Both peaks contained only GRP-10 GRP-27 was present only as a minor component associated with very dense particulate material of uncertain provenance. Further work will be required to establish whether the particles in peak VII are the actual precursors of those in peak VIII or whether GRP-10 is repackaged after synthesis

Storage in Electromotor Neurons

Hitherto only VIP (2) and its accompanying product of posttranslational modification, PHI (6), have been found in the cholinergic electromotor neurone, both are packaged (in equimolar amounts) in a synaptophysin-positive dense storage particle (6) We have now found a second, unrelated peptide, immunochemically and chromatographically almost identical to porcine GRP-27 in the axons of such neurones and also in the electric lobes and electric organ, regions where the cell bodies and nerve terminals of these neurones are respectively concentrated. As such terminals account for only 1–2% of the electric organ, the concentration of GRP immunoreactivity there could be quite high—even higher than in the lobe

The density gradient and chromatographic behavior of the GRP-like immunoreactivity in cytoplasmic extracts of electric organs indicated that the peptide is stored in particulate material in this model cholinergic neurone as in those of the MPLM However, the storage particles appear to be more heterogeneous than those storing VIP.

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