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IDENTIFICATION OF GASTRIN RELEASING PEPTIDE-RELATED SUBSTANCES IN GUINEA PIG AND RAT BRAIN

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SUMMARY: Rat and guinea pig brain extracts were examined for the occurrence of gastrin-releasing peptide (GRP)-like substances by sequence specific radioimmunoassays interfaced with gel filtration and reversed phase high performance liquid chromatography (RP-HPLC). Tryptic digestion of the immunoreactive peptides followed by RP-HPLC was used to further characterize GRP-related peptides in brain. Using these analytical techniques it was found that guinea pig brain extracts contained a peptide with characteristics identical to authentic GRP (27 amino acid residues long). A carboxyterminal fragment with the characteristics of GRP(18-27) as well as a respective aminoterminal fragment with the characteristics of GRP(1-16) were also present in guinea pig brain extracts. The GRP(18-27) seems to correspond to the bombesin related material that has been described previously in mammalian brain extracts.

Rat brain extracts also contained a peptide with the characteristics of GRP(18-27). The corresponding aminoterminal fragment, however, behaved differently on RP-HPLC from authentic GRP(1-16) and it was not recognized by antibodies directed to the aminoterminal tridecapeptide fragment of authentic GRP. Similarly the GRP-like peptide from rat brain did not comigrate on RP-HPLC with authentic GRP and was unreactive to antibodies directed toward the aminoterminus of GRP.

Recent studies by several different investigators have identified peptides in mammalian brain that are immunologically similar to bombesin, a tetradecapeptide isolated from amphibian skin (1). Because bombesin, when injected into the brain, has potent effects on thermoregulation, eating and anterior pituitary hormone release (2-4), it was suggested that it may be a peptide neurotransmitter or neuromodulator in mammalian nervous systems (5, 6). No precise information regarding the exact molecular nature of the bombesin immunoreactive material in mammalian tissues is available although

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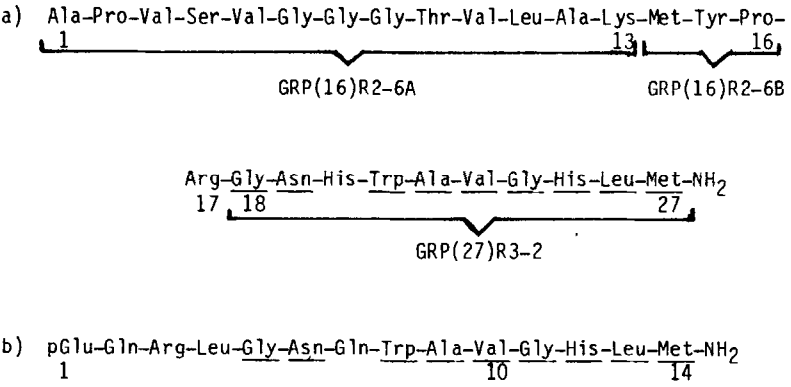

Figure 1. Amino acid sequences of gastrin releasing peptide (GRP) (a) and bombesin (b). Homologous amino acid sequences are underlined. Trypsin susceptible sites are marked by arrows. The recognition sites for the three GRP antisera are indicated by brackets.

some evidence has been presented that brain bombesin behaves similarly to authentic bombesin in several chromatography systems (7). Recently, McDonald et al. (8) have isolated a 27 amino acid peptide from porcine gut with a heptapeptide sequence at the carboxyterminus that is identical to the carboxyterminal seven amino acids of bombesin (Fig. 1). This peptide stimulates the release of gastrin from the stomach mucosa and has been termed gastrin-releasing peptide (GRP). Immunohistochemical studies have shown that GRP immunoreactive material is present in various regions of the rat brain where it is often colocalized with bombesin related immunoreactive material in the same neurons (9). We now present evidence that authentic bombesin is not present in guinea pig or rat brain and that the bombesin related material is likely to be the carboxyterminal fragment of GRP, GRP(18-27).

METHODS

Radioimmunoassays (RIA)

Three GRP antisera were generated in rabbits against synthetic porcine GRP and GRP(1-16) (Peninsula Laboratories, San Carlos, CA) linked by a water soluble carbodiimide to bovine throglobulin as described previously (10). RIA conditions have been described (11). Three GRP RIA's were utilized in this study, the specificity of these RIA's is indicated in Table 1. The GRP carboxyterminal antiserum crossreacted with GRP, GRP(18-27), and bombesin but not GRP(1-16). The GRP aminoterminal RIA crossreacted with GRP, GRP(1-13), GRP(1-16), but not GRP(18-27). The GRP(1-16) carboxyterminal antisera cross-reacted with GRP(1-16) but had little crossreactivity with GRP, or GRP(1-13).

Table 1. Crossreactivity of Gastrin Releasing Peptide Radioimmunoassays
Percent Crossreactivity in RIA

Synthetic Peptide	GRP(1-27) Code R3-2	GRP(1-16) Code R2-6A	GRP(1-16) Code R2-6B
GRP	100	100	03
GRP(1-13)	0	100	03
GRP(1-16)	0	100	100
GRP(18-27)	60	0	0
α-N-acetyl-GRP(20-27)	60	0	0
Bombesin	30	0	0

An antisera dilution of 1:20,000 for the GRP(1-27) R3-2 antisera and a dilution of 1:15,000 for the GRP(1-16) R2-6A were used for RIA. For these RIA's GRP(1-27) was used as ^{125}I-trace and standard. An antisera dilution of 1:20,000 for GRP(1-16) R2-6B and GRP(1-16) trace and standard were used in the GRP(1-16) carboxyterminal assay. Crossreactivity was based on the amount of unlabeled peptide needed to obtain a 50 percent displacement of ^{125}I-trace from the antisera. The highest concentration of unlabeled peptide tested was 1 μM.

Synthetic peptides were synthesized by standard solid phase methods and purified by gel-filtration and partition chromatography. Purity of the peptides was shown by RP-HPLC and amino acid analysis. GRP(18-27) was made by trypsin digestion of GRP(14-27) followed by RP-HPLC purification. GRP(1-13) was generated by trypsin digestion of GRP(1-16) followed by RP-HPLC purification.

Characterization of Immunoreactive GRP-like Peptides

The GRP immunoreactive material from rat and guinea pig brain was characterized by gel filtration and reverse phase high performance liquid chromatography (RP-HPLC). Whole brains (without cerebellums) from 15 rats and from 10 guinea pigs were extracted in acid acetone (acetone:water:12 N HCl; 40:6:1). Acid acetone extracts of rat brains and guinea pig brains were delipidized with heptane as described previously (11), evaporated under nitrogen and resuspended in 2 ml of 50 percent acetic acid and placed on a 120 x 2.5 cm Sephadex G-50 column and eluted with 50 percent acetic acid. 5 ml fractions were collected and aliquots were evaporated under reduced pressure and assayed in the three GRP RIA's. Aliquots of the immunoreactive peak fractions were subjected to RP-HPLC analysis as described previously (11). The large MW peak of GRP carboxyterminal immunoreactivity in rat and guinea pig brain was also trypsin digested prior to RP-HPLC. For trypsinization aliquots from the large MW peak were evaporated under nitrogen and resuspended in 500 μl of 0.2 M Tris/HCl buffer pH 8.1. About 10,000 cpm of ^{125}I-dynorphin(1-8) and 10 μl of 1 mg/ml TPCK treated trypsin (Millipore) were added. The ^{125}I-dynorphin (1-8) served both as an internal standard for the chromatography and as a marker for monitoring the completeness of the trypsin digestion. The reaction was stopped after 2 hours by addition of 100 μl of glacial acetic acid and the sample injected on the column. HPLC fraction aliquots were evaporated under reduced pressure and analyzed by RIA.

RESULTS

In gel filtration of guinea pig brain extract two peaks of GRP carboxy-terminal immunoreactivty were observed (Figure 2A). The large molecular weight (MW) MW peptide had an identical size, and RP-HPLC retention time as porcine GRP (Figure 4'). Upon trypsin digestion this material liberated an equimolar amount of a peptide which co-chromatographed with GRP(18-27)

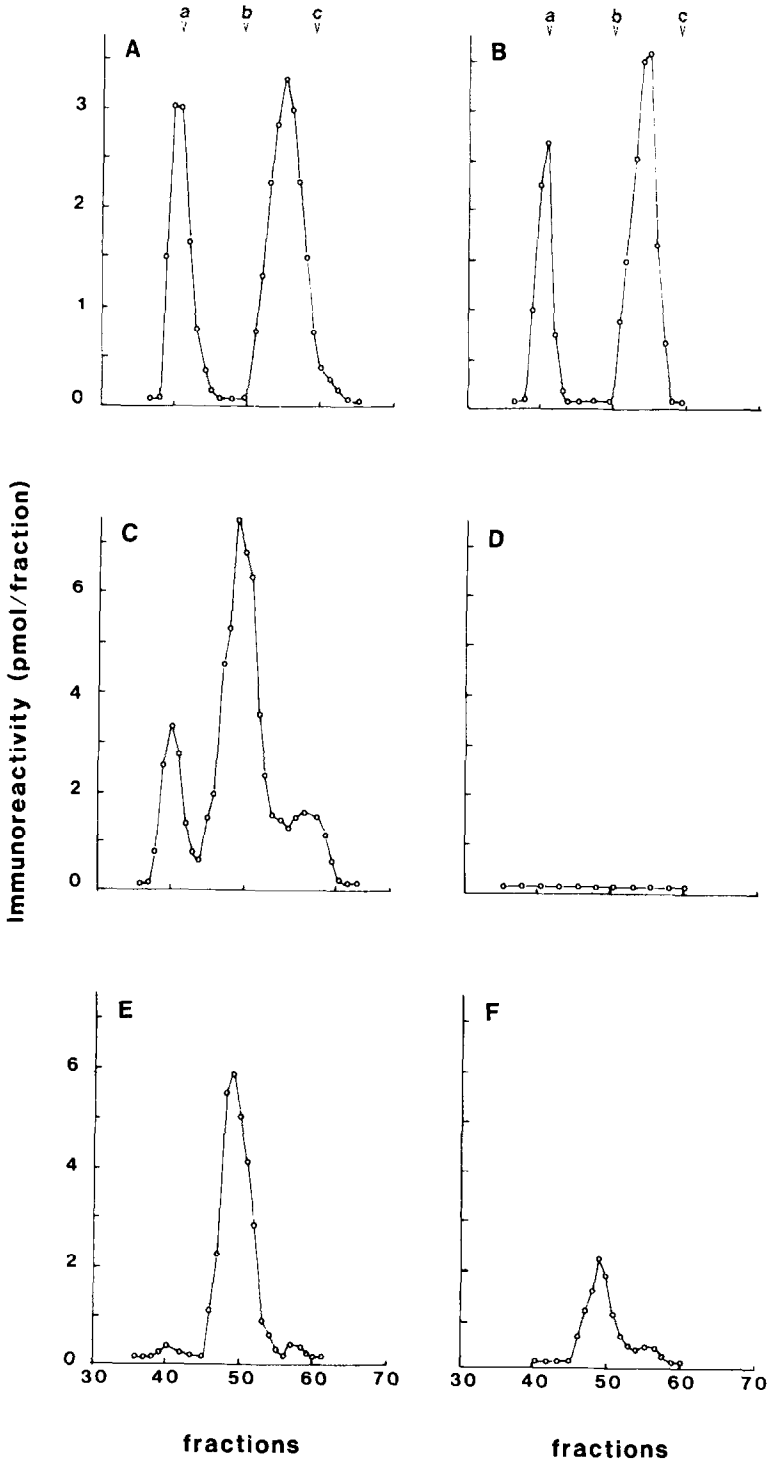

Figure 2. Gel-filtration chromatography profiles of GRP carboxyterminal (A,B), GRP aminoterminal (C,D), and GRP(1-16) carboxyterminal (E,F) immunoreactivity in guinea pig (A,C,E) and rat brain (B,D,F). The column was calibrated with (a) ^{125}I-GRP, MW=2800, (b) ^{125}I-α-melanocyte stimulating hormone, MW=1770, (c) ^{125}I-met-enkephalin-Arg^{6}-Phe^{7}, MW=880).

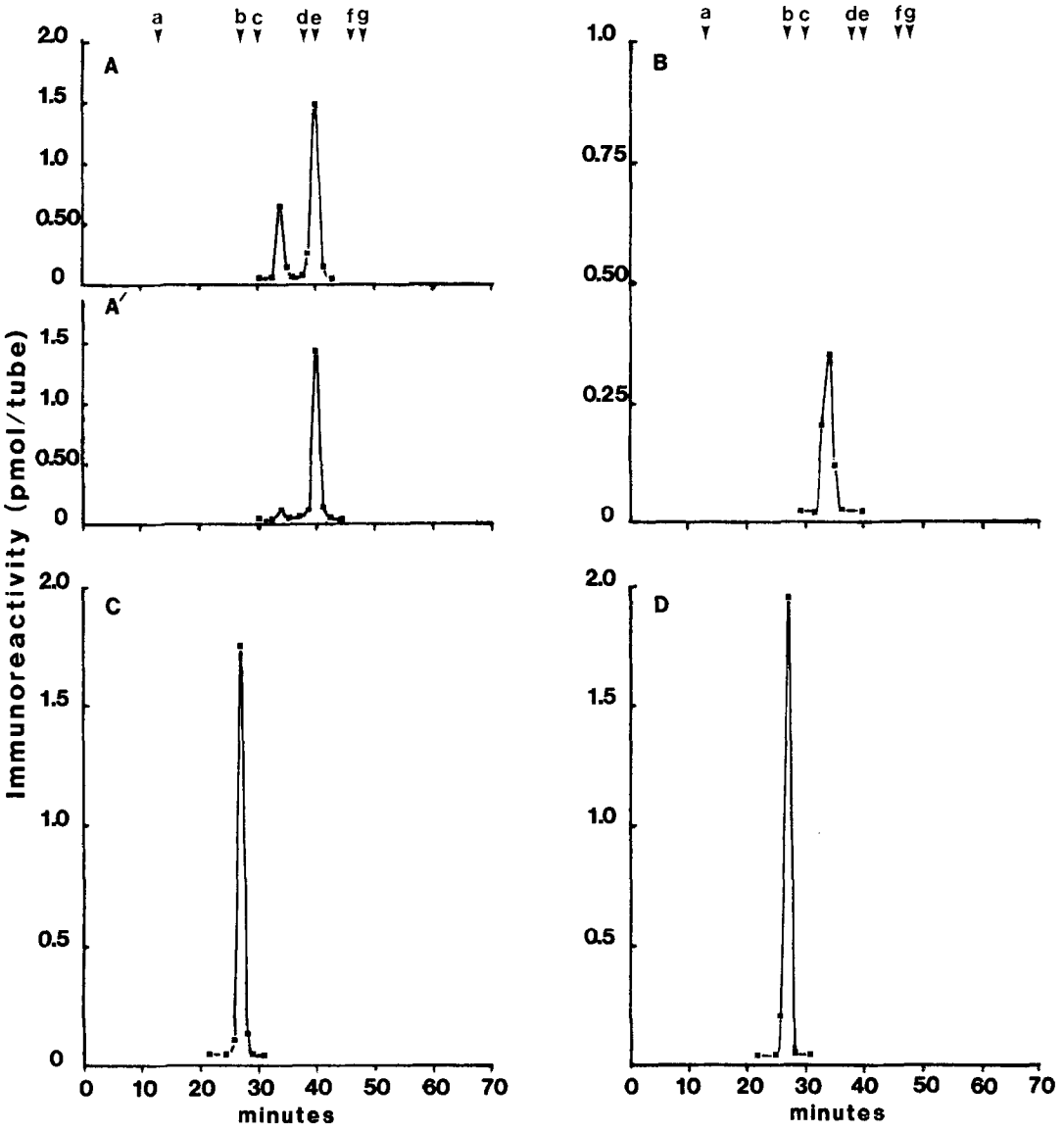

Figure 3. RP-HPLC profiles of the lower MW GRP immunoreactive peaks from gel filtration of guinea pig and rat brain. (A) GRP aminoterminal and (A') GRP(1-16) carboxyterminal immunoreactivity from guinea pig brain gel filtration fractions 48,49,50. (B) GRP(1-16) carboxyterminal immunoreactivity from rat brain gel filtration fractions 48,49,50. GRP carboxyterminal immunoreactivity from gel filtration fractions 54,55,56 from guinea pig (C) and rat brain (D). Chromatography was performed on an Altex Ultrasphere ODS column (250 mm x 4.6 mm, particle size 5 μm). Two Altex HPLC pumps and a Beckman gradient mixing computer were used to generate an acetonitrile gradient of 0-17.5 percent in 5 minutes followed by a 17.5-35 percent gradient in 75 minutes. HPLC buffer consisted of 50 mM monosodium phosphate, 1 mg ml^{-1} phosphoric acid and 5 percent methanol, pH 2.7. The flow rate was 1.25 ml min^{-1} and 1 min fractions were collected. ~10,000 cpm of ^{125}I-α-neo-endorphin and ^{125}I-dynorphin(1-8) were added as internal standards. Radioactivity in the eluate was monitored with a continuous flow through γ-counter (Isoflo, Nuclear Enterprises).
Markers: (a) GRP(1-13), (b) GRP(18-27), (c) methionine sulfoxide bombesin, (d) bombesin, (e) GRP(1-16), (f) trypsinized bombesin, (g) GRP(1-27).

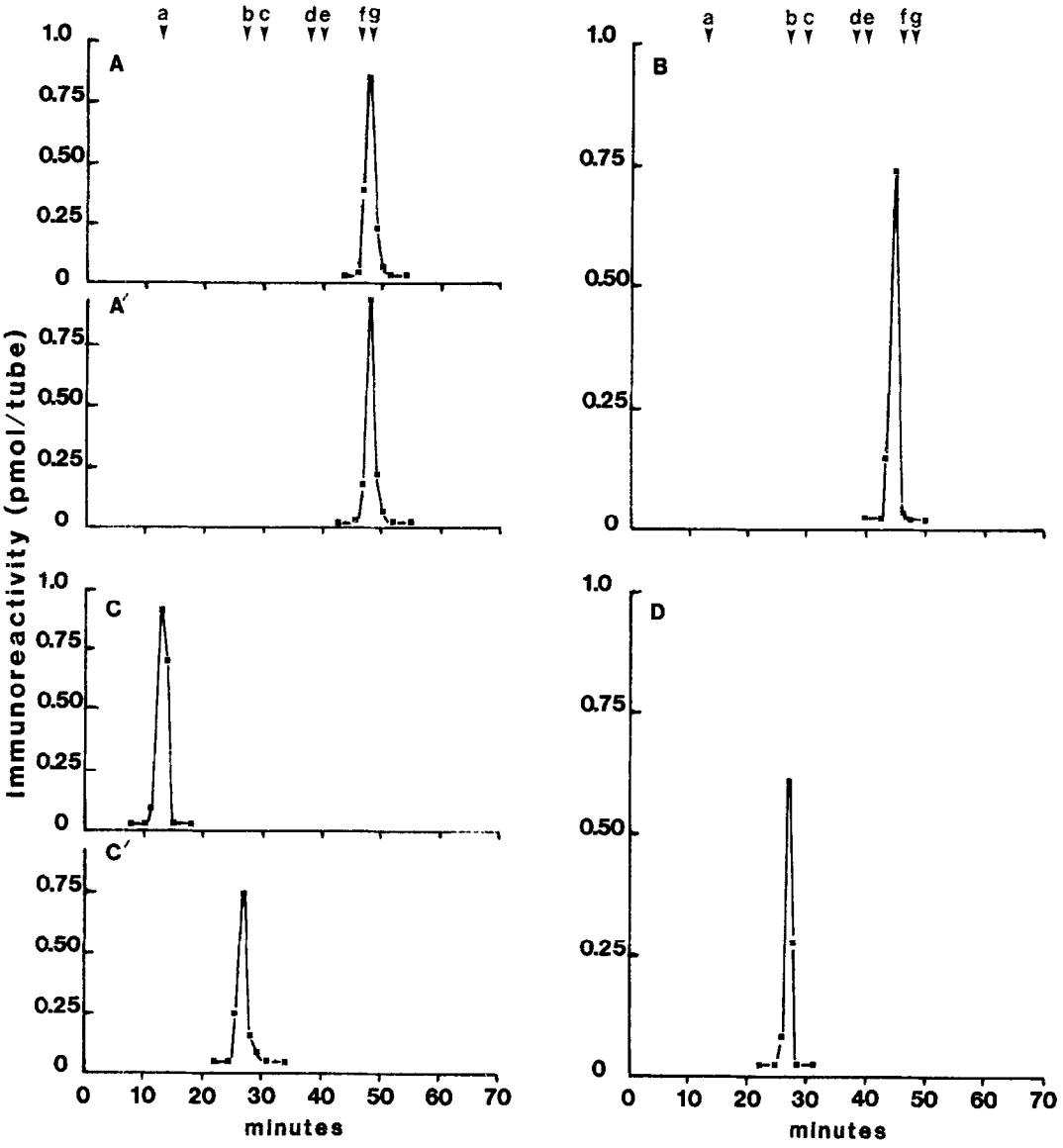

Figure 4. RP-HPLC profile of the larger MW GRP immunoreactive peak from gel filtration of guinea pig and rat brain. (A) GRP aminoterminal and (A') GRP carboxyterminal immunoreactivity from guinea pig brain gel filtration fractions 39,40,41. (B) GRP carboxyterminal immunoreactivity from rat brain gel filtration fractions 39,40,41. (C) GRP aminoterminal immunoreactivity and (C') GRP carboxyterminal immunoreactivity of trypsin digested guinea pig gel filtration fractions 39,40,41. (D) GRP carboxyterminal immunoreactivity of trypsin digested rat brain gel filtration fractions 39,40,41.
Markers: (a) GRP(1-13), (b) GRP(18-27), (c) methionine sulfoxide bombesin, (d) bombesin, (e) GRP(1-16), (f) trypsinized bombesin, (g) GRP(1-27).

(Figure 4C'). The smaller MW peak of GRP carboxyterminal immunoreactivty had an identical size and RP-HPLC retention time as GRP(18-27) (Figure 3C).

Upon gel filtration chromatography of guinea pig brain extracts, the GRP aminoterminal RIA recognized two peaks of immunoreactivity (Figure 2C). The

large MW peptide had an identical size and RP-HPLC retention time as GRP (Figure 4A). The GRP aminoterminal and carboxyterminal RIA's measured equimolar amounts of this material, the GRP(1-16) carboxyterminal RIA did not recognize this material. Upon trypsin digestion of this material, the GRP aminoterminal RIA recognized an equimolar amount of material which co-chromatographed with GRP(1-13) (Figure 4C). The smaller MW material had a similar size and RP-HPLC retention time as GRP(1-16) (Figure 3A). In both gel filtration and RP-HPLC this peak of immunoreactivity was detected in approximately equimolar amounts by the GRP carboxyterminal RIA (Figure 2E, 3A').

These results indicate the presence in guinea pig brain of GRP, GRP (1-16), and GRP(18-27)-like peptides which have identical immunodeterminants, size, and RP-HPLC characteristics as their respective porcine GRP fragments.

In gel chromatographs of rat brain the GRP carboxyterminal RIA revealed two peaks of immunoreactivity (Figure 2B). The larger MW peak had a size similar to GRP, however, in RP-HPLC this material eluted several minutes earlier than GRP (Figure 4B). Upon trypsin digestion an equimolar amount of a peptide which co-chromatographed with GRP(18-27) was formed (Figure 4D). The smaller MW peak had a similar size and retention time as GRP(18-27) (Figure 3D).

The GRP aminoterminal RIA revealed no peaks of immunoreactivity (Figure 2D). The GRP(1-16) carboxyterminal RIA identified a single peak of immunoreactivity of similar size as GRP(1-16) (Figure 2F). On RP-HPLC this material eluted several minutes earlier than GRP(1-16) (Figure 3B).

These findings suggest the presence in rat brain of a GRP(18-27)-like peptide of similar size and RP-HPLC retention time as GRP(18-27). A GRP, and a GRP(1-16)-like peptide are present in rat brain, however, these compounds are not identical to their porcine counterparts since they elute earlier on RP-HPLC and they are not recognized by a GRP aminoterminal RIA.

DISCUSSION

Our results suggest that bombesin does not exist in rat or guinea pig brain. Instead, in both species a peptide of similar size and RP-HPLC characteristics as porcine GRP(18-27) was found. In guinea pig brain GRP and GRP(1-16)-like peptides with identical characteristics as their porcine counterparts were found. In rat brain although GRP and GRP(1-16)-like peptides were found, these materials were not identical to porcine GRP and GRP(1-16). These results suggest that the GRP(18-27) sequence has been conserved across these species; the aminoterminal sequence of GRP, however, has not been strictly conserved. It is interesting that the biological actions of bombesin can be produced by GRP which is homologous to bombesin in its carboxyterminal region (12,13).

The presence of GRP(1-16) and GRP-(18-27)-like peptides in brain implies proteolytic processing of the parent molecule at a single arginine residue (position 17, Fig. 1). Recently a number of endogenous neuropeptides have been shown to be generated from their precursors by proteolytic processing at single arginine residues. Both dynorphin(1-8) and dynorphin-B, two naturally occurring opioid peptides, seem to be liberated from their precursors by cleavage at single arginine residues (14-16). A glycopeptide derived from the pro-arginine vasopressin/neurophysin II precursor, the carboxyterminal octapeptide of cholecystokinin, and somatostatin-28 are also generated from their respective precursors by cleavages at single arginine residues (17-19). Thus, the results reported here demonstrate a fifth major neuropeptide system that seems to utilize the single arginine cleavage to generate peptide fragments. Future studies on neuropeptide processing in brain must therefore take into account that proteolytic processing pathways are not restricted to cleavage at double basic amino acid residues. Here, the single arginine cleavage gives rise to a carboxyterminal fragment of GRP that is similar in structure and size to amphibian bombesin. This is possibly the peptide that other investigators have characterized as bombesin in mammalian brain (7).

Our results clearly demonstrate that authentic bombesin is not present in guinea pig or rat brain. Future studies must now address the question whether GRP(18-27) can mimic the pharmacological actions of bombesin on eating, thermoregulation and anterior pituitary hormone release (2-4) and therefore would be a candidate as an endogenous mediator of these effects.

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