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The Regional Distribution and the Chemical, Chromatographic, and Immunologic Characterization of Motilin Brain Peptides: The Evidence for a Difference between Brain and Intestinal Motilin-immunoreactive Peptides[1]

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Abstract

Motilin-like immunoreactive peptides (MLIPs) have been detected in the brain by radioimmunoassay (RIA), (Yanaihara, C., H. Sato, N. Yanaihara, S. Naruse, W. G. Forssman, V. Helmstaedter, T. Fujita, K. Yamaguchi, and K. Abe (1977) Adv. Exp. Biol. Med. *106:* 269–283; Chey, W. Y., R. Escoffery, F. Roth, T. M. Chang, and H. Yajima (1980) Regul. Pept. Suppl. *1:* 519; O'Donohue, T. L., M. C. Beinfeld, W. Y. Chey, T. M. Chang, G. Nilaver, E. A. Zimmerman, H. Yajima, H. Adachi, M. Roth, R. P. McDevitt, and D. M. Jacobowitz (1981) Peptides *2:* 467–477). Previous studies (O'Donohue et al., 1981) demonstrated that MLIPs in rat brain probably differ chemically from porcine intestinal motilin (PIM), the first motilin peptide isolated. The possibility that this rat-pig difference represents a species difference was not examined in the previous study (O'Donohue et al., 1981), neither was the question of the cross-species distribution of MLIP. This study was initiated to examine brain MLIP distribution by RIA in three additional species: cow, pig, and guinea pig. The question of rat-pig species differences was addressed by characterizing MLIP in the brains of these species in comparison to PIM.

By RIA, MLIPs were widely distributed in the brains of all species examined. MLIP concentration was highest in rat brain and lowest in pig brain. Some motilin antisera consistently detected less or no MLIPs in some brain regions of all species. Rat pituitary, pineal gland, and retina had substantially higher MLIP concentrations than did brain. MLIPs were abundant throughout the rat gastrointestinal tract and in some other peripheral organs.

Rat, cow, and pig brain extracts separated by gel filtration chromatography contain a peak of MLIP similar in size to PIM. MLIP immunoreactivity in these fractions from rat and cow were largely or completely peptidase sensitive. On high pressure liquid chromatography (HPLC), MLIP similar in size to PIM from rat, cow, and pig brain elutes as a discrete peak, well separated from PIM. Other MLIPs, smaller in size than PIM, also separate from PIM on HPLC and are much less protease sensitive.

In conclusion, a brain MLIP similar in size to PIM is present in the brains of several vertebrate species. However, based on the immunological and chromatographic evidence presented, the sequence of brain MLIP probably differs from that of PIM (or it has been post-translationally modified). The extent of this difference and its implication for the functional role of MLIP in brain remain to be determined.

Motilin was originally isolated from porcine intestine by Brown et al. (1971) and was named for its ability to contract isolated stomach pouches. It is a 22-amino acid peptide, which is weakly homologous with the vasoactive intestinal peptide (VIP) family of peptides, in particular with growth hormone-releasing hormone (Guillemin et al., 1982; Rivier et al., 1982).

The physiological role of motilin in the gastrointestinal tract is unknown, although in some species it is thought to be involved in the migrating motor complex. The literature on the physiological role of motilin in the gastrointestinal tract has been reviewed recently (Fox, 1984).

Motilin-like immunoreactive peptides (MLIPs) were detected in the brains of several vertebrate species by radioimmunoassay (RIA) (Yanaihara et al., 1977; Chey et al., 1980; Nagai et al., 1980; O'Donohue et al., 1981) and immunocytochemistry (Chey et al., 1980; Chan-Palay et al., 1981; Jacobowitz et al., 1981; Nilaver et al., 1982). MLIPs were first reported in canine and monkey brains, being particularly abundant in anterior pituitary, pineal gland, and hypothalamus (Chey et al., 1980). Motilin-containing cells were visualized in cerebellum, anterior pituitary, hypothalamus, cerebral cortex, and pineal gland (Yanaihara et al., 1977; Chey et al., 1980). Human cerebrospinal fluid also contains high MLIP levels (Chey et al., 1980).

Within the cerebellum the motilin-like staining was confined to the cerebellar Purkinje neurons (Chan-Palay et al., 1981; Nilaver et al., 1982), some of which are also GABAergic as judged by the presence of glutamic acid dehydrogenase staining (Chan-Palay et al., 1981). Motilin staining was quite dense in the median eminence (Jacobowitz et al., 1981; Loftus et al., 1983). Motilin-containing cells, some of which also stain for growth hormone, have been observed in the anterior lobe of the pituitary of rats and humans (Loftus et al., 1983). Motilin is able to release growth hormone *in vitro*, possibly due to its structural similarity with growth hormone-releasing hormone (Samson et al., 1982). Motilin is a potent excitatory agent when applied iontophoretically to neurons in the cerebral cortex and spinal cord (Phillis and Kirkpatrick, 1979). In a more recent study, motilin was found to be inhibitory on neurons of the lateral vestibular nucleus (Chan-Palay et al., 1982).

Previous studies based on immunological criteria (Shin et al., 1980; Yanaihara et al., 1980) have indicated that the sequence of

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duodenal motilin may not be well conserved. Canine duodenal motilin was found to differ in 5 of 22 amino acids (Poitras et al., 1983), whereas human duodenal motilin was found to be identical to porcine intestinal motilin (J. Reeve, personal communication). In another study (O'Donohue et al., 1981), based on immunological criteria and high pressure liquid chromatography (HPLC) separations, rat brain MLIPs were found to differ from porcine intestinal motilin (PIM). An immunocytochemical study (Nilaver et al., 1982) noted that motilin-like staining in rat cerebellum could be blocked only partially, even at high PIM concentrations, suggesting possible differences in motilin structure between rat and pig motilin.

These early studies did not chromatographically characterize MLIPs in any species other than the rat; therefore, the possibility that the pig-rat difference may be a species difference was not confirmed. Furthermore, the distribution of MLIPs was determined in only a few species. This current study was initiated to examine brain MLIPs in a number of species and to determine whether MLIP in any of these species, including the pig, resembles PIM.

Materials and Methods

Motilin RIA

The motilin RIA was performed as previously described (O'Donohue et al., 1981; Beinfeld et al., 1983). Four antisera were utilized: (*1*) N1–8 (called 1–4 in O'Donohue et al., 1981; courtesy of Dr. G. Nilaver); (*2*) no. 13 (courtesy of Dr. W. Chey); (*3*) no. 9 (courtesy of Dr. Chey); and (*4*) GP (courtesy of Dr. H. Adachi).

The cross-reactivity of these antisera with various motilin fragments has been previously described (O'Donohue et al., 1981). Antisera N1–8, 13, and GP are middle- to C-terminal directed, while antiserum 9 is N-terminal directed. In addition, none of these motilin antisera cross-react with motilin 1–10 or 9–16. Additional characterization of N1–8 was performed to determine whether antiserum N1–8 cross-reacts with any members of the VIP family to which PIM is weakly homologous. Antiserum N1–8 did not cross-react with human growth hormone-releasing hormone, VIP, peptide histidine isoleucine amide, secretin, or glucagon tested at 1 mg/ml. Natural gastric inhibitory peptide (GIP) provided by Robert T. Jensen, M.D. (Digestive Diseases Branch, National Institute of Arthritis, Metabolic and Digestive Diseases), cross-reacted less than 0.01%. It is possible that this natural GIP preparation is very slightly contaminated with motilin, as it has been shown to be slightly contaminated with cholecystokinin-33 (Jensen et al., 1982).

Tissue dissection and extraction

Rats and guinea pigs were killed by decapitation, and their brains were removed quickly and dissected on a chilled glass Petri dish. Cow and pig brains were obtained at slaughterhouses and were either dissected immediately and frozen on dry ice or packed in wet ice, transported to the laboratory, and dissected in the cold, and then frozen on dry ice until extraction. The dissections were aided by published atlases (Paxinos and Watson 1982; Yoshikawa, 1968).

For the regional dissection studies a combined boiling water and acetic acid extraction was used. The brain pieces were weighed, placed in boiling water for 10 min, homogenized with a Tekmar Ultra-Turrax homogenizer, and clarified by centrifugation, and the supernatant was saved. The pellet was re-extracted by homogenization with 0.5 M acetic acid at room temperature followed by centrifugation. The combined supernatants were clarified further if necessary, aliquoted, and dried prior to the RIA. This extraction was found to be the most efficient for extracting motilin peptides (O'Donohue et al., 1981).

For the Sephadex chromatography experiments, larger quantities of brain were extracted with an acid/acetone extraction procedure modified from a procedure for substance P (Sherwood et al., 1983). Frozen brains were blended with 0.05 M HCl (1 ml/gm of original tissue weight) in a large Waring blender until liquefied. To the liquid in a glass chromatography jar, acetone was added (4 ml/gm), and the mixture was stirred with an air-driven stirrer in a fume hood at room temperature for 2 hr. The mixture was filtered through a Whatman no. 1 filter and the filter cake was re-extracted with 4 ml/gm of acetone:0.01 M HCl (80:20, v/v). To the clarified liquid was added petroleum ether (2 ml/gm), the mixture was stirred and the petroleum ether was aspirated off. The petroleum ether extraction was repeated three more times. The resulting aqueous extract was left in the hood overnight to allow any residual acetone to evaporate. Following this, the extract was reduced in volume in a Savant vacuum centrifuge. Both of these extraction methods yielded about the same amount of MLIP per gram of tissue weight.

Protease digestion

Trypsin. Neutralized Sephadex fractions containing rat or bovine MLIP or synthetic motilin were incubated at 37°C in 10 mM sodium phosphate, pH 7.5. The reaction was started by addition of the trypsin (Sigma bovine pancreas type III, 0.5 mg/ml). Aliquots were removed prior to the trypsin addition and at 1, 5, 10, and 30 min after. The aliquots were boiled for 10 min and then cooled; then, motilin assay buffer was added to dilute the samples prior to motilin RIA with antiserum N1–8.

Pronase. The pronase digestion was similar to the trypsin digestion with the following exceptions. Pronase (from *Streptomyces griseus*, Sigma type

TABLE I

Motilin distribution in rat brain, pituitary, pineal gland, and retina

Region	Motilin Concentration[a] (ng/gm of tissue weight)			
	N1–8	Ab13	Ab9	AbGP
Brain Region				
Cerebellum	4.7 ± 0.39[b]	0.5 ± 0.1	1.2 ± 0.1	0.51 ± 0.06
Olfactory bulbs	2.8 ± 0.65	0.4 ± 0.03	1.9 ± 0.9	0.2 ± 0.07
Hypothalamus	1.9 ± 0.15	0.7 ± 0.1	0.8 ± 0.2	ND,[c] <0.13
Septum	1.6 ± 0.10	0.6 ± 0.1	1.3 ± 0.2	ND, <0.14
Striatum	1.5 ± 0.11	0.5 ± 0.06	0.8 ± 0.2	ND, <0.1
Thalamus	1.5 ± 0.13	0.4 ± 0.03	ND, <0.4	0.2 ± 0.02
Cerebral cortex	1.4 ± 0.29	0.4 ± 0.03	0.4 ± 0.1	0.3 ± 0.02
Hippocampus	1.3 ± 0.21	0.3 ± 0.05	ND, <0.4	0.2 ± 0.03
Pons	1.3 ± 0.31	0.27 ± 0.03	ND, <0.4	0.2 ± 0.05
Midbrain	1.1 ± 0.14	0.31 ± 0.06	0.5 ± 0.1	0.22 ± 0.05
Spinal cord	1.0 ± 0.33	1.2 ± 0.13	2.2 ± 0.6	ND, <0.19
Medulla	0.7 ± 0.08	0.3 ± 0.04	ND, <0.4	ND, <0.09
Anterior intermediate lobe of the pituitary	38.5 ± 6.0	7.6 ± 1.7	38.5 ± 12.1	4.8 ± 0.8
Pineal gland	25.1 ± 2.5	24.6 ± 2.3	ND, <2.5	20.9 ± 4.0
Retina	8.1 ± 2.1	1.4 ± 0.1	ND, <1.3	1.3 ± 0.4
Neural lobe of the pituitary	4.4 ± 0.3	n. det.[d]	n. det.	n. det.

[a] The motilin concentration in rat brain regions as determined by RIA with motilin antisera N1–8, 13, 9, and GP.

[b] Data are expressed as mean ± SEM, $N = 4$ to 6, with data from 40-day-old rats.

[c] ND, not detectable.

[d] n. det., not determined.

XIV) was used at 1 mg/ml and the reaction was allowed to proceed up to 120 min. One sample contained motilin but no pronase, as did some of the Sephadex fraction samples, to test the stability of the peptides to 37°C incubation and boiling steps. The motilin immunoreactivity of the synthetic motilin and MLIP pools were unaffected by the incubation and boiling steps. One sample also contained the pronase or trypsin but no motilin and was processed as usual. This was included to ensure that the boiled trypsin did not alter the RIA. At the protease concentrations used, the boiled proteases did not interfere with the motilin RIA.

Chromatography

Sephadex

Extracts of rat, bovine, and porcine brain were prepared as previously described and were chromatographed on 5 × 100 cm columns of Sephadex-G25-fine run in 1 M acetic acid at room temperature. Fractions of 20 ml were collected, and aliquots were dried in a Savant vacuum centrifuge and assayed for motilin (as previously described) or VIP immunoreactivity (Eiden et al., 1982). Sephadex fractions high in motilin immunoreactivity were dried and used for protease digestions and were further characterized by HPLC.

HPLC

Extracts were run on Alltech C18 columns in two systems.

TEAP. The buffer was triethylamine-phosphoric acid (TEAP), pH 3.25: 17 ml of 85% HPLC grade phosphoric acid were added to distilled, deionized water treated further with a Technics Lab 5 polisher, and Pierce Chemical Co. Sequanal grade triethylamine was added until the pH was 3.25. The final volume was then adjusted to 1:1. The samples were applied in 20% acetonitrile, 80% TEAP and eluted with a linear gradient to 60% acetonitrile, 40% TEAP in 40 min. The flow rate was 1 ml/min and 1-ml fractions were collected.

TFA. Two buffers were utilized for this system: buffer A (0.09% trifluoroacetic acid (TFA) (Pierce Sequanal grade) in the water previously described, and buffer B (acetonitrile:water, 90:10 (v/v) in 0.09% TFA). The samples were applied in 80% buffer A, 20% buffer B and eluted with a linear gradient to 40% buffer A, 60% buffer B, over 60 min.

TABLE II

Motilin distribution in guinea pig brain, pituitary, and duodenum

Region	Motilin Concentration[a]		N
	Nanograms/gram	Picograms/region	
Brain			
Septum	1.57 ± 0.24[b]	36 ± 5	6
Hypothalamus	1.1 ± 0.08	54 ± 5	6
Diencephalon	0.81 ± 0.18	234 ± 71	6
Medulla and trapezoid body	0.78 ± 0.14	205 ± 42	6
Olfactory bulbs	0.69 ± 0.05	32 ± 5	6
Cerebral cortex	0.63 ± 0.09	1067 ± 182	6
Pons	0.62 ± 0.06	43 ± 2	6
Striatum	0.54 ± 0.06	74 ± 7	6
Thalamus	0.44 ± 0.06	68 ± 10	6
Hippocampus	0.42 ± 0.03	123 ± 18	6
Cerebellum	0.41 ± 0.034	180 ± 18	5
Spinal cord	0.30 ± 0.065	35 ± 6	6
Whole pituitary	5.55 ± 0.72	84 ± 7	6
Duodenum	1.07 ± 0.06	921 ± 72	6

[a] Motilin concentration determined with motilin antiserum N1-8.

[b] Values are mean ± SEM.

Results

Homology with rabbit skeletal tropomyosin (TM)

The possibility that brain MLIPs are some known peptide(s) homologous to motilin which cross-reacts with many motilin antisera was tested by having the sequence of PIM searched by the National Institute of Arthritis, Metabolic and Digestive Diseases data bank. This search revealed that a portion of PIM 9–17 is strongly homologous to a portion of rabbit skeletal muscle TM as shown below:

Motilin 9–17:	Glu-Leu-Gln-Arg-Met-Gln-Glu-Lys-Glu
TM 142–150:	Glu-Leu-Gln-Glu-Met-Gln-Leu-Lys-Glu

However, it is unlikely that MLIP is TM or a TM fragment, as none of the motilin antisera cross-react with either native TM (Sigma) or boiled TM tested to 3×10^{-6} M in the RIA. This is also consistent with our observation that synthetic motilin 9–16 (which is most of the region of homology with TM) does not cross-react with any of the motilin antisera tested. No other homologies were revealed by the computer search.

Distribution studies

The regional distribution of MLIPs in rat brain as determined by RIA with the four motilin antisera is shown in Table I. As previously reported (O'Donohue et al., 1981), MLIPs are widely distributed in rat brain and are particularly concentrated in cerebellum and forebrain, but are less abundant in hindbrain and spinal cord. MLIPs are more abundant in pineal gland, retina, and pituitary than in brain. The presence of MLIPs in pituitary and pineal gland has been reported previously (Yanaihara et al., 1977; Chey et al., 1980; Nagai et al., 1980). The concentration of MLIPs in the gastrointestinal tract and adrenal gland of the rat is similar to or in some cases higher than the MLIP concentration in the cerebellum (data not shown). Motilin antiserum N1-8 detects higher levels of MLIPs in rat brain than any of the other motilin antisera. This is also true for cow and pig brain (see Tables III and IV).

The regional distribution of MLIPs in guinea pig brain as determined by RIA with motilin antiserum N1-8 is shown in Table II. Like rat brain, MLIPs in guinea pig brain are widely distributed, and MLIPs

TABLE III

Motilin distribution in pig brain

Brain Region	Motilin Concentration[a] (ng/gm of tissue weight)			
	N1-8	Ab13	Ab9	AbGP
Striatum	1.00 ± 0.20[b]	0.17 ± 0.01	0.21 ± 0.13	<0.15
Midbrain	0.96 ± 0.12	0.19 ± 0.04	<0.12	<0.22
Cerebral cortex	0.80 ± 0.07	0.21 ± 0.02	0.27 ± 0.04	0.28 ± 0.02
Hippocampus	0.68 ± 0.14	0.22 ± 0.01	0.18 ± 0.04	<0.13
Pons	0.60 ± 0.12	0.19 ± 0.02	0.18 ± 0.06	<0.14
Septum	0.64 ± 0.14	0.38 ± 0.06	0.58 ± 0.14	0.39 ± 0.20
Hypothalamus	0.33 ± 0.07	0.35 ± 0.05	<0.06	<0.12
Thalamus	0.33 ± 0.08	0.25 ± 0.04	<0.05	<0.10
Medulla	0.31 ± 0.03	0.06 ± 0.01	<0.03	<0.06
Cerebellum	0.26 ± 0.02	0.52 ± 0.03	0.28 ± 0.08	0.08 ± 0.02

[a] Motilin concentration in pig brain regions as determined by RIA with motilin antisera N1-8, 13, 9, and GP.

[b] Data are expressed as mean ± SEM. $N = 4$ to 6.

TABLE IV
Motilin distribution in cow brain[a]

Brain Region	Motilin Concentration[a] (ng/gm of tissue weight)			
	N1-8	Ab13	Ab9	AbGP
Hippocampus	0.85 ± 0.09[b]	0.36 ± 0.03	0.79 ± 0.12	0.12 ± 0.02
Septum	0.75 ± 0.05	0.32 ± 0.02	0.63 ± 0.09	0.06 ± 0.01
Cerebellum	0.73 ± 0.19	0.34 ± 0.05	0.90 ± 0.10	0.13 ± 0.02
Striatum	0.69 ± 0.07	0.41 ± 0.02	0.76 ± 0.12	0.09 ± 0.01
Frontal cortex	0.63 ± 0.05	0.31 ± 0.03	0.38 ± 0.06	0.08 ± 0.01
Hypothalamus	0.62 ± 0.09	0.27 ± 0.02	0.58 ± 0.04	0.11 ± 0.02
Occipital cortex	0.57 ± 0.05	0.30 ± 0.03	0.53 ± 0.06	0.09 ± 0.02
Pons	0.56 ± 0.06	0.24 ± 0.02	0.87 ± 0.07	ND,[c] <0.05
Midbrain	0.50 ± 0.04	0.29 ± 0.05	0.62 ± 0.06	0.09 ± 0.01
Temporal cortex	0.49 ± 0.07	0.24 ± 0.03	0.76 ± 0.13	0.08 ± 0.01
Putamen	0.49 ± 0.03	0.26 ± 0.02	0.52 ± 0.11	ND, <0.07
Olfactory bulb	0.36 ± 0.02	0.24 ± 0.02	0.62 ± 0.07	ND, <0.05
Medulla	0.34 ± 0.04	0.22 ± 0.03	0.74 ± 0.11	ND, <0.04
Spinal cord	0.31 ± 0.01	0.35 ± 0.03	0.35 ± 0.12	0.09 ± 0.002
Thalamus	0.13 ± 0.04	0.19 ± 0.02	0.45 ± 0.10	ND, <0.07

[a] The motilin concentration in rat brain regions as determined by RIA with motilin antisera N1-8, 13, 9, and GP.
[b] Data are expressed as mean ± SEM; $N = 6$ to 8.
[c] ND, not detectable.

in whole pituitary are about 3 times higher than in the septum, the region with the highest MLIP concentration in guinea pig brain. Rat brain MLIP concentrations are consistently higher than guinea pig MLIP concentrations. The guinea pig duodenal MLIP concentration is about the same as that of brain MLIP.

The regional distribution of MLIPs in porcine brain as determined by RIA with four motilin antisera is shown in Table III. MLIPs in porcine brain, like the other species examined, are widely distributed but are less abundant than rat brain MLIP.

The regional distribution of MLIPs in bovine brain as determined by RIA with four motilin antisera is shown in Table IV. MLIPs in cow brain are present in all of the brain regions examined and, as in guinea pig and pig brain, cow MLIPs are less abundant than rat brain MLIPs.

Sephadex chromatography of brain MLIP.

Acid/acetone extracts (procedure described under "Materials and Methods") of rat, cow, and pig brain were characterized by chromatography on a preparative scale Sephadex-G25 column. The elution of MLIPs was detected with motilin antiserum N1-8 (Fig. 1, *A* to *C*), and VIP-like immunoreactivity in the porcine brain extract (Fig. 1*D*) was detected with a VIP RIA (Eiden et al., 1982). Porcine brain VIP, which is identical to porcine intestinal VIP (Carlquist et al., 1982), makes a convenient internal molecular weight marker in these extracts. In all of these extracts a broad peak of MLIPs was observed (peak I) which was similar in size to that of VIP (peak I) and another broad peak (peak II) which elutes in the position of salt on the column. The relative proportion of peak I in relationship to peak II varies in different species, with the rat having the highest peak I/II ratio and the pig the lowest.

Pooled peak I and peak II fractions from these Sephadex chromatography steps were used for further HPLC characterization and protease digestion studies.

HPLC Chromatography. In Figure 2, the results of HPLC separation of rat and cow MLIPs in peak I pools from Figure 1 are shown in comparison with PIM. MLIP in the HPLC fractions was determined with the motilin RIA (using antiserum N1-8) in comparison to the elution of PIM detected optically at 210 nm and with the motilin RIA. Bovine and rat brain peak I MLIP fractions elute from this C18 column as a single major peak, with some additional minor components eluting before. Both rat and cow MLIPs separate from PIM by more than 20 min. The recovery of MLIP in these peaks is high, between 70 and 98% of the MLIP applied to the column. In Figure 3, the results of HPLC chromatographic separation of the pig brain peak I pool from Figure 1 is shown. Utilizing the TEAP system, on which rat brain MLIPs elute after PIM (O'Donohue et al., 1981), pig brain peak I MLIPs also elute after PIM.

Protease digestion studies of rat and cow peak I and II Sephadex pools. Peak I and II fractions from rat and bovine Sephadex chromatography were pooled and subjected to digestion with either trypsin (Table V) or pronase (Table VI), in comparison with PIM. As can be seen in Tables V and VI, PIM is very sensitive to treatment with either trypsin or pronase. The motilin immunoreactivity in the rat peak I pool was decreased by almost 80%, after 30 min of trypsin treatment. Rat peak II fractions, by comparison, were decreased by only 35% by trypsin. Motilin immunoreactivity in bovine peak I was decreased by about 65% by trypsin, whereas motilin immunoreactivity in bovine peak II was completely trypsin insensitive. Pronase treatment, in contrast, completely eliminated motilin immunoreactivity in rat peak I pools, although it did so more slowly than it eliminated PIM. Rat peak II fractions were also more completely eliminated by pronase than by trypsin, as were bovine peak I pools, although neither was completely eliminated. Bovine peak II is also pronase insensitive.

In Figure 4, the displacement of ^{125}I-labeled motilin from antiserum N1-8 by increasing concentrations of PIM is compared to the displacement caused by increasing volumes of pig brain extracts. The data are displayed on a log/log plot which makes visual comparison easier. The two lines are clearly not parallel, which suggests that pig brain MLIP differs from PIM.

Discussion

MLIPs, as detected by RIA, were widely distributed in the brains of all species examined. Rat brain consistently had the highest concentration of MLIPs of any of the species examined, whereas pig brain had the lowest. MLIPs were also abundant throughout the gastrointestinal tract of the rat and, in addition, were detectable in adrenal gland, liver, and kidney but very low or undetectable in heart or skeletal muscle (data not shown). In comparing the MLIP concentration in specific brain regions among different species, certain

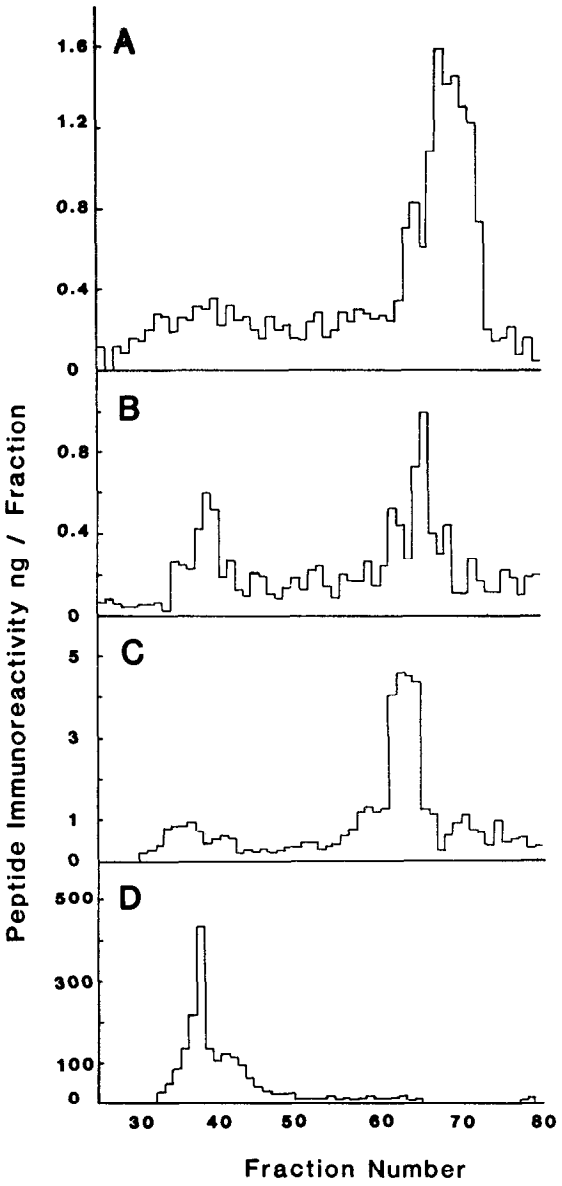

Figure 1. Sephadex-G25-fine (5 × 100 cm) chromatography of MLIP peptides in pig (*A*), rat (*B*), and cow (*C*) brain extracts as determined by motilin RIA with antiserum N1–8. The elution of VIP-like immunoreactivity from chromatogram *A* is shown in *D* for comparison.

areas were consistently among the highest (hypothalamus, cerebral cortex, and septum), whereas other areas were consistently lower (thalamus, pons, spinal cord, and medulla). Other areas like the cerebellum, olfactory bulbs, hippocampus, and midbrain were among the area with either the highest or the lowest MLIP concentration.

Although MLIPs are widely distributed in brain, their actual concentration in any area is substantially lower than that of other neuropeptides like cholecystokinin (Beinfeld et al., 1981) and VIP (Eiden et al., 1982).

Motilin antiserum N1–8 consistently detects more MLIPs than does antiserum 9 or GP, and it frequently detects more than does antiserum 13. The inability of antisera GP and 9 (both able to detect 1 to 5 pg of PIM/assay tube) to detect MLIPs in most of the porcine brain regions examined is further evidence that porcine brain MLIP is not the same as PIM.

The MLIPs in brain extracts of the species examined, when characterized by Sephadex chromatography, contain two broad peaks of motilin immunoreactivity. In these acid/acetone extracts, little high molecular weight motilin (which would have eluted in fractions 20 to 30) was observed, as was previously reported for rat brain (O'Donohue et al., 1981) and human intestine and plasma (Christofides et al., 1981). This high molecular weight MLIP may not be efficiently extracted with the acid/acetone method, or it may not be resolved on Sephadex-G25 in 1 M acetic acid. That no high molecular weight MLIP was observed in rhesus monkey cerebral cortex and cerebellum samples (Beinfeld and Bailey, 1985) prepared as by O'Donohue et al. (1981) (with a combination aqueous and acid extract), but chromatographed in 1 M acetic acid instead of 60 mM phosphate buffer, pH 7.4, containing 2% human serum albumin (as in O'Donohue et al., 1981; Christofides et al., 1981), suggests that the high molecular peaks observed in rat brain extracts by O'Donohue et al. (1981) could have been aggregates of MLIP or MLIP bound to proteins in the extract.

The peak of MLIPs similar in size to PIM in both rat and cow brain extracts (peak I) is either entirely or largely protease sensitive, indicating that it is mainly peptidic in nature. Peak II fractions were less protease sensitive than peak I fractions. Based on their molecular weight and their consistent elution on HPLC in the void volume, even in the absence of any acetonitrile in the mobile phase (data not shown), it is likely that only a portion of the motilin immunoreactivity in peak II is peptidic, the bulk being salt or other low molecular weight compounds interfering with the motilin assay. When peak I fractions from rat, cow, and pig brain Sephadex runs were subjected to HPLC, the motilin immunoreactivity never eluted in the same position as PIM. Depending upon what chromatographic system was utilized, peak I MLIP emerges either before or after PIM. In all instances peak I MLIP emerges as a single major peak, but it is accompanied by multiple minor peaks.

One control was performed to eliminate the possibility that our extraction method was altering brain motilin peptides and causing them to elute at a different retention time than that for PIM. To a piece of frozen porcine cerebal cortex was added a small amount of PIM, prior to extraction, drying, and HPLC. The HPLC elution of this added PIM was not altered by the tissue handling, and the recovery of added motilin immunoreactivity in the peak was high (80 to 100%).

The ability of extracts from pig brain to inhibit the motilin RIA in comparison with PIM was examined, and one such experiment is shown in Figure 4. The inhibition caused by the pig brain extract is not parallel to that of PIM. To test whether pig brain extracts might contain substances which interfered with motilin's ability to inhibit antisera binding, pig brain extracts were spiked with PIM before and after boiling, and the parallelism was examined in comparison with PIM and with aliquots of PIM boiled without pig brain extracts. All of these samples to which PIM had been added gave identical parallel inhibition curves, indicating that a lack of parallelism was probably caused by chemical nonidentity between pig brain MLIP and PIM (data not shown).

In summary, MLIP appears to be widely distributed in the brains of all of the vertebrate species examined. In addition, MLIPs are even more abundant in rat and guinea pig pituitary, rat pineal gland and retina than in brain. Based on the immunologic and chromatographic evidence presented, even in pig brain, the species from which PIM was isolated, brain MLIPs appear to differ chemically from PIM. Whether this difference is a minor amino acid substitution in PIM or a post-translational modification like sulfation, acetylation, or cleavage, or whether MLIPs are not closely related chemically but share some immunological determinants with PIM remains to be determined.

The recent findings that brain MLIPs are present very early in development (Beinfeld et al., 1983; Korchak et al., 1984) and the discovery that rat brain MLIPs are located in the cell nucleus and

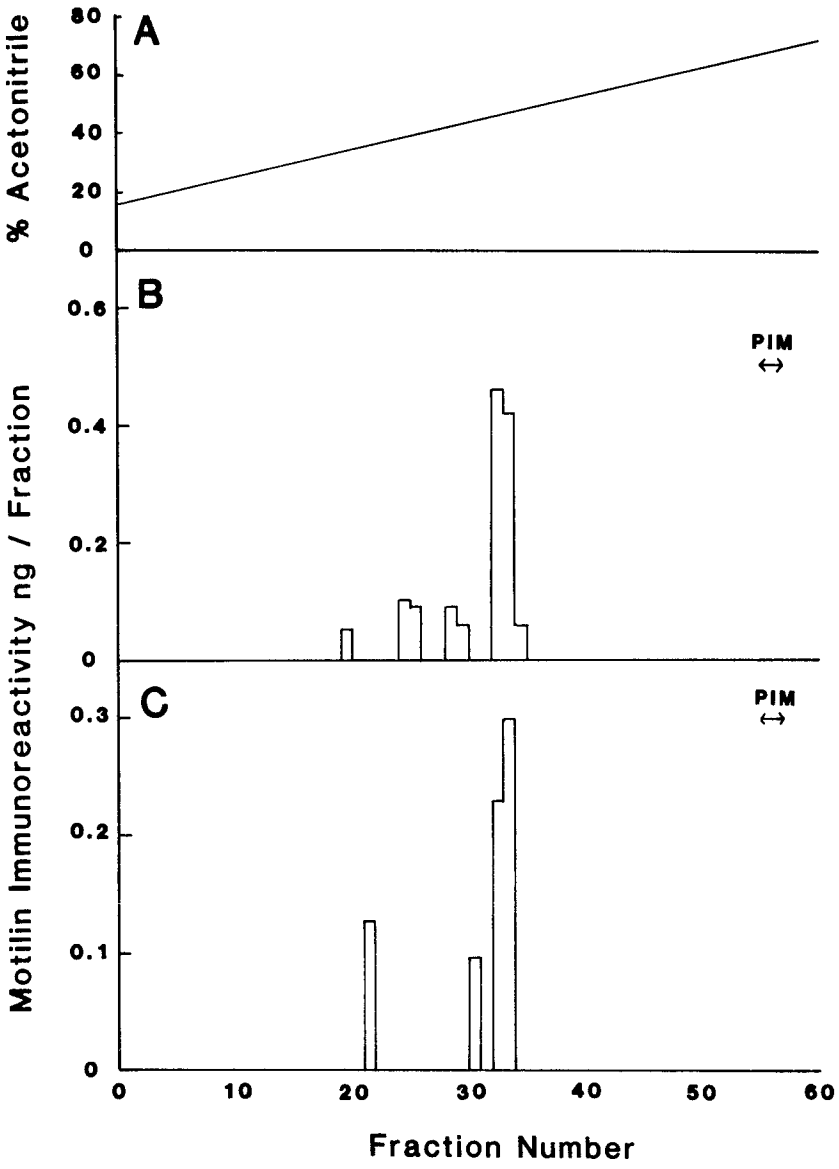

Figure 2. HPLC separation of motilin-like immunoreactivity in bovine (*B*) and rat (*C*) peak I fractions in comparison with PIM detected optically at 210 nm and with the motilin RIA using antiserum N1–8. In *A* the gradient of acetonitrile used to elute the peptides is shown. The acetonitrile was mixed with 0.09% TFA. The HPLC column was an Alltech C18, 2.5 × 350 mm. The flow rate was 1 ml/min and 1-ml fractions were collected.

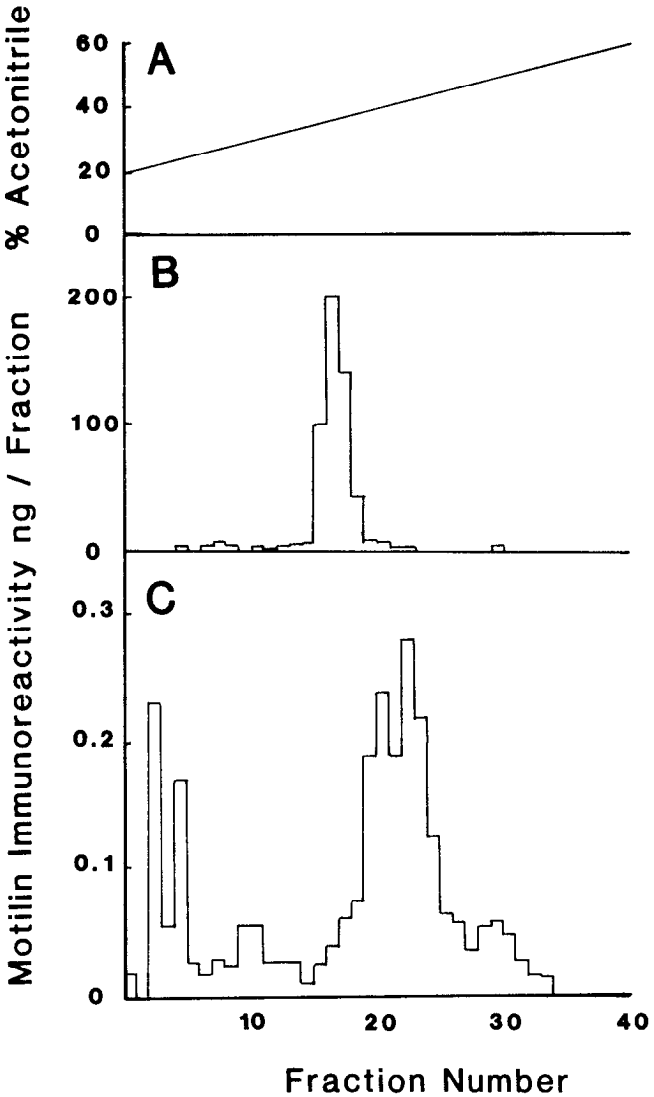

Figure 3. HPLC separation of pig brain MLIP (*C*) in comparison with PIM (*B*). The gradient used to elute is shown in *A*. The HPLC column, flow speed, and fraction size were the same as in Figure 2, but the buffer was TEAP, pH 3.25. The elution of MLIP was detected with antiserum N1-8.

TABLE V

Effect of trypsin on PIM and rat and bovine brain peak I and II motilin immunoreactivity[a]

	Percentage of Initial Motilin Immunoreactivity Remaining[b]			
	1 min	5 min	10 min	30 min
PIM	0	0	0	8.0
Rat peak I	ND[c]	ND	42.9	23.4
Rat peak II	ND	ND	65.2	67.9
Bovine peak I	ND	ND	45.6	34.1
Bovine peak II	ND	ND	99.6	227.8

[a] See "Materials and Methods" for experimental details.
[b] Motilin immunoreactivity was determined with motilin antiserum N1-8.
[c] ND, not determined.

not in vesicles (Korchak et al., 1985) raises some new questions about the possible functional role of MLIPs. The elucidation of the sequence of brain MLIP (which is currently in progress) would be the first step in determining whether MLIP does function as a DNA-regulatory protein and whether this function is related to the development of the brain.

TABLE VI

Effect of pronase on PIM and rat and bovine brain peak I and II motilin immunoreactivity[a]

	Percentage of Initial Motilin Immunoreactivity[b]				
	After pronase				With no pronase added (120 min)
	10 min	30 min	60 min	120 min	
PIM	0.8	0	ND[c]	0	118.5
Rat I	ND	81.0	0	0	ND
Rat II	ND	99.4	ND	35	83.4
Bovine I	ND	27.4	53.7	27.4	105
Bovine II	ND	132.5	97.2	125.3	134.9

[a] See "Materials and Methods" for experimental details.
[b] Motilin immunoreactivity was determined with motilin antiserum N1-8.
[c] ND, not determined.

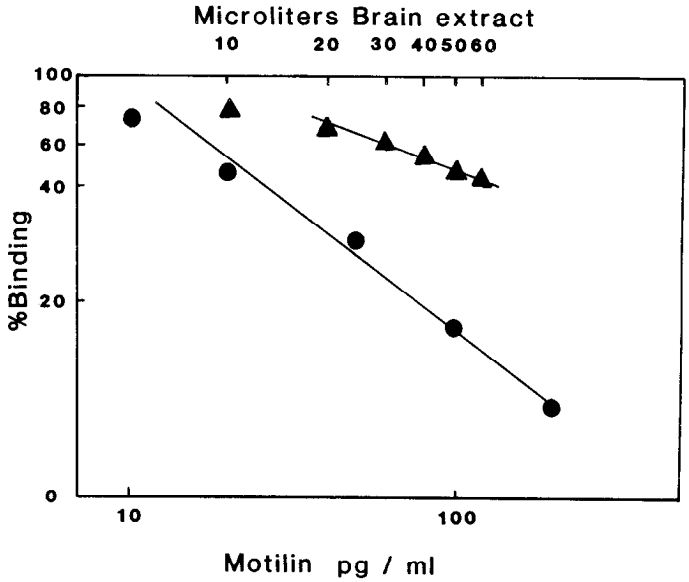

Figure 4. Comparison of the displacement of antiserum N1-8 with PIM (●) with increasing aliquots of pig brain extract (▲). The data are plotted on a log/log scale to linearize the data for easier comparison.

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