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There Are Three Distinct Forms of Bombesin

IDENTIFICATION OF [Leu^{13}]BOMBESIN, [Phe^{13}]BOMBESIN, AND [Ser^{3},Arg^{10},Phe^{13}]BOMBESIN IN THE FROG *BOMBINA ORIENTALIS**

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Srinivasa R. Nagalla‡, Brenda J. Barry‡, Arnold M. Falick§, Bradford W. Gibson¶, John E. Taylor‖, Jesse Z. Dong‖, and Eliot R. Spindel‡**

From the ‡Division of Neuroscience, Oregon Regional Primate Research Center, Beaverton, Oregon 97006, §PerSeptive Biosystems, South San Francisco, California 94080, the ¶Department of Pharmaceutical Chemistry, School of Pharmacy, University of California, San Francisco, California 94143, and ‖Biomeasure Inc., Milford, Massachusetts 01757

Amphibian bombesin is the prototypic peptide that defines the bombesin-like peptide family. In this paper we show that in the frog *Bombina orientalis*, there are actually 3 distinct forms of bombesin, and each of these peptides is an agonist with differing affinities for the known bombesin receptors. Oligonucleotides complementary to the 5′- and 3′-untranslated regions of the bombesin mRNA were used to amplify bombesin-related cDNAs from the skin, brain, and gut of *B. orientalis*. Three classes of cDNAs were found. One class encoded the previously characterized form of bombesin which has a Leu at position 13 ([Leu^{13}]bombesin). The other two classes, respectively, encoded new bombesin-like peptides which we have designated as [Phe^{13}]bombesin and [Ser^{3},Arg^{10},Phe^{13}]bombesin ([SAP]bombesin). The existence of [SAP]bombesin in skin was confirmed by tandem mass spectrometry. Polymerase chain reaction analysis of genomic DNA showed the mRNAs for [Leu^{13}]bombesin, [Phe^{13}]bombesin, and [SAP]bombesin most likely arise from separate genes. Polymerase chain reaction analysis showed different patterns of tissue-specific expression for each form. [Leu^{13}]Bombesin and [SAP]bombesin were predominantly expressed in skin, brain, and gut; [Phe^{13}]bombesin was expressed only in brain, and [Leu^{13}]bombesin predominated in oocytes. [SAP]Bombesin contained a cleavage site between residues 4 and 5, which if used would yield the peptide [SAP]bombesin(5–14) which has the sequence [Gln^{3},Arg^{6}]neuromedin B. Thus a frog homolog of NMB could derive from the [SAP]bombesin prohormone. [Phe^{13}]Bombesin, [SAP]bombesin, and [SAP]bombesin(5–14) were synthesized and their affinities for the mammalian bombesin-like peptide (GRP and NMB) receptors determined. These peptides acted as agonists for the GRP and NMB receptors, with relative potencies for the GRP receptor of [Leu^{13}]bombesin > [Phe^{13}]bombesin > [SAP]bombesin(5–14) > [SAP]bombesin and for the NMB receptor of [Phe^{13}]bombesin > [SAP]bombesin(5–14) > [Leu^{13}]bombesin > [SAP]bombesin. None of these peptides demonstrated high affinity binding for the BRS-3 receptor. The different receptor affinities and tissue distribution of these peptides suggests distinct physiologic roles and raises the possibility of as yet uncharacterized mammalian homologs of these new amphibian peptides.

Bombesin, a tetradecapeptide, was isolated from the skin of the frog *Bombina bombina* by Anastasi *et al.* (1) in 1971 (Fig. 1). The bombesin-related peptides ranatensin and [Leu^{8}]phyllolitorin were subsequently isolated from the skin of *Rana* and *Phyllomedusa* species by Nakajima *et al.* (2) and by Yasuhara *et al.* (3) (Fig. 1). Amphibian bombesin was found to have multiple effects in mammals (4, 5) and bombesin-like immunoreactivity was observed in mammalian brain, GI tract, and lung (6–8). In 1979, using gastrin release as a bioassay McDonald and co-workers (9) isolated from porcine stomach a 27-amino acid peptide homologous to the carboxyl terminus of bombesin and named it gastrin-releasing peptide (GRP)[1] (Fig. 1). GRP is widely distributed in mammals; acts as a neurotransmitter in brain, a paracrine hormone in GI tract, and a growth factor in developing lung (10–12). Neuromedin B (NMB) is a second mammalian bombesin-like peptide (Fig. 1). NMB was originally isolated from porcine spinal cord (13) and like GRP is widely distributed in brain and GI tract. Frogs have both GRP and bombesin (14) indicating they are distinct peptides. In frogs GRP predominates in gut, bombesin in skin and brain (14). Phyllogenetic analysis of the prohormones for GRP (14–16), NMB (17, 18), ranatensin (17), bombesin (19), and phyllolitorin (20) shows the bombesin-like peptides can be divided into three distinct branches. The peptides originally found in skin, bombesin, ranatensin, and phyllolitorin form one branch which we have designated as the bombesin-related peptide (BRP) branch; GRP and NMB form the other two branches (20). To date the BRP family has only been characterized in amphibians, although given that the three branches appear to have diverged prior to the vertebrate radiation, mammalian homologs of the BRP peptides may exist (20).

Four subtypes of receptors for bombesin-like peptides have been characterized to date. A GRP-preferring subtype was cloned by Spindel *et al.* (21) and by Battey *et al.* (22); a neuro-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) U49450 and U49451.

** To whom correspondence and reprint requests should be sent: Div. of Neuroscience, Oregon Regional Primate Research Center, 505 NW 185th Ave., Beaverton, OR 97006. Tel.: 503-690-5512; Fax: 503-690-5384; E-mail: spindele@ohsu.

[1] The abbreviations used are: GRP, gastrin-releasing peptide; NMB, neuromedin B; BRP, bombesin-related peptide; RT-PCR, reverse transcription-polymerase chain reaction; [SAP]bombesin, [Ser^{3},Arg^{9},Phe^{13}]bombesin; MH^{+}, protonated molecular ion; PSD, post source decay; MALDI, matrix-assisted laser desorption ionization; TOF, time-of-flight; CID, collision-induced dissociation.

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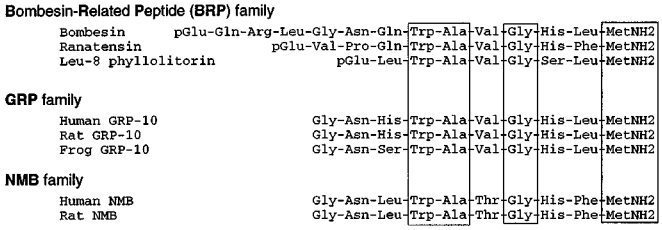

FIG. 1. **The three subfamilies of bombesin-like peptides.** Representative members of each subfamily are shown. Bombesin, ranatensin, and phyllolitorin are amphibian, GRP and NMB are from the species shown. GRP-10 is the COOH-terminal decapeptide of GRP and contains full biological activity of GRP.

medin B receptor was cloned by Wada *et al.* (23); and a third subtype whose ligand is still unknown, designated BRS-3, was cloned by Fathi *et al.* (24) and by Gorbulev *et al.* (25). Our laboratory recently characterized a fourth class of bombesin receptors (designated BB4) which appear to constitute the receptors for the bombesin-related peptides as they have higher affinity for bombesin than GRP (26). A second nomenclature system for the bombesin receptors is also in use. In this system, the NMB receptor is known as the BB1 receptor, the GRP receptor is the BB2 receptor, and the BRS-3 receptor is the BB3 receptor (27, 28).

While bombesin-related peptides have been described in multiple species of frogs, it has previously been believed that a given species has one primary BRP, *e.g.* bombesin in *Bombina orientalis*, ranatensin in *Rana pipiens*, and litorin in *Litoria aurea* (29), and that these peptides will have either a Leu or a Phe as the penultimate residue. An exception to this rule was *Phyllomedusa sauvagei* which expresses both [Leu^8]-phyllolitorin and [Phe^8]phyllolitorin. In cloning the cDNAs encoding [Leu^8]phyllolitorin and [Phe^8]phyllolitorin we observed that the cDNAs differed by only a single nucleotide. This suggested that other species of frogs might also express multiple BRP-encoding cDNAs that may previously have not been detected because of their high homology. To examine this, reverse transcription-PCR (RT-PCR) was used to amplify bombesin-related cDNAs in *B. orientalis*. Sequence analysis then revealed three different bombesin-related cDNAs, each encoding different subtypes of bombesin. The bombesin subtypes have different distributions and receptor affinities which suggests different physiologic roles for these multiple peptide forms.

MATERIALS AND METHODS

Animals and Reagents—Frogs (*B. orientalis*) were obtained from California Zoological Supply (Santa Ana, CA). Enzymes were purchased from New England Biolabs (Beverly, MA), Stratagene (La Jolla, CA) or Promega (Madison, WI). Isotopes were purchased from DuPont NEN.

RT-PCR—Total RNA was prepared by homogenization of frog tissues in guanidine thiocyanate followed by centrifugation through CsCl (30). 5 μg of total RNA was reverse transcribed with 25 pmol of oligo(dT^{18}), 200 units of Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories), 5 × buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM $MgCl_2$, 50 mM dithiothreitol, 2.5 mM dNTPs) in a 20-μl total volume at 37 °C for 1 h. The entire reverse transcription was used in a 100-μl PCR reaction using 100 pmol of the 5′ primer and 3′ primers (shown in Fig. 2) selected to amplify the entire coding region of the *B. orientalis* bombesin mRNA as described previously by Spindel *et al.* (19). PCR conditions were one cycle of 92 °C × 2 min, 50 °C × 2 min, 72 °C × 5 min for second strand synthesis followed by 35 cycles of 92 °C × 1 min, 55 °C × 1 min, 72 °C × 2 min, using 2.5 units of *Taq* polymerase (Promega). 20 μl of the reaction was separated on a 1% agarose gel, blotted, and hybridized to an internal ^{32}P-end labeled oligonucleotide probe. Hybridizing products were subcloned into PGEM-T vector (Promega) and sequenced as described previously (14).

In order to differentiate between related bombesin cDNAs, RNA was reverse transcribed as above, then amplified with primers common to all cDNAs. 10 μl of the reaction was then electrophoresed in triplicate on 1% agarose, blotted, and hybridized to 20- or 21-base oligonucleotides specific for each cDNA. Hybridization conditions were as described by Sommer *et al.* (31) using 25% formamide, 5 × SSC, 40 °C; washing was in 0.5 × SSC, 55 °C. Specificity of hybridization was checked by including samples of each cDNA on each gel. Sequence of primers used is shown in Fig. 2.

Mass Spectrometry—Dorsal skin from *B. orientalis* was homogenized in 4% trifluoroacetic acid, then lyophilized. The lyophilized crude skin secretion was dissolved in 5 ml of 0.1% trifluoroacetic acid and loaded onto two C-18 Sep-Pak Plus cartridges (Waters, MA). The peptide fraction was eluted with 1.4 ml of 70% CH_3CN containing 0.08% trifluoroacetic acid after the cartridges were washed with 2 ml of 0.1% trifluoroacetic acid. The peptide mixture was dried on a Savant Speed Vac and redissolved in aqueous 0.1% trifluoroacetic acid for subsequent HPLC separation. HPLC separation was carried out on a Rainin gradient HPLC system using a Vydac C-18 analytical column (25 cm × 4.4 mm, inner diameter). Peptides were eluted with a linear gradient of 0.1% trifluoroacetic acid/H_2O to 70% CH_3CN/0.08% trifluoroacetic acid in 100 min.

Individual HPLC fractions (average volume 0.4 ml) were analyzed by matrix-assisted laser desorption ionization (MALDI) mass spectrometry (32), using a Voyager MALDI time-of-flight (TOF) mass spectrometer (PerSeptive Biosystems, Framingham, MA). For these experiments, a 1-μl aliquot of each HPLC fraction was mixed with 0.5 μl of a matrix solution, placed on 1 well of a 100-well stainless steel sample plate and allowed to dry at room temperature. The matrix solution was a 10 mg/ml solution of α-cyano-4-hydroxycinnamic acid in CH_3CN/ethanol (1/1). Samples were irradiated with a pulsed 337-nm N_2 laser and 20–50 scans were summed. The instrument was calibrated using phosphokemptide and oxidized insulin β chain as external standards, and typical mass measurement accuracy was better than 0.1%.

Fraction 22, which contained the molecular ion of interest (m/z 1643, average MH^+), was further analyzed by post source decay (PSD) on a Voyager RP MALDI-TOF instrument with a reflector (PerSeptive Biosystems, Framingham, MA). Under PSD conditions (33), one observes fragmentation of protonated peptides that are very similar to those described for high and low energy collision-induced dissociation (CID) (34). In addition, this same fraction was analyzed by liquid secondary ion mass spectrometry under CID conditions to obtain additional sequence information. To carry out this latter experiment a small aliquot of the HPLC fraction (20 μl) was dried down onto a stainless steel probe tip and dissolved in a mixture of glycerol/thioglycerol (2/1). The samples were then acidified with 0.1 μl of 1% trifluoroacetic acid and inserted into the source of a Kratos Concept IIHH mass spectrometer as described in detail elsewhere (35).

Xenopus Oocyte Expression—Functional expression of bombesin receptors in oocytes was performed as described previously (36, 37). In brief, *Xenopus laevis* oocytes were co-injected with 5 ng of *in vitro* transcribed receptor RNA and the calcium photo-protein aequorin then incubated for 18 h in nutritive medium (21). Single oocytes were placed in a luminometer (Berthold LB253) and challenged with various ligands at a concentration from 10^{-6} M to 10^{-10} M. Ligand-induced increases in intracellular calcium cause a proportional increase in light emission by aequorin which is recorded by the luminometer. The human GRP receptor cDNA was as described previously (37), the human NMB and BRS-3 receptor expression vectors were generously provided by Jim Battey (24, 38).

Receptor Binding Assay—Methods were as described previously (39, 40). In brief, membranes for the GRP receptor binding assay were obtained from rat AR42J pancreas cells, membranes for NMB receptor were obtained from rat olfactory bulb. Tissues were homogenized in 50 mM Tris, pH 7.4, centrifuged twice at 39,000 × *g*, then resuspended in Tris + 0.1% bovine serum albumin and 0.1 mg/ml bacitracin. For GRP receptor binding, aliquots (0.4 ml) were incubated with 50 μl of [^{125}I-Tyr^4]bombesin (New England Nuclear) (0.05 nM), for NMB receptor binding with [^{125}I-Tyr^4]bombesin. After a 30-min incubation (4 °C), bound peptide was separated from free by rapid filtration through GF/B filters which had been previously soaked in 0.3% polyethyleneimine. Specific binding was defined as the total ^{125}I-peptide bound minus that bound in the presence of 1 μM unlabeled peptide.

RESULTS

RT-PCR of RNA from skin, brain, and stomach of *B. orientalis* with primers spanning the coding region of the bombesin cDNA resulted in bands clearly visible by staining with ethidium bromide. These bands were subcloned and multiple clones from skin, brain, and stomach were sequenced. As

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shown in Fig. 2, three classes of cDNAs were obtained. These cDNAs were highly homologous, but the minor nucleotide differences resulted in new forms of bombesin. One class of cDNAs was the cDNA for [Leu^{13}]bombesin as described previously by our laboratory (19). The second class of cDNAs contained a G to C change in the codon at the Leu^{13} position of bombesin, such that the cDNA encoded the new bombesin-like peptide, [Phe^{13}]bombesin. The third class contained three nucleotide changes in the bombesin coding region to produce the new peptide, [Ser^{3},Arg^{10},Phe^{13}(SAP)]bombesin. The sequences of the three forms of bombesin are shown in Fig. 2*B*. Interestingly, the Ser^{3} substitution in [SAP]bombesin creates another copy of the Ser-Leu motif that signals cleavage of bombesin from its precursor. If this cleavage site is utilized, then the peptide [Gln^{3}-Arg^{6}]neuromedin B which resembles mammalian neuromedin B could be produced from the [SAP]bombesin

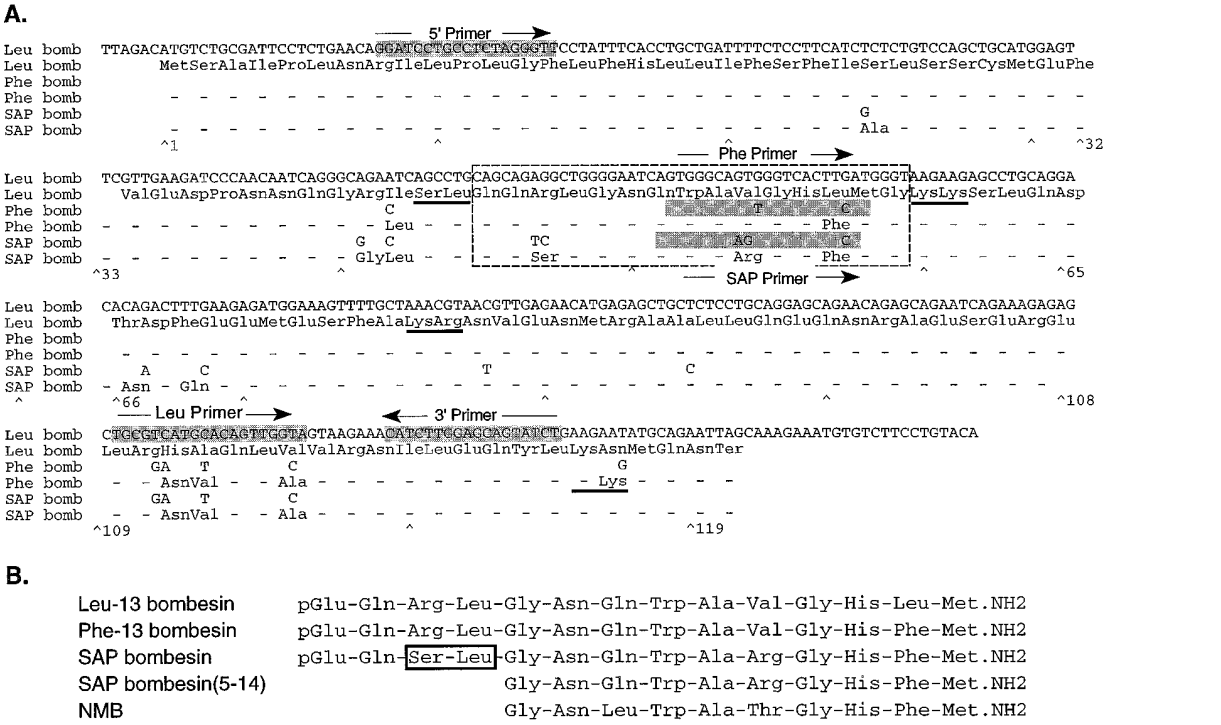

FIG. 2. *A*, nucleotide and amino acid sequence of the 3 bombesin mRNAs. Nucleotide sequence derived from sequencing at least 4 independent clones of each form. Coding region for bombesin, [Phe^{13}]bombesin, and [Leu^{13}]bombesin is *boxed*. The proteolytic cleavage sites for the bombesin-like peptides are *underlined*. The location of the common 5′ and 3′ primers used to amplify the 3 cDNAs for the distribution studies are as shown. The location of the [Phe], [Leu], and [SAP]bombesin specific primers for differential hybridization are as shown. *B*, the sequences of the three forms of bombesin are as shown. [SAP]Bombesin(5–14) would be produced if cleavage of [SAP]bombesin occurred at the internal Ser-Leu motif that is used for cleavage of bombesin.

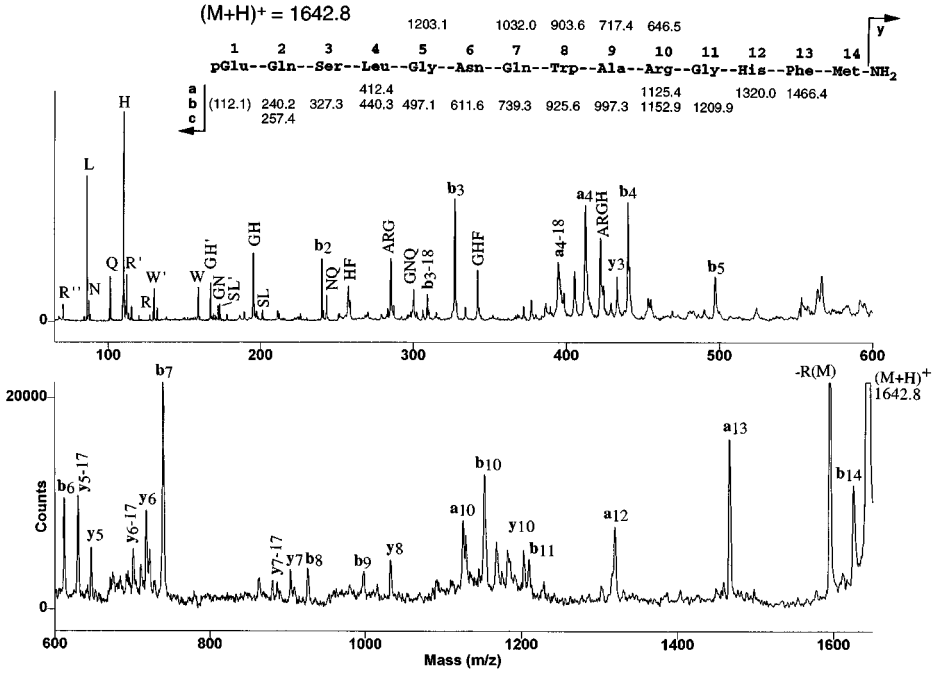

FIG. 3. **Post source decay MALDI-TOF spectrum of the selected *m/z* 1642.8 (average) ion with sequence ions labeled according to the accepted nomenclature for peptide fragmentation (43).** Additional ions were also observed that are consistent with double cleavage reactions forming a series of internal peptide ions. These latter ions are labeled according to single letter amino acid codes; *e.g.* GH is Gly-His at *m*/*z* 195, where the prime refers to the additional loss of 28 Da (CO).

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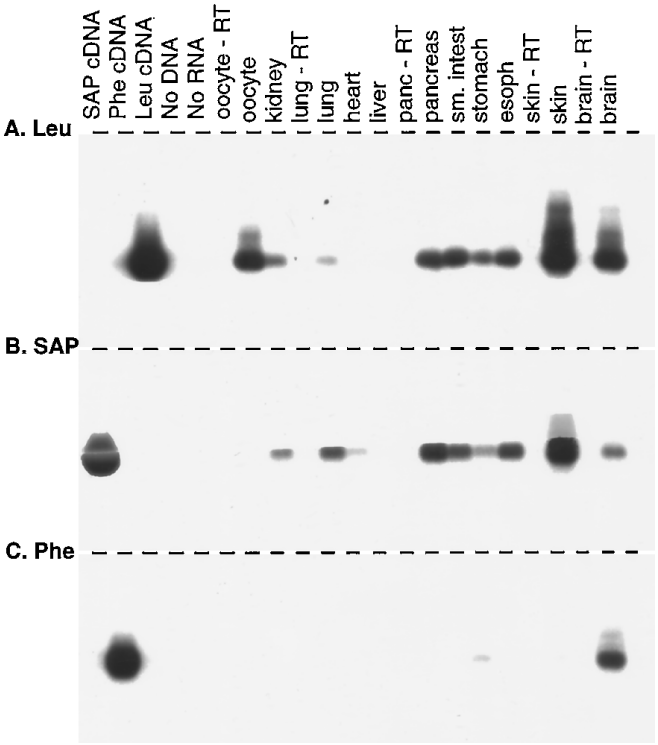

FIG. 4. **RT-PCR analysis of the expression of the [Leu^{13} bombesin (*Leu*), [SAP]bombesin (*SAP*), and [Phe^{13}]bombesin (*Phe*) mRNAs in the frog tissues shown.** Tissues were reverse transcribed with the 5′ and 3′ primers shown in Fig. 2*A*, then hybridized with the specific primers shown in Fig. 2*A*. Controls were reverse-transcription reactions lacking reverse transcriptase (−*RT*) from skin, brain, pancreas (*panc*), lung, and oocytes. Controls also included reverse transcription reaction with no input RNA (*No RNA*) and PCR amplification with no DNA (*No DNA*). Positive controls to show specificity were cDNAs amplified from the [Leu^{13}]bombesin cDNA, [Phe^{13}]bombesin cDNA, and [SAP]bombesin cDNA.

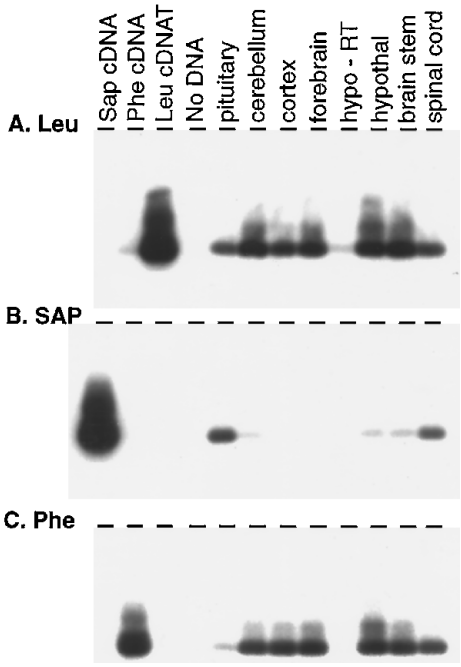

FIG. 5. **RT-PCR analysis of dissected brain regions as shown.** *Hypo*, hypothal = hypothalamus. Primers and controls as for figure 3.

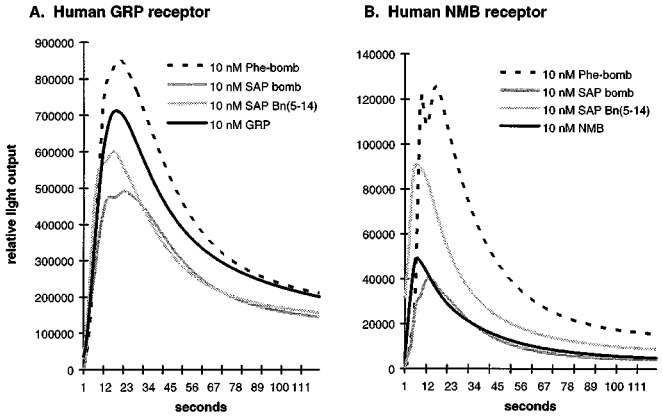

FIG. 6. **Luminometric assay of the response of *Xenopus* oocytes expressing the human GRP and NMB receptors to [Phe^{13}]bombesin, [SAP]bombesin, and [SAP]bombesin(5–14).** Oocytes were co-injected with 5 ng of human GRP or NMB receptor RNA transcribed from a cDNA encoding the receptor and the calcium photoprotein aequorin (21). After 24 h the eggs were challenged with 10 nM concentration of ligand and the light output was measured over 120 s in a luminometer. Results show averages of three independent injected oocytes for each compound and receptor. The overall lower responses seen in oocytes injected with the NMB receptor reflect a smaller amount of injected transcript. The relatively low response to NMB itself may reflect partial oxidation of the peptide.

precursor (Fig. 2*B*). To determine the representation of the three cDNA forms in genomic DNA, the same primers were used to amplify genomic DNA prepared from *B. orientalis* liver. Sequence analysis of the resulting product showed roughly equal representation of the three forms suggesting the existence of multiple genes.

Given that sequence analysis of multiple clones amplified from both genomic DNA and reverse-transcribed cDNA showed the identical 3 classes of cDNAs, the possibility of PCR artifact is very unlikely. To definitively prove this, advantage of the high levels of peptide present in skin was taken and mass spectrometry was performed to show the existence of [SAP]-bombesin in skin. HPLC separation of the *B. orientalis* skin extract was performed as previously reported (41). Mass spectrometric profiling of the individual HPLC fractions allowed for the identification of a fraction eluting at about 25% CH_3CN (Fraction 22). The MALDI mass spectrum of this fraction contained a peak at *m/z* 1643, that was close to the expected value for [SAP]bombesin, *i.e.* $MH^+_{(avg)}$ at *m/z* 1642.8 as well as peaks 16 and 32 Da higher at *m/z* 1659 and 1675. Subsequent analysis of this same fraction at a higher resolving power ($M/\Delta M \approx 2,500$) using the Voyager RP TOF mass spectrometer resulted in the mass resolution of the isotopic pattern of this molecular ion and the assignment of the ^{12}C-containing monoisotopic mass at *m/z* 1641.8, agreeing exactly with the expected value of *m/z* 1641.8. The two species at slightly higher mass are likely oxidized versions of this same peptide containing methionine sulfoxide (+16 Da, O) and sulfone (+32 Da, O_2), as their relative abundance increased during storage (data not shown).

Sequence analysis by PSD and high energy CID of the (non-oxidized) peptide yielded information supported the identity of this peptide as the [SAP]bombesin analog. The PSD spectrum shown in Fig. 3 contains a number of low mass immonium ions, $^+NH_2$ = CHR (42) and/or related ions for the expected amino acids Trp (*m/z* 159 and 130), Arg (*m/z* 112, 70), His (*m/z* 110), Gln (*m/z* 101), and Leu (*m/z* 86). In addition, a strong **b**-ion series dominates the rest of the PSD spectrum, resulting from cleavage at the amide nitrogens with charge retention at the NH_2 terminus (43). These ions, along with some **a**- and **y**-ions, defined 13 of the 14 amino acids of the [SAP]bombesin sequence (see Fig. 3, *inset*). The high energy CID spectrum (not shown) added additional support for the composition, containing distinctive immonium ions for all amino acids except for Gly. In addition, a partial sequence of (pGlu,Gln)-Ser-Leu-Gly-

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Asn-Gln-Trp-(Ala,Arg)-Gly-His-Phe-Met-amide could be determined based on abundant **a**, **b**, and **c**-type ions. Taken together, the PSD and high energy CID data provided sufficient sequence data to confirm the identity of this peptide as that for [SAP]bombesin as predicted from the cDNA. This also confirms that processing at the atypical Ser-Leu site takes place as predicted to yield [SAP]bombesin.

The relative distribution of each bombesin subtype was examined by RT-PCR. Common primers were used to amplify all three bombesin cDNAs, then primers specific for each form were used for specific hybridization. As shown in Fig. 4, [Leu^{13}]bombesin was present in most tissues (not heart or liver), with highest levels in skin and brain. [Leu^{13}]Bombesin was the predominant form of bombesin present in oocytes. [SAP]Bombesin was also widely distributed, with highest levels in brain. Interestingly, [SAP]bombesin was also present in lung. [Phe^{13}]Bombesin was present only in skin. The distribution in brain was further examined in dissected brain regions (Fig. 5). [Leu^{13}]Bombesin was present in all regions examined. [Phe^{13}]Bombesin was also present throughout brain, although little was present in pituitary. [SAP]Bombesin had a more limited distribution, with higher levels in spinal cord and pituitary, and lower levels in other brain regions. The tissue-specific patterns of distribution suggests different physiologic functions for each peptide. This is further supported by the different affinities of the peptides for the known bombesin receptors.

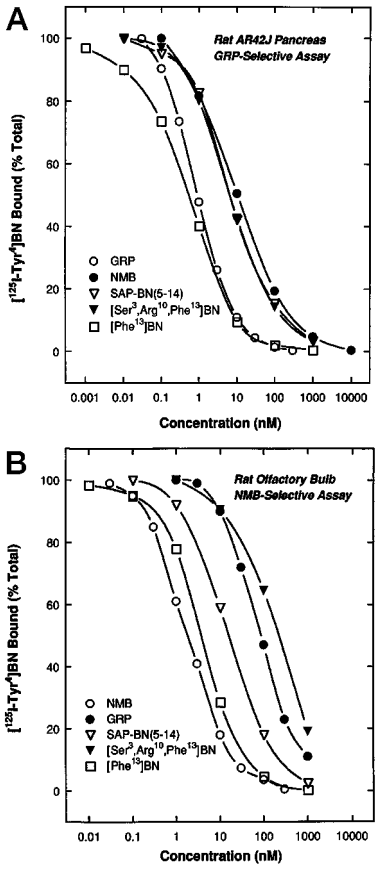

FIG. 7. **Displacement curves for GRP receptor selective assay (*A*) and NMB selective assay (*B*) for the peptides shown.** Data shown is representative of 3–4 independent determinations.

The ability of these new bombesin-like peptides to act as agonists at the known bombesin receptors was tested in *Xenopus* oocytes expressing the GRP, NMB, and BRS-3 receptors. As shown in Fig. 6, the new bombesin-like peptides, [Phe^{13}]bombesin, [SAP]bombesin, and [SAP]bombesin(5–14) were potent agonists for the GRP and NMB receptors. [Phe^{13}]Bombesin is a particularly potent agonist both for the GRP and NMB receptor. None of these peptides showed agonist activity for the BRS-3 receptor (data not shown). While expression in oocytes provides an index of agonist potency, it is not quantitative. Thus K_i values to displace binding of ^{125}I-labeled bombesin to GRP and NMB receptors in AR42J and rat olfactory membranes, respectively, was measured (Fig. 7, Table I). GRP receptor binding was measured by displacement of ^{125}I-bombesin (concentration = 0.05 nM) from membranes prepared from AR42J cells and NMB receptor binding was measured by displacement of ^{125}I-bombesin from membranes prepared from rat olfactory bulb. Nonspecific binding was consistently less than 5%. In both tissues, [Phe^{13}]bombesin showed highest affinity, and [SAP]bombesin relatively lower affinity. Thus these new peptides are clearly agonists at the known bombesin receptors, but have differing affinities.

DISCUSSION

The complexity of the bombesin-like peptide family is becoming apparent. Phylogenetic analysis of bombesin-like peptides identifies three subfamilies, the GRP family, the NMB family, and the BRP family (Fig. 1). The BRP family is composed of bombesin, ranatensin, and phyllolitorin, the bombesin-like peptides that occur in frog skin. Different species of frogs have different BRP's; *B. orientalis* have bombesin, *R. pipiens* have ranatensin, and *P. sauvagei* have phyllolitorin; but all have highly related prohormones. GRP and bombesin are clearly distinct peptides as frogs have both GRP and bombesin. Previous Northern blot analyses by our laboratory (14, 19) and Richter *et al.* (44, 45) has shown that in frogs, bombesin occurs at highest levels in skin, brain, and oocytes, while GRP occurs at highest levels in the GI tract (14).

Our observation that [Leu^{8}]phyllolitorin and [Phe^{8}]phyllolitorin are encoded by mRNAs differing by only a single nucleotide led us to determine if there might be multiple forms of bombesin also encoded by homologous mRNAs. As shown in Fig. 2, in *B. orientalis* there are 3 forms of bombesin encoded by 3 highly homologous mRNAs. The three forms of bombesin are [Leu^{13}]bombesin, [Phe^{13}]bombesin, and [Ser^{3},Arg^{9},Phe^{13}]-

TABLE I
Inhibition constants for bombesin-like peptides for the GRP and NMB receptors

The K_i in nanomolar of the following peptides to compete for binding to membranes prepared from tissues rich in GRP and NMB receptors. GRP receptor binding was measured by displacement of [^{125}I-Tyr^{4}]bombesin binding to membranes prepared from AR42J cells. NMB binding was measured by displacement of [^{126}I-Tyr^{4}]bombesin to membranes prepared from rat olfactory bulb.

Peptide	K_i (nM) ± S.E.	
	GRP-receptor (AR42J)	NMB-receptor (rat olfactory bulb)
Porcine GRP	1.55 ± 0.29	247 ± 14
[Leu^{13}]Bombesin	1.88 ± 0.23	16.7 ± 3.1
[Phe^{13}]Bombesin	0.63 ± 0.0.03	2.24 ± 0.75
[Ser^{3},Arg^{9},Phe^{13}]Bombesin	5.44 ± 1.36	148 ± 17
NMB	15.8 ± 2.8	1.71 ± 0.25
[SAP]Bombesin (5–14)	5.55 ± 1.43	9.55 ± 2.18

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bombesin. These different peptides are produced by a surprisingly limited number of nucleotide changes. It thus appears that most frogs will have multiple forms of bombesin or bombesin-related peptides. In *P. sauvagei*, phyllolitorin occurs in both a Phe^8 form and a Leu^8 form. In *X. laevis*, Wechselberger *et al.* (45) showed the existence of a Phe form of bombesin. As will be described elsewhere,[2] we have also identified both Phe and Leu forms bombesin in *Xenopus*. Thus in *B. orientalis*, *P. sauvagei*, and *X. laevis* there are multiple forms of bombesin. This conservation of multiple forms of bombesin suggests there will be conserved unique physiologic forms for these multiple peptides.

As would be expected given the high nucleotide homology between the cDNAs, the three bombesin prohormones are highly homologous. The [Phe^{13}]bombesin prohormone contains an additional dibasic cleavage site creating the potential for an additional processed peptide. Bombesin is cleaved from its precursor at its amino terminus following Ser-Leu residues (44). Ser-Leu also occurs within the sequence of [SAP]bombesin. If this second Ser-Leu motif is used for cleavage, then a peptide homologous to mammalian NMB, [SAP]bombesin(5–14) with the structure [Gln^3-Arg^6]neuromedin B would be produced. Whether this cleavage actually takes place remains to be determined.

The 3 forms of bombesin could arise by independent genes, alternate splicing, or RNA editing. While RNA editing is uncommon, because RNA editing is responsible for producing [Leu^8]phyllolitorin and [Phe^8]phyllolitorin (20), it is important to consider that possibility. Amplification of genomic DNA followed by sequence analysis, showed the presence of all 3 forms in genomic DNA at approximately equal proportions. This suggests that the three forms of bombesin arise from independent genes and not by alternate splicing or RNA editing. The high homology between the three forms suggests a relatively recent gene duplication or gene conversion event. This also shows that the genes for bombesin do not have introns within the coding region as do the genes for GRP and NMB (16, 17, 46). Alternate splicing of large exons which include the entire amplified region cannot be ruled out, although seems unlikely. The observation of the identical DNA sequence in the genomic clones and the clones generated by RT-PCR would tend to rule out the possibility of a PCR artifact generating the multiple forms. This is further supported by the different distributions discussed below and the confirmation of the presence of [SAP]bombesin by tandem mass spectrometry.

To further rule out the possibility that the multiple observed forms could be a PCR artifact and to prove translation and processing of the SAP prohormone, mass spectrometry was used to identify [SAP]bombesin in skin. In these experiments, the skin extract was first separated by HPLC and each fraction was analyzed by MALDI mass spectrometry to first identify the correct mass (M_r 1642) and then by PSD and high energy CID analysis to establish the amino acid sequence. In both cases, the mass assignments and MS fragmentation data were consistent with the expected [SAP]bombesin analog. The identification of the existence of [SAP]bombesin in skin also confirms that SAP is cleaved adjacent to the Ser-Leu residues at position 43,44 despite the fact that the Arg residue at position 41 which is conserved in a similar cleavage site in relaxin (47) is changed to a Gly in the SAP prohormone. This loss of the Arg residue may, however, decrease efficiency of processing, and could increase the presence of other forms of the [SAP]bombesin prohormone.

The distribution of the three forms of bombesin was examined by RT-PCR. As stated above, previous Northern blot analyses by our laboratory (14, 19) and Richter *et al.* (44, 45) indicate that in frogs, bombesin occurs at highest levels in skin, brain, and oocytes, while GRP occurs at highest levels in GI tract (14). [Leu^{13}]Bombesin and [SAP]bombesin were widely distributed. [Leu^{13}]Bombesin was present in all tissues examined except heart and liver. Notably, [Leu^{13}]bombesin was the predominant form of bombesin present in oocytes. As will be reported elsewhere,[2] a Leu form of bombesin also predominates in *Xenopus* oocytes. Both [SAP]bombesin and [Leu^{13}]bombesin were present in lung. This is significant given the findings of Aguayo *et al.* (48) and Sunday and co-workers (49, 50) that bombesin and phyllolitorin stimulate lung development in mice. Thus a [SAP]bombesin or [Leu^{13}]bombesin homolog could be present in rodent lung. [Phe^{13}]Bombesin was found only in brain. This is consistent with the observation that [Phe^8]-phyllolitorin is the predominant form of phyllolitorin in *P. sauvagei* brain (20) and a Phe form of bombesin was isolated from *Xenopus* brain (45). Thus in amphibian brain, a bombesin-related peptide with Phe in the penultimate COOH-terminal position appears to be frequently expressed.

The distribution within brain was examined in dissected brain region. [Phe^{13}]Bombesin and [Leu^{13}]bombesin were found in all brain regions. Interesting [SAP]bombesin was most abundant in pituitary and spinal cord. Relatively little [Phe^{13}]bombesin was present in pituitary. The expression of a novel bombesin-like peptide in pituitary is interesting given the many effects of bombesin-like peptides that have been reported on pituitary function (51, 52).

Our laboratory has recently cloned the apparent receptor for [Phe^{13}]bombesin from *B. orientalis* brain (26). This receptor is expressed only in brain and has a K_d for [Phe^{13}]bombesin of .2 nM, and defines a new class (BB4) of bombesin receptors. The existence of this receptor reinforces the potential importance of [Phe^{13}]bombesin as a brain neuropeptide. Interestingly in rats, the amphibian "Phe form" bombesin-like peptide, [Phe^8]-phyllolitorin, has potent effects on scratching behavior (53) raising the possibility that mammals have homologous peptides. Specific receptors for [Leu^{13}]bombesin and [SAP]bombesin likely exist and remain to be characterized.

If [Phe^{13}]bombesin and [SAP]bombesin have physiologic roles distinct from [Leu^{13}]bombesin, they should have different receptor affinities. As shown in Figs. 5 and 6 and Table I, [Phe^{13}]bombesin and [SAP]bombesin are agonists at both the GRP and NMB receptors. Neither of these peptides had any significant affinity for the BRS-3 receptor (24, 25), thus their structures give no hint as to the identity of the BRS-3 ligand. [SAP]Bombesin has relatively lower affinity for the known bombesin receptors; thus its receptor may be quite distinct from the [Phe^{13}]bombesin receptor.

[SAP]Bombesin(5–14), an NMB agonist, could potentially be produced by cleavage at the internal Ser-Leu motif (position 47,48) of [SAP]bombesin. Whether this peptide is produced *in vivo* in frog brain remains to be determined. A key question remaining to be answered is if frogs have NMB or if, instead, [Phe^{13}]bombesin or [SAP]bombesin perform some of the same functions in frog brain as does NMB in mammalian brain.

The existence of multiple forms of bombesin within a single species of frog implies a more complicated physiologic role for bombesin-like peptides than previously realized. This appears to be generalizable as this occurs in multiple species of frogs. The distinct distributions and affinities for each peptide further suggests distinct physiologic roles. Given that the bombesin-related peptides diverged from GRP and NMB prior to the vertebrate radiation (20) it is quite likely that BRPs distinct

[2] S. R. Nagalla, B. J. Barry, C. J. Prasad, and E. R. Spindel, unpublished data.

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from GRP will also exist in mammals, and quite likely in multiple forms. Given the high affinity of [Phe^{13}]bombesin both for its native receptor (26) and for the GRP and NMB receptors and given the prevalence of [Phe]bombesin in amphibian brain, a BRP with Phe as the penultimate residue may also exist in mammals and may mediate some of the effects ascribed to bombesin-like peptides in mammals.

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