Neuropeptide FF (FLFQPQRF-NH_2) and its Fragments Bind to $\alpha_2\delta$ Subunit of Voltage-Gated Calcium Channels

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ABSTRACT—Purpose: Gabapentin, a drug for neuropathic pain, exerts its therapeutic effect via inhibition of the $\alpha_2\delta$ subunit of N-type Ca^{2+} channels. Thus, finding peptides that specifically displace gabapentin from its binding site may lead to the development of new drugs. **Methods:** Displacement of bound [^{3}H]-gabapentin in membrane preparations of rat cerebral cortex and of human Cav2.2/β_3/$\alpha_2\delta_1$ expressed in CHO cell line. **Results:** Neuropeptide FLFQPQRF-NH2 specifically displaced bound [^{3}H]-gabapentin in membrane preparations from rats and CHO cells. Truncation of the C-terminus of FLFQPQRF-NH2 by three amino acid residues to produce FLFQP-NH_2 improved the displacement of gabapentin. FLFQP-NH_2 displaced bound [^{3}H]-gabapentin with IC_{50} and K_i values of 2.7 μM and 1.7 μM, respectively. Deletion of two amino acid residues (FQ) in the middle of the FLFQP-NH_2 sequence yielded FLP-NH_2 that displaced bound [^{3}H]-gabapentin with a lower affinity. IC_{50} and K_i values were 11.9 μM and 7.8 μM, respectively. Neutral binding cooperativity existed when of FLFQP-NH_2, FLP-NH_2 and gabapentin when incubated together. FLFQPQRF-NH_2 but not FLFQP-NH_2 displaced bound [^{3}H]-gabapentin to membrane preparations of human Cav2.2/β_3/$\alpha_2\delta_1$ expressed in CHO cells. **Conclusion:** FLFQPQRF-NH_2, FLFQP-NH_2 and FLP-NH_2 displace bound gabapentin in membrane preparations of rat cerebral cortex. Binding cooperativity was detected when GBP/FLFQP-NH_2/FLP-NH_2 were incubated together. These novel binding sites may provide new approaches to modulate L-type Ca^{2+} channels.

INTRODUCTION

Human neuropeptide FF gene encodes several peptides containing a common carboxy-terminal motif that consists of an arginine (R) and an amidated terminal phenylalanine residue (RF-amide peptides): NPFF (FLFQPQRF-amide), NPAF (AGEGLSSPFWSLAAPQRF-amide) and NPSF (SLNFEELKDWGPKNVIKMSTPAVNKMPHSF ANLPLRF-amide). These three peptides are derived from a precursor gene present in all vertebrate species (1). NPFF binds with high affinity to two receptors (NPFF1 and NPFF2) that modulate nociception (1, 2, 3). FLFQPQRF-NH_2 (NPFF without the amidation of the terminal phenylalanine residue) is highly localized in the spinal cord of many vertebrates including humans (4). The peptide exerts morphine (5, 6) and blood pressure (7) modulating activity.

Voltage-gated calcium channels (Ca_V1.x, Ca_V2.x, and Ca_V3.x) are an important route of **calcium** entry into the cell. The channels consist of a pore protein and four types of auxiliary subunits: α_1, α_2, β, δ, and in some tissues an additional γ subunit (8, 9). $\alpha_2\delta$ has been implicated in the development of neuropathic pain caused by damage or disease of the somatosensory nervous system. The α_2 protein is an extracellular glycoprotein connected through disulfide bonds to the δ protein (10, 11). $\alpha_2\delta_1$ of the N-type channel (Ca_V2.2) is a target for treatment of neuropathic pain (12, 13). Three Cav2.2 antagonists, gabapentin (GBP, Neurontin), pregabalin (Lyrica), and ziconotide are approved drugs for chronic pain. α_1 of L-type channel (Ca_V1.2) also seems to be involved in pain signaling and chronic pain (14).

Gabapentin with a chemical structure of 1-(aminomethyl)-cyclohexaneacetic acid and its analog pregabalin (3-(aminomethyl)-5-methylhexanoic acid; 3-isobutyl; GABA) bind to $\alpha_2\delta_1$ and $\alpha_2\delta_2$ subunits but not to $\alpha_2\delta_3$ or $\alpha_2\delta_4$ (15-19). Their binding decreases the level of Ca_V2.2 in the cell membrane but it does not block the channel pore. Both compounds are common drugs for neuropathic pain (20, 21). GBP binds to an RRR (arginine residue) motif located at the N-terminal of a VWA (von Willebrand factor) domain in the α_2 protein (21). Replacing a single arginine residue to form RRA motif abrogates GBP binding to the α_2

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protein and consequently, GBP does not alleviate neuropathic pain in a pain model in mice (22).

The present study demonstrates that FLFQPQRF-NH_2 and its fragments (FLFQP-NH_2 and FLP-NH_2) at µM concentrations displace bound [^{3}H]-GBP in membrane preparations of rat cerebral cortex and human Cav2.2 expressed in CHO cells. However, no displacement of bound [^{3}H]-GBP) was observed when GBP/FLFQP-NH_2/FLP-NH_2 were incubated together suggesting a novel binding site(s) that cross interacts with GBP receptor $\alpha_2\delta_1$.

METHOD AND MATERIALS

Materials

FLFQPQRF-NH_2, Bovine, NPAF (AGEGLSSPFWSLAAPQRF-NH_2) and NPSF (SLNFEELKDWGPKNVIKMSTPAVNKMPHSF ANLPLRF-NH_2) were purchased from AnaSpec Inc. (Fremont, CA, USA). [^{3}H]- GBP (90-120 Ci/mmol) was purchased from ARC Inc. (St. Louis, MO, USA) and unlabeled GBP from Millipore Sigma (St. Louis, MO, USA).

Peptide synthesis

Four peptides (FLFQP-NH_2, FLP-NH2, FLHyp-NH_2 and FQP-NH_2) were custom synthesized with purity greater than 95% using a standard solid-phase method (AnaSpec Inc., Fremont, CA, USA). The peptides were synthesized from all L-amino acids. *Cis*-4-hydroxy-L-proline residue was used in the synthesis of FLHyp. The peptides were purified by reversed phase HPLC using a C_{18} column (Acclaim™ 120 C_{18} Column; Thermo Fisher Scientific Inc. Waltham, MA, USA) coupled to a linear ion trap mass spectrometer (LTQ XL, Thermo Fisher Scientific Inc.). Mass spectra at the range of m/z values of 200 to 2000 were used to calculate the molecular mass of the peptides (Fig. 1). A singly charged peptide ion was the most intense peak in the mass spectra of FLFQP-NH_2 at m/z value of 651 (A). FLP-NH_2 was present as a singly charged ion at m/z value of 376.2 (B). FLHyp-NH_2 (C) and FQP-NH_2 (D) were also present as singly charged ions at m/z values 392 and 391, **respectively**. Dimeric ions and other charged ions commonly formed in the gas phase environment of the mass Spectrometer were also detected. Sodium adduct peaks were observed at 22 m/z units above the peptide ions. The deviation of the calculated molecular masses from the theoretical masses was less than 1 Da. After purification and spectrum analysis, the peptides were lyophilized and kept dry at 4ºC.

Membranes preparation of rat brain cortex

Wistar male rats (175 ±25 g body weight) were sacrificed by CO_2 overexposure and the cerebral cortex was dissected and used for membrane preparations (BioLASCO Taiwan Co., Taipei, Taiwan). All procedures were done at 4°C as described by Glee et al (16).

Brain tissue was removed from the skull into buffer A (5 mM Tris-HCl, pH 7.4, containing 0.32 M sucrose). Cerebral cortex tissue was removed from the brain tissue and homogenized in 10 volumes (v/w) of buffer A in a glass-Teflon homogenizer using ~10-15 up-and-down strokes. The homogenate was centrifuged at 1,000g for 10 min. The resulting pellet was washed twice with buffer A and the supernatants were collected and centrifuged at 21,500g for 20 min. The supernatant was removed and the resulting crude membrane pellet was resuspended in 5 mM Tris-HCl, pH 8.0 and stirred for 1 h at 4ºC. The suspension was centrifuged at 40,000g for 30 min and the pellet was resuspended in buffer B (5 mM Tris-HCl, pH 7.4, containing 1.2 M sucrose). The suspension was layered onto the top of a discontinuous sucrose density gradient. The gradient was centrifuged at 100,000g for 90 min. The fraction containing the cerebral cortex membranes was recovered from the 0.9 M –1.2 M sucrose interface and resuspended in 5 mM Tris-HCl, pH 7.4. The suspension was centrifuged at 40,000g for 30 min and the membrane fraction was kept at -80°C until use. Membrane fractions were prepared by Eurofins Scientific (Taiwan).

Membranes preparation of CHO cells expressing Cav2.2/β_3/$\alpha_2\delta_1$ and Cav1.2/β_2/$\alpha_2\delta_1$

Cell lines

Stable cell lines expressing (under tetracycline induction) human N- and L-type Cavs in CHO (Chinese hamster ovary) cells were constructed as described (23).

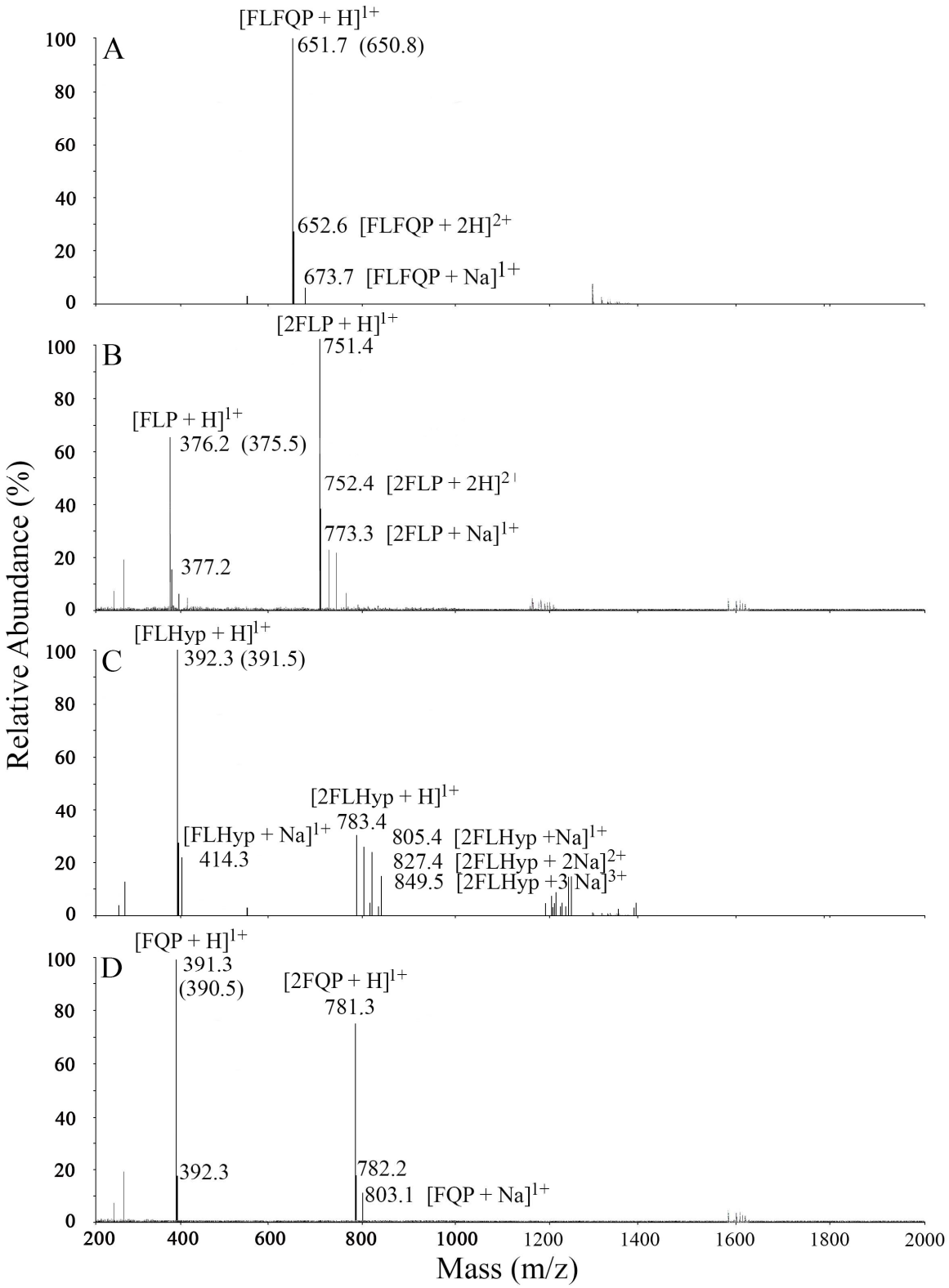

Figure 1. Linear ion trap mass spectra of FLFQP-NH_2, FLP-NH_2, FLHyp-NH_2 and FQP-NH_2. The four most intense ions were the peptides. Experimentally and theoretical (in parenthesis) values for the peptides are given above the peaks. Theoretical values are based on the chemical composition derived from the amino acid sequence of the peptides. Dimeric form of the peptides and multi charged ions as well as sodium adducts are shown. Hyp is the abbreviation for *cis*-4-hydroxy-L-proline.

One stable cell line expressed Cav2.2/β_3/$\alpha_2\delta_1$. Neuronal CACNA1B gene that encodes the pore-forming subunit of N-type channel was co-expressed with its β_3 and $\alpha_2\delta_1$. The other cell line expressed Cav1.2/β_2/$\alpha_2\delta_1$. Neuronal CACNA1C gene that encodes the pore-forming subunit of L-type channel was co-expressed with its β_2 and $\alpha_2\delta_1$. Cell lines were produced by ChanTest corp (OH, USA).

Preparation of Membranes

Confluent cells (75-85% confluency) were washed and harvested in cold DPBS (Dulbecco's phosphate buffered saline without Ca^{2+} or Mg^{2+}) by scrapping from the plates. Cell pellets were prepared by centrifugation at 100g for 10 min. The cells were resuspended in 10 mM Tris-HCl pH 7.5, 2 mM EDTA and a protease inhibitor cocktail (1 tablet per 250 ml buffer, cOmplete, Roche Diagnostics Corp., IN, USA). Subsequently, the cells were homogenized in a Polytron homogenizer. The homogenate was centrifuged at 38,000g for 20 min. This procedure was repeated twice and the membrane pellets were combined and transferred into a glass Dounce homogenizer. The membrane pellet was homogenized in a minimal volume of 10 mM Tris-HCl (pH 7.5) containing 10% sucrose using 10 up-and-down strokes. The resulting membrane suspension at a protein concentration of 2.5 mg/ml was aliquot and kept at -80ºC until use. Membrane suspensions were prepared by ChanTest corp (OH, USA).

Displacement of bound [^{3}H]-gabapentin by peptides in membrane preparations

Binding of [^{3}H]-GBP (90-120 Ci/mmol) to membrane preparations was performed as previously described (16, 24). Briefly, a 20 µg aliquot of membrane protein was incubated with 20 nM [3H]-GBP in 96-well microplates (U96 PP-0.5 ml, Thermo Fischer Scientific Inc.) in the absence or presence of increasing concentrations of a specific peptide in 10 mM HEPES, pH 7.4. The peptides were either dissolved in the incubation buffer or in the incubation buffer containing 1% DMSO. The membrane preparations were filtered and washed with 100 µM unlabeled GBP in 10 mM HEPES, pH 7.4 to remove non-specific binding. The filters were then counted using a microplate scintillation counter (TopCount NXT, Packard Instrument Company, CT, USA) to determine bound [^{3}H]-GBP. Math-IQ software was used for curve fitting and calculation of various binding parameters (IDBS, MA, USA).

Under optimized assay conditions (incubation at 25ºC for 30 min) the maximum signal-to-noise ratio was ~20% (specific binding of [^{3}H]-GBP; Fig. 2). Signal-to-noise ratio was determined from total binding (receptor + [^{3}H]-GBP + incubation buffer) divided by nonspecific binding (receptor + [^{3}H]-GBP + 100 µM unlabeled GBP + incubation buffer). Binding assays were performed by Eurofins Scientific (Taiwan).

RESULTS

Displacement of bound [^{3}H]-gabapentin by FLFQPQRF-NH_2 and its fragments in rat cerebral cortex membranes.

[^{3}H]-Gabapentin bound to membrane preparations of rat cerebral cortex with high affinity and produced a steep monophasic curve (Fig. 2). The Kd and Bmax values were 38 nM and 6.8 pmol/mg protein, respectively and in agreement with published data. The saturation binding data were compatible with a single high-affinity binding site (Hill coefficient = 0.9) with IC_{50} and K_i values of 40 nM and 26 nM, respectively. Thus, the displacement of GBP from its high affinity-binding site was used to screen for peptide ligands.

FLFQPQRF-NH_2 was capable of displacement of [^{3}H]-GBP from its binding site in the membrane preparations. FLFQPQRF-NH_2 at 10 µM displaced 78% of bound [^{3}H]-GBP (Table 1). The related peptides, NPAF-NH_2 and NPSF-NH_2 did not displaced bound [^{3}H]-GBP.

FLFQPQRF-NH_2 was truncated at the C-terminus to form FLFQP-NH_2 and at the N- and C-terminal to form FQP-NH_2. Ten µM of FLFQP-NH_2 displaced 88% of bound [^{3}H]-GBP, a slight improvement in the displacement capacity relative to FLFQPQRF-NH_2. Ten µM of FQP-NH_2 displaced 71% of bound [^{3}H]-GBP. These results revealed that the two truncated peptides retain the capability to displace bound [^{3}H]-GBP from $\alpha_2\delta$. However, FLFQP-NH_2 seemed to be the most efficient (Fig. 2). The displacement of bound [^{3}H]-GBP resulted in a shallow, rightward shift of the curve in a concentration-dependent manner. The displacement curve was monophasic suggesting an interaction with a single binding site. The IC_{50} and K_i values for the displacement of bound GBP by FLFQP-NH_2 were 2.7 µM and 1.7 µM, respectively. FLFQP-NH_2 is a weaker ligand than GBP (~67 fold differences in IC_{50} and K_i). It may suggest that FLFQP-NH_2 (molecular weight of 651) interacts with a large

surface of $\alpha_2\delta$ whereas GBP (molecular weight of 171) interacts with only one arginine residue (R^{217}).

Table I. Displacement of bound [^{3}H]-GBP by neuropeptides and fragments in membrane preparations of rat cerebral cortex.

Peptide	Displacement (%)
NPSF-NH_2	43
NPAF-NH_2	47
FLFQPQRF-NH_2	78
FLFQP-NH_2	88
FQP-NH_2	71

Membrane preparations (20 µg protein) were incubated with a mixture of 20 nM [^{3}H]-GBP and one of the unlabeled peptides at a concentration of 10 µM for 30 min at 25ºC. Specific binding of [^{3}H]-GBP without any peptide was ~5,650 cpm and nonspecific binding was ~710 cpm. Data are expressed as a percentage of displacement of bound [^{3}H]-GBP. Displacement greater than 50% is considered specific and significant. Results are an average of duplicates.

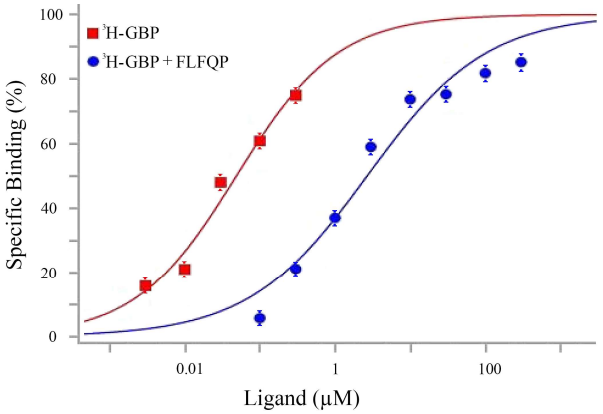

Figure 2. Displacement of bound [^{3}H]-GBP by unlabeled FLFQP-NH_2 in membrane preparations of rat cerebral cortex. Displacement curve of bound [^{3}H]-GBP by unlabeled 100 µM GBP. (red line). Displacement of bound [^{3}H]-GBP by unlabeled FLFQP-NH_2 (blue line). [^{3}H]-GBP and FLFQP-NH_2 were added to the incubation medium at the same time. Each point represents the percentage of maximum specific binding (total binding minus nonspecific binding) at a certain concentration and is the mean of two determinations. Error bars indicate the range of data for the duplicates.

In another sets of experiments FLP-NH_2 displaced bound [^{3}H]-GBP in a concentration dependent manner (Fig. 3). Two amino acid residues (FQ) from the middle of FLFQP-NH_2 sequence were omitted to form FLP-NH_2. The IC_{50} and K_i values were 11.9 µM and 7.8 µM, respectively. These values were ~ 4-fold lower than the values obtained for FLFQP-NH_2. Removal of the sequence in the middle (FQ) reduced the binding affinity to the to the GBP binding site (Table 2). It suggests that the two amino acid residues make a significant contribution to the binding of FLFQP-NH_2 to $\alpha_2\delta_1$. Replacing of proline residue in FLP-NH_2 with hydroxyproline (*cis*-4-hydroxy-L-proline) residue decreased the displacement of bound [^{3}H]-GBP suggesting that the hydroxyl group of hydroxyproline interferes with the binding of FLP-NH_2 (Table 2).

Bound [^{3}H]-GBP was not displaced when FLFQP-NH_2 + FLP-NH_2 were added concomitantly to the incubation medium (Table 2). At 10 µM concentration each of the peptide displaced ~ 40-50% bound [^{3}H]-GBP (Fig. 2). About 100% displacement was expected but instead the displacement efficiency was zero. One plausible explanation is that the peptides bind to a site distinct from the GBP binding site and cooperativity exists between these binding sites. As a consequence of this interaction bound [^{3}H]-GBP was not displaced.

Displacement of bound [^{3}H]-gabapentin by FLFQPQRF-NH_2 in membranes obtained from CHO cells expressing human Cav2.2/β3/α2δ1

[^{3}H]-gabapentin binds to the $\alpha_2\delta_1$ subunit of the human N-type channel (Cav2.2/β3/α2δ1) expressed in CHO cells with IC_{50} value of 94 nM is in good agreement with IC_{50} of 40 nM in rat cortex membrane preparations. FLFQPQRF-NH_2 displaced bound [^{3}H]-GBP from this membrane preparation with a similar affinity observed in the membrane preparations of rat cerebral cortex (Table 3). On the other hand, FLFQP-NH_2 exhibited a low displacement capability of bound [^{3}H]-GBP compared to that observed in membrane preparations of rat cerebral cortex (Table 1). One reason for changes in the displacement ability of FLFQP-NH_2 is the differences in membrane constituents and environments. Similar results were obtained with membrane preparations of human L-type channel, Cav1.2/β2/α2δ1 expressed in CHO cells (data not shown). The IC_{50} value for [^{3}H]-GBP in membrane preparations of Cav1.2/β2/α2δ1 was 29 nM.

DISCUSSION

Interpretation of binding data

Gabapentin exists in two distinct conformations in solution with three elements for interaction.

Alkylamino and carboxylate charged groups and a hydrophobic hydrocarbon ring (25). α_2/δ interacts with GBP and with the amino acid leucine that closely resembles gabapentinoid (26). FLFQPQRF-NH_2, FLFQP-NH_2 and FLP-NH_2, on the other hand, can adopt many conformations in solution and they probably interact with a larger surface of α_2/δ than GBP (27). The data may also suggest that FLFQPQRF-NH_2 and its fragments interact with $\alpha_2\delta$ at distinctly different sites than GBP. The peptides may interact with another binding site, an allosteric site, and in such a case the peptides may have identified a novel allosteric site sites in α_2/δ. However, it has been shown that a single binding site interacts with structurally different ligands (30). Further studies will determine the peptides binding site(s).

Study of the cross-interactions between GBP/FLFQP-NH_2/FLP-NH_2 binding sites in α_2/δ can provide new information for design a better drug. The muscarinic acetylcholine receptors, for example, have two distinct allosteric sites that modulate sensitivity to ligands (31). Cooperativity with an orthosteric site can switch between negative, positive, and neutral cooperativity depending on the presence or absence of a specific ligand. Allosteric modulation of the GBP binding site in α_2/δ may affect its efficacy and thus its analgesic and anti-epileptic properties. It has been shown that spermine (28) and Ruthenium red (29) bind to allosteric sites in α_2/δ that is distinguished from GBP binding site. Thus, it is possible that FLFQPQRF-NH_2, FLFQP-NH_2 and FLP-NH_2 bind to additional distinguished binding

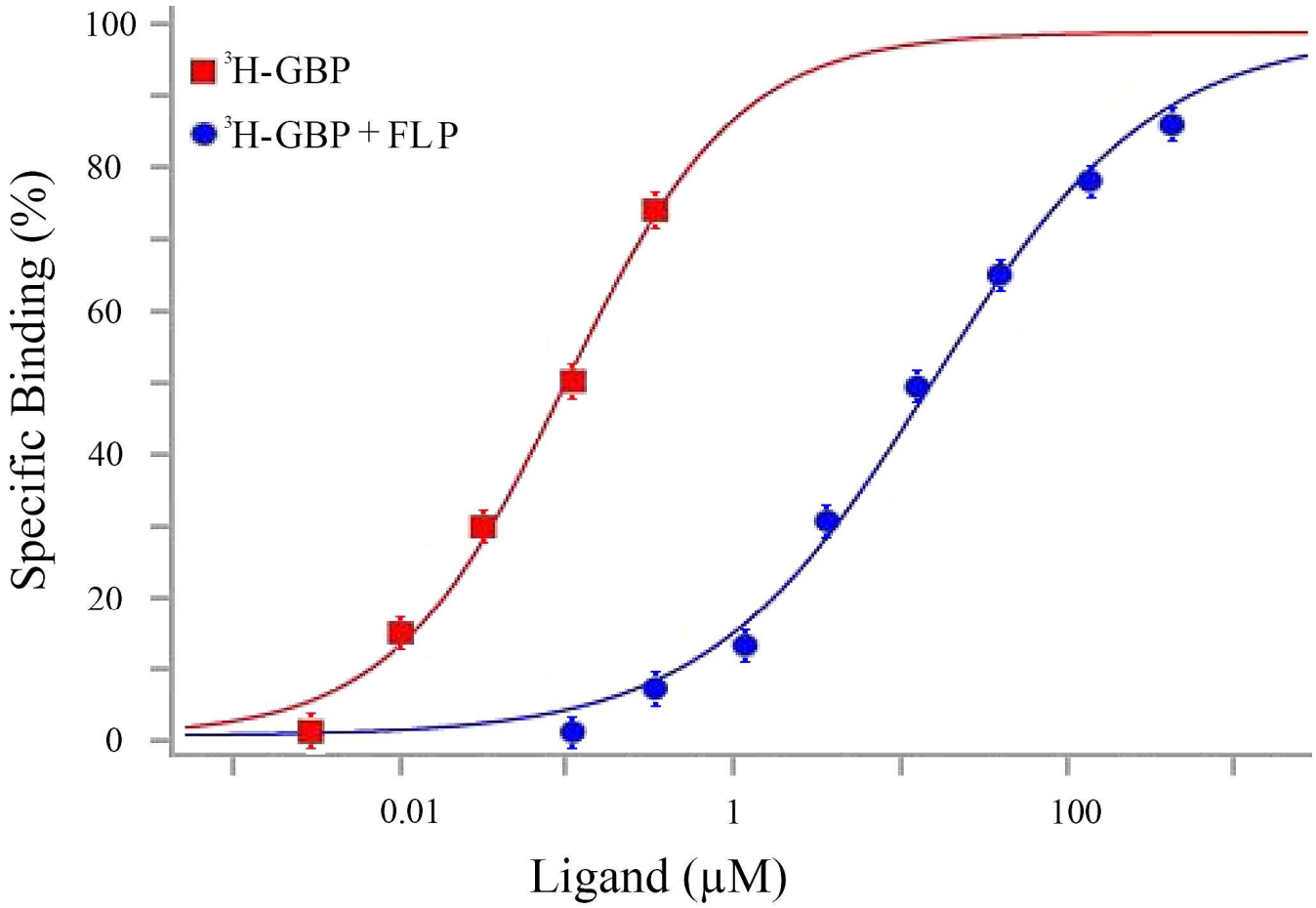

Figure 3. Displacement of bound [^{3}H]-GBP by unlabeled FLP-NH_2 in membrane preparations of rat cerebral cortex. Figure legend as in Figure 2.

Table 2. Displacement of bound [^{3}H]-GBP by FLP-NH_2 in membrane preparations of rat cerebral cortex.

Compound	Concentration (μM)	Displacement (%)
FLP-NH_2	100	99
FLHyp-NH_2	100	87
FLFQP-NH_2 + FLP-NH_2	10	-3

Membrane preparations (20 μg protein) were incubated with a mixture of 20 nM [3H]-GBP and one or two peptides at a concentration of 10 μM for 30 min at 25ºC. Specific binding of [^{3}H]-GBP without any peptide was ~5,650 cpm and nonspecific binding was ~710 cpm. The data are expressed as percentage of specific binding. Displacement greater than 50% was considered significant. Results are an average of duplicates. Hyp is the abbreviation for *cis*-4-hydroxy-L-proline.

Table 3. Displacement of bound [^{3}H]-GBP by FLFQPQRF-NH_2 and its fragments in membrane preparations of CHO cells expressing human Cav2.2/β3/α2δ1.

Compound	Displacement (%)
FLFQPQRF-NH_2	79
FLFQP-NH_2	56
FLP-NH_2	62

Membrane preparations of CHO cells expressing human Cav2.2/β3/α2δ1 were incubated for 30 min at 25ºC in 10 mM HEPES, pH 7.4 with 20 nM [^{3}H]-GBP and 10 μM of each peptide. The counts per minute value for 100% binding for [^{3}H]-GBP alone were 19,335±3,600 cpm and for non-specific binding were 428±100 cpm (mean±S.D. of 6 determinations). Results are an average of duplicates. Displacement greater than 50% was considered significant.

Potential ligands of α_2/δ

FLFQPQRF-NH_2, FLFQP-NH_2 and FLP-NH_2 may be putative endogenous ligands of the α2. One cleavage product of NPFF, QRF-NH_2, was detected in mouse brain (32). It is possible that more cleavage sites exist in NPFF to allow the production of various peptides. Whether the FLFQP-NH_2 and FLP-NH_2 exist under physiological conditions is an unanswered question. Further structure and binding relationship studies have to be performed and the peptides' biological activity has to be determined. Whether these shorter peptides are functionally relevant in human brain biology remains unknown.

GBP binding affinity to the α2/δ subunit expressed in CHO cells was similar to its binding to membrane preparations of rat cerebral cortex. FLFQPQRF-NH_2 displaced bound [^{3}H]-GBP from the two different preparations suggesting the presence of a common binding site. However, the membrane environment of CHO cells affected FLFQP-NH2 binding and it did not displace bound [^{3}H]-GBP. The reason for this is unknown at the moment. The peptides can provide preliminary information on novel binding sites within $\alpha_2\delta$ and they may serve as a template for the development of new drugs for neuropathic pain (33).

NPFF (FLFQPQRF-amide) and its fragments and pain homeostasis

NPFF at a cellular level exerts antinociceptive, anti-opioid activity via its receptors, NPFF1R and NPFF2R (34, 35). NPFF can modulate nociception depending on the pharmacological dosage and locations of administration (36-38). NPFF suppressed pain in a mouse model of neuropathic pain depending on the site of administration. The mRNA level of NPFF remained unchanged in a model of carrageenan induced inflammatory pain (2). The analgesic effects of FLFQP-NH_2 and FLP-NH_2 in a range of animal models of pain have to be studied to determine their involvement in the elevation of pain.

Currently, the GBP binding site in $\alpha_2\delta$ has no endogenous ligand. GBP affects the α_2/δ recycling and thus prevents neuropathic pain (39, 40). It is possible that FLFQPQRF-NH_2, FLFQP-NH_2 and FLP-NH_2 are endogenous ligands that modulate acute and neuropathic pain sensitivity and therefore involved in keeping homeostasis of pain sensation (41). These preliminary results provide a starting point for the identification of the binding sites of FLFQPQRF-NH_2, FLFQP-NH_2 and FLP-NH_2 and their biological involvement in pain and other diseases such as epilepsy.

CONCLUSION

Understanding of the interactions between α_2/δ subunit of the L-type Calcium channels and the peptides may facilitate the discovery of more selective analgesic drugs with reduced side effects.

Conflict of Interest

The author states no conflict of interest

Research Involving Human Participants and/or Animals

This article does not contain studies with human participants or animal performed by the author.

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