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Recognition of Neuropeptides FMRFamide and LPLRFamide by Chicken Cerebellum Avian Pancreatic Polypeptide Binding Sites[1] (42562)

KANJANA GANESHAN,* MICHAEL O. PERLMAN,* JANET M. PERLMAN,* MARTIN L. ADAMO,† ROBERT L. HAZELWOOD,† AND DOUGLAS F. DYCKES*

Departments of Chemistry, and †Biology, University of Houston–University Park, Houston, Texas 77004

Abstract. Binding isotherms were constructed for the binding of synthetic tetrapeptide and pentapeptide fragments to membranes prepared from chicken cerebellar tissue. Both the tetrapeptide (FMRFamide), which was originally isolated from ganglia of mollusks, and the pentapeptide (LPLRFamide) previously isolated from chicken brain are known to increase blood pressure and modulate brain neurons in rats. The C-terminal dipeptide sequences of the two peptides are identical and both show similarity to the dipeptide sequence established for the pancreatic polypeptide (PP) family. Specific high-affinity binding sites exist for the latter peptide, sites which are competed for (though with less affinity) by neuropeptide Y (NPY). Affinity for cerebellar membranes was virtually equivalent for the synthetic peptide LPLRFamide and FRMFamide; the binding affinities (IC_{50}) of all fragments tested (C-terminal pentapeptides of avian PP and NPY, and FMRFamide and LPLRFamide) fell in the same approximate range. Since the N-terminal residues of FMRFamide and LPLRFamide are not homologous with equivalent residues of APP or NPY, our results indicate that only Arg–Tyr-NH_2 or Arg–Phe-NH_2 sequences are necessary for binding of the carboxy terminus peptides of the PP family. In this respect, these sequences are functionally equivalent. © 1987 Society for Experimental Biology and Medicine.

Two neuropeptides from the tissues of two widely separate animal species appear to be the first known members of a new family of neurotransmitters (1). The first of these, isolated as a molluscan cardioexcitatory factor from ganglia of the clam *Macrocallista nimbosa,* was found to be a tetrapeptide amide (2). It was named FMRFamide, based on the single letter code for its amino acid sequence (phenylalanyl-methionyl-arginyl-phenylalanine amide). A related peptide, LPLRFamide (leucyl-prolyl-leucyl-arginyl-phenylalanine amide), was isolated later from chicken brain (3). Both peptides showed parallel effects in increasing arterial blood pressure and in modulating brain neurons in the rat (1, 3). Recently, a third related peptide, pQDPFLRFamide (pyroglutamyl-aspartyl-prolyl-phenylalanyl-leucyl-arginyl-phenylalanine amide), has been isolated from ganglia of the snail *Helix aspersa* (4). Based on its relative potency and detected blood levels, pQDPFLRFamide may be an invertebrate cardioregulatory hormone (4).

The initial detection of LPLRFamide came as a result of its cross-reactivity with antibodies raised to FMRFamide (3). Similarly, pQDPFLRFamide was isolated using a protocol which detected its presence through cross-reactivity with antiserum to an extended form of FMRFamide (YGGFMRFamide) (4). All of these molecules possess an identical C-terminal dipeptide amide sequence, i.e., –Arg–Phe-NH_2.

Peptides belonging to the pancreatic polypeptide (PP) family are 36 amino acid residues in length. They are characterized by a highly conserved C-terminal dipeptide sequence (5), namely –Arg–Tyr-NH_2. The similarity between this dipeptide and that found in the FMRFamide-like peptides has been noted (3); they differ only in that the side chain of the tyrosyl residue possesses a phenolic hydroxyl group. Indeed, the only PP which has been found not to possess the sequence –Arg–Tyr-NH_2 at its C-terminus is alligator pancreatic polypeptide (6), which terminates in –Arg–Phe-NH_2. Recent studies carried out in our laboratories indicate that these two terminal sequences may be functionally equivalent.

In surveying a wide range of chicken tissue membranes, we found that the chicken brain possesses specific, high-affinity binding sites for

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avian pancreatic polypeptide (APP) (7). These APP binding sites recognized synthetic pentapeptide amides corresponding to the C-terminal segments of APP and bovine PP (7), but these peptides competed with much weaker affinities. Subsequently, chicken brain APP binding was found to be primarily cerebellar; similar APP binding sites were detected in a variety of avian species (8).

One member of the PP family, neuropeptide Y (NPY), was actually isolated from extracts of the porcine brain (9). Antibodies raised to NPY have revealed an extensive immunoreactive neuronal system in the rat brain (10), and similar studies on human brain have indicated that NPY immunoreactive molecules therein exceed in quantity any previously discovered neuropeptide (11).

NPY binds specifically to the cerebral cortex of the rat (12). We have found that it also is capable of competing for chick cerebellar APP binding sites, but with only about one-tenth the affinity exhibited by APP (13). The C-terminal pentapeptide amide of NPY competes for APP binding sites in the chick cerebellum with an affinity approximating that of the corresponding APP segment.

A series of analogs of the C-terminal segment of NPY has been synthesized and their binding affinities measured. Of the six functional groups at the five residue positions studied, only the side chain guanidinium group of the penultimate arginyl residue and the C-terminal aromatic side chain and carboxamide functions are essential for binding (13). In particular, the analog terminating in $-Arg-Phe-NH_2$ bound with the same affinity as did the one with the native $-Arg-Tyr-NH_2$ sequence. This suggested strongly that the neuropeptides of the FMRFamide group would show affinity for APP binding sites. The results of binding studies for FMRFamide and LPLRFamide are reported here.

Materials and Methods. *Peptides.* LPLRF-amide was prepared by solid phase synthesis using the methods described in detail in the previous NPY study (13). It was purified by chromatography on Sephadex G-15 and Sephadex G-25 and by ion-exchange chromatography (SP-Sephadex C-25). The product was homogeneous by thin-layer chromatography (butanol:acetic acid:water, 1:1:1, R_f = 0.34) and by thin-layer electrophoresis (pyridine:acetic acid:water, 1:10:89, pH 3.3; mobility vs arginine = 0.74). Amino acid analysis; Leu, 2.0; Phe, 0.9; Arg, 1.0.

FMRFamide was purchased from Sigma Chemical Co. (St. Louis, Mo). It was homogeneous by thin-layer chromatography (R_f = 0.68) and thin-layer electrophoresis (mobility vs arginine = 0.68) in the same systems used to analyze LPLRFamide. Amino acid analysis: Met, 1.0; Phe, 2.2; Arg, 0.8.

Binding assays. Inhibition of specific binding of [^{125}I]APP to chick cerebellar membranes by the test peptides was measured as previously described in detail (7). Nonspecific binding was determined from the counts bound in the presence of 1.2 μM unlabeled APP. This value was subtracted from the counts bound at each concentration of the competitor to determine specifically bound [^{125}I]APP. These values, expressed as a percentage of the counts bound specifically in the absence of added unlabeled APP, were used to plot binding isotherms from which the binding affinity (IC_{50}, the concentration of competitor required to inhibit 50% of the specific [^{125}I]APP binding) was deduced.

Results and Discussion. The binding isotherms of FMRFamide and LPLRFamide in

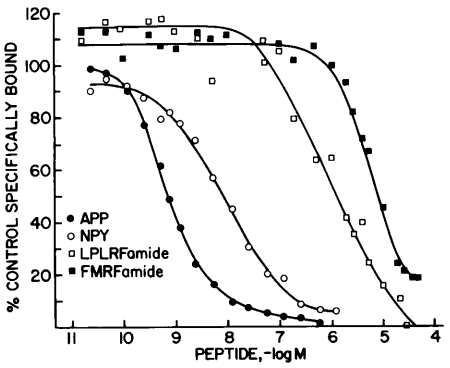

FIG. 1. Binding of synthetic peptide fragments to cerebellar membranes. Competition plots presented for intact, unlabeled APP and NPY are taken from ref. (13) and are compared with isotherms of synthetic LPLRF-amide and FMRF-amide of the present study. All were added in increasing concentrations of chick cerebellar membranes (40 μg protein). Inhibition of specific [^{125}I]APP binding is plotted vs the negative log of the molar peptide concentration. Number of observations: APP was mean of 20–24 determinations at each point; NPY was mean of duplicate or triplicates on two separate assays; see Table 1 for the two synthetic fragment preparations.

a chicken brain membrane assay system are shown in Fig. 1. The isotherms of APP and NPY obtained from earlier studies (13) also have been plotted for comparison. Table I lists the binding affinities determined for FMRFamide and LPLRFamide from these isotherms and the equivalent binding affinities for the C-terminal pentapeptide amides of APP and NPY determined in earlier work. These values represent the concentration of each peptide required to inhibit by 50% the specific ^{125}I-labeled APP ([^{125}I]APP) binding to cerebellar membranes.

The binding affinities of all of the peptides in Table I fall in the same approximate range, but the neuropeptide actually isolated from chicken brain, LPLRFamide, appears to exhibit the strongest affinity. This affinity is low compared with that of APP; however, the peptide is binding to the specific APP-binding site. Analogues of NPY_{32-36} which lack the guanidinium function of arginine-35 show no ability to compete with [^{125}I]APP for the brain binding sites (13).

The N-terminal residues of FMRFamide and LPLRFamide show no homology to the equivalent residues of the APP and NPY segments. The fact that all of these peptides bind with approximately equivalent affinity supports the postulate that only the -Arg-Tyr-NH_2 or -Arg-Phe-NH_2 sequences are necessary for binding of carboxy terminus peptides of PP and that these sequences are functionally equivalent in that respect.

The N-terminal residues of these peptides, although not possessing any functional groups essential for binding, do have an effect on binding affinity. Residues that increase the hydrophobicity of the N-terminus tend to increase the binding affinity. Extension of the C-terminal peptide of APP towards the N-terminus by addition of the native sequence, Leu-Asn-Val-Val-, generates a nonapeptide amide (APP_{28-36}) which binds six times more strongly (0.8 μM) (14) than does the corresponding pentapeptide amide. On the other hand, the NPY tetrapeptide amide (NPY_{33-36}) has less binding affinity (10 μM) (13) by a factor of three than does the corresponding pentapeptide amide. Improved binding is not simply a matter of chain length; LPLRFamide exhibits an affinity approaching that of the nonapeptide. However, a simple extension of our argument based on hydrophobicity cannot explain the much stronger affinity of APP_{1-36}. In the case of the native peptide, conformational and other factors undoubtedly play a major role.

Finally, the most important question is: Does this binding of the two neuropeptides to brain membrane preparations have any physiological significance? The present consideration is restricted to LPLRFamide, the avian homolog. While APP binds with an affinity which is compatible with its circulating plasma concentrations (in chickens), and pQDPFLRFamide shows similar high levels in snail blood, there has been no report of LPLRFamide in general circulation. When injected at doses of 50–200 nmol g^{-1}, LPLRFamide was found to be effective in raising the blood pressure of rats (1, 3), but the total brain content of LPLRFamide has been estimated at 5–8 nmol g^{-1} (3). The latter figure is not compatible with the 1 μM affinity of LPLRFamide for the APP binding site in chicken brain. However, it is entirely possible that because it is endemic to the chicken brain,

TABLE I. AFFINITIES FOR BINDING TO CHICKEN BRAIN MEMBRANES BY PEPTIDE AMIDES RELATED TO THE C-TERMINUS OF APP

Sequence	Identity	IC_{50} (μM)	Reference
Thr-Arg-His-Arg-Tyr-NH_2	APP_{32-36}[a]	5.0	(7)
Thr-Arg-Gln-Arg-Tyr-NH_2	NPY_{32-36}[b]	2.5	(13)
Phe-Met-Arg-Phe-NH_2	FMRFamide[c]	7.0	(this work)
Leu-Pro-Leu-Arg-Phe-NH_2	LPLRFamide[c]	1.0	(this work)

[a] Results of the mean of duplicate assays run on four membrane preparations.
[b] Mean of three separate synthetic preparations assayed in duplicate or triplicate.
[c] Mean of duplicate assays run on a single preparation. SEM for FMRFamide was 3.4% and for LPLRFamide was 7.4%.

local concentrations of LPLRFamide could reach the high levels required for it to bind to the putative APP receptor. Specific LPLRFamide receptors may exist in chicken brain or, alternatively, the peptide may exert its effects via APP (or NPY) receptors. A more precise answer to the significance of these data can be given only when the distribution of LPLRFamide in brain, and the exact location of the sites which bind these peptides, have been better defined.

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