0026-895X/97/050816-09$3.00/0

MOLECULAR PHARMACOLOGY, **51**:816–824 (1997).

Interaction of [^{3}H]Orphanin FQ and ^{125}I-Tyr14-Orphanin FQ with the Orphanin FQ Receptor: Kinetics and Modulation by Cations and Guanine Nucleotides

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Received October 3, 1996; Accepted February 3, 1997

SUMMARY

The heptadecapeptide orphanin FQ (OFQ) has been identified as the endogenous ligand for a G protein-coupled receptor (OFQ-R), which, despite its high degree of sequence similarity to opioid receptors, fails to bind opioid ligands. We developed two radioligands for the OFQ-R: a tritiated native OFQ peptide ([^{3}H]OFQ) and a radioiodinated form in which Leu14 was substituted by tyrosine (^{125}I-Tyr14-OFQ). Their binding properties were examined in human embryonic kidney (HEK) 293 and Chinese hamster ovary (CHO) cells heterologously expressing the OFQ-R at different levels (HEK 293 expressed 40-fold more OFQ-R than did CHO). Both ligands exhibited rapid, monophasic association kinetics in each cell line. Dissociation of both ligands from OFQ-R expressed in HEK 293 cells was biphasic, whereas dissociation of ^{125}I-Tyr14-OFQ from OFQ-R expressed in CHO cells was monophasic and slow. Saturation binding analysis revealed two affinity states in HEK 293 cells with binding parameters in accord with those determined kinetically. In CHO cells, ^{125}I-Tyr14-OFQ detected a single affinity state with an intermediate K_d value of 54 pM. Optimal binding of the radioligands required 1–5 mM $MgCl_2$, whereas millimolar concentrations of $ZnCl_2$, $CaCl_2$, $MnCl_2$, and NaCl reduced specific binding of both ligands. A nonhydrolyzable GTP analog [guanosine-5′-(β,γ-imido)triphosphate] reduced the affinity of both OFQ ligands to their receptor without significant changes in the total binding capacity, indicating functional interactions between the OFQ-R and G proteins. In rat brain membranes, specific, saturable binding of ^{125}I-Tyr14-OFQ was demonstrated to be pharmacologically identical to the heterologously expressed OFQ-R. Taken together, these results indicate that ^{125}I-Tyr14-OFQ and [^{3}H]OFQ exhibit virtually identical characteristics and are suitable for the pharmacological analysis of the OFQ-R.

The molecular cloning of G protein-coupled receptors has served to vastly increase our understanding of the biology of this class of proteins over the past several years. Although the receptors for most known neurotransmitters and neuropeptides have been identified, a large number of putative receptor sequences have also been cloned for which the natural ligands are still not known; thus, these proteins have been referred to as "orphan" receptors. As a first step toward elucidation of novel neurotransmitter systems, we searched for the endogenous ligand for an opioid receptor-like orphan receptor (LC132) (1). Although this protein shares a high degree of sequence homology to the cloned μ-, δ-, and κ-opioid receptors, no opioid ligands have been found that interact with receptor LC132 below micromolar concentrations (2). Recently, we (3) and others (4) isolated a novel neuropeptide from mammalian brain that activates the LC132 receptor with high potency. We termed this peptide OFQ and its receptor OFQ-R.

To further characterize this system, it is desirable to have tools that allow the pharmacological and biochemical characterization of this receptor and the determination of receptor expression in different preparations. For these purposes, we developed two peptidergic radioligands: one is a tritiated version of the native peptide ([^{3}H]OFQ), and the other is a radioiodinated derivative, based on a biologically active OFQ peptide in which Leu14 is substituted by tyrosine (^{125}I-Tyr14-OFQ). We have previously shown that both Tyr14-OFQ and iodo-Tyr14-OFQ are full agonists with potencies identical to the parent peptide and that ^{125}I-Tyr14-OFQ binds in a saturable manner to the OFQ-R expressed in CHO cells (3, 5). However, the utility of both [^{3}H]OFQ and ^{125}I-Tyr14-OFQ as radioligands depends on their ability to bind appropriately to a receptor site that exhibits properties consistent with G protein-coupled receptors, including saturability, reversibility, and modulation by environmental factors

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ABBREVIATIONS: OFQ, orphanin FQ; OFQ-R, orphanin FQ receptor; CHO, Chinese hamster ovary, HEK, human embryonic kidney; Gpp(NH)p, guanosine-5′-(β,γ-imido)triphosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

such as ions and guanine nucleotides. The results of the current study demonstrate that these radioligands bind in a reversible, saturable, and high affinity manner to OFQ-Rs heterologously expressed in HEK 293 or CHO cells. Furthermore, this interaction is characterized in terms of its modulation by monovalent and divalent cations and guanine nucleotides. Finally, we demonstrate that ^{125}I-Tyr14-OFQ can be used to specifically label sites in rat brain membranes that exhibit nearly identical pharmacological properties as the OFQ-R expressed in CHO cells.

Materials and Methods

Radioligands. All peptides were synthesized at Research Genetics (Huntsville, AL). Iodination and purification of Tyr14-OFQ, in which the leucine residue at position 14 of native OFQ is replaced by tyrosine, was carried out at Amersham International PLC (Buckinghamshire, UK) using the chloramine-T method (5, 6). The product was purified by reverse-phase chromatography, yielding a specific activity of 2000 Ci/mmol (74 TBq/mmol). Eighty-two percent of the labeled product was monoiodinated on Tyr14. [^{3}H]OFQ was prepared by Amersham via catalytic hydrogenation of dehydro-Leu14-OFQ, followed by reverse-phase high performance liquid chromatography purification, yielding a specific activity of 110 Ci/mmol.

Cell culture. CHO dhfr$^-$ cells, which were stably transfected with the rat OFQ-R as previously described (3), were grown in α modified Eagle's medium containing 5% fetal calf serum and 500 μg/ml geneticin (G418) in a 5% CO_2 atmosphere at 37°. HEK 293 cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum in 5% CO_2 at 37°. For high level expression, the rat OFQ-R was subcloned into the eukaryotic expression vector pCEP4 (InVitrogen, San Diego, CA) and transfected into HEK 293 cells using Lipofectamine (Life Technologies, Gaithersburg, MD) according to the manufacturer's recommendations. Transfected cells were selected in the presence of 100 units/ml hygromycin (Calbiochem, San Diego, CA), and OFQ-R-expressing clones were identified by the ability of 10 nM OFQ to inhibit forskolin-stimulated cAMP accumulation as previously described (3). All cell culture reagents were obtained from Life Technologies.

Membrane preparation. CHO and HEK 293 cells were harvested by incubation in phosphate-buffered saline plus 1 mM EDTA. Cells were washed three times with phosphate-buffered saline before resuspension in buffer A (50 mM Tris·HCl, pH 7.8, 5 mM $MgCl_2$, 1 mM EGTA) and disruption with a tissue homogenizer (30 sec, setting 4, Pt 20, Kinematica, Kriens-Lucern, Switzerland). A total membrane fraction was obtained by centrifugation at 49,000 × g at 4°. This procedure was repeated twice, and the pellet was resuspended in buffer A. Aliquots were stored at −70°, and protein concentrations were determined using the BCA Protein Assay Reagent (Pierce, Rockford, IL) following the manufacturer's recommendations. Membranes from various rat brain regions were prepared essentially as for cultured cell membranes with the exception that the following protease inhibitors were added to buffer A: 10 μg/ml aprotinin, 0.1 mg/ml phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml pepstatin (all protease inhibitors were obtained from Boehringer-Mannheim Biochemica, Mannheim, Germany).

Binding assays. ^{125}I-Tyr14-OFQ binding assays were carried out in deep-well 96-well plates (Beckman Instruments, Fullerton, CA). The binding assay consisted of 18 μg (CHO cells) or 14 μg (rat brain tissues) of membrane protein and ^{125}I-Tyr14-OFQ in a final volume of 0.2 ml of buffer A containing 0.1% bovine serum albumin (Sigma Chemical, St. Louis, MO). Saturation analysis of ^{125}I-Tyr14-OFQ binding to 293/OFQ-R membranes was carried out in a final volume of 0.5 ml using 1 μg of membrane protein. [^{3}H]OFQ competition and dissociation studies were carried out with 77 μg of membrane protein (HEK 293 cells) in a final assay volume of 0.5 ml of buffer A plus 0.1% bovine serum albumin and 0.01% bacitracin (Boehringer-Mannheim). For [^{3}H]OFQ association and saturation studies, 6–7 μg of protein was used, and the final assay volume was increased to 2 ml to avoid binding >10% of the added [^{3}H]OFQ ligand. For both [^{3}H]OFQ and ^{125}I-Tyr14-OFQ, 50 nM unlabeled OFQ was used to define the nonspecific binding, and a 1-hr incubation time at room temperature was found to be optimal for saturation and competition assays. The assays were terminated by filtration through Whatman GF/C filters (Brandell, Gaithersburg, MD) [pretreated with 0.3% polyethylenimine (Sigma) and 0.1% bovine serum albumin (Sigma) for 1 hr] on a Brandell multichannel harvester, and the filters were washed six times with 1 ml of ice-cold 50 mM Tris·HCl, pH 7.5. In some cases, to avoid excessive nonspecific binding of ^{125}I-Tyr14-OFQ to filter materials, saturation assays were terminated by rapid cooling to 4°, followed by centrifugation at 14,000 × g for 15 min at 4°. After a brief rinse with buffer A and centrifugation for 5 min at 14,000 × g (4°), the membrane pellets were counted in a gamma counter. Binding parameters determined using this separation method did not differ from those determined using separation by filtration when nonspecific filter binding was not experienced. The association rates of ^{125}I-Tyr14-OFQ (0.1 nM) and [^{3}H]OFQ (0.2 nM) were determined by incubating the assays for increasing lengths of time after the addition of membrane suspension. For determination of dissociation kinetics, assays containing 10 pM ^{125}I-Tyr14-OFQ or 0.2 nM [^{3}H]OFQ were allowed to equilibrate for 30 min at room temperature. Then, 50 nM OFQ was added to the assay, and the incubation was continued for increasing lengths of time before termination by filtration.

Results of kinetic experiments were analyzed using the program Kinetic (Biosoft, Cambridge, UK) (7). Saturation data were analyzed by nonlinear regression analysis using the program LIGAND (Biosoft) (8), whereas inhibition binding data were fit to the four-parameter logistic equation using the nonlinear curve-fitting function of the program Kaleidagraph. All experiments were carried out in duplicate or triplicate and repeated at least three times.

Results

Association and dissociation kinetics of [^{3}H]OFQ and ^{125}I-Tyr14-OFQ. To identify appropriate conditions for conducting equilibrium binding studies of [^{3}H]OFQ and ^{125}I-Tyr14-OFQ, the kinetics of association and dissociation were investigated using an HEK 293 cell line stably expressing the OFQ-R (293/OFQ-R). Under the conditions used for these assays, < 10% of the radioligand was bound at equilibrium, and with the assumption that these ligands interact with only one class of binding sites, the kinetics of association can be considered of pseudo-first-order. The association of both [^{3}H]OFQ and ^{125}I-Tyr14-OFQ was rapid and monophasic, with a $t_{1/2}$ of 201 sec for 0.2 nM [^{3}H]OFQ (Fig. 1A), in close agreement with the $t_{1/2}$ of 117 sec obtained with 0.1 nM ^{125}I-Tyr14-OFQ (data not shown). The apparent association rate constants (k_{obs}) were determined at four concentrations of [^{3}H]OFQ (40–400 pM) (data not shown), and the association rate constant (k_a) was determined from the slope of a plot of k_{obs} versus [^{3}H]OFQ concentration (9) (Table 1). The value for k_a calculated in this manner did not differ significantly from the k_a value calculated assuming pseudo-first-order conditions. Thus, k_a values for ^{125}I-Tyr14-OFQ binding to 293/OFQ-R and CHO/OFQ-R membranes were calculated using the pseudo-first-order simplification (9) (Table 1). Once bound, both ligands were found to dissociate in a biphasic manner, as shown for [^{3}H]OFQ in Fig. 1B, and curve fitting to two exponentials resulted in a significantly better fit than fitting to one exponential. The $t_{1/2}$ values for the two components were 7.6 and 218 min in a proportion of 55% and 45%,

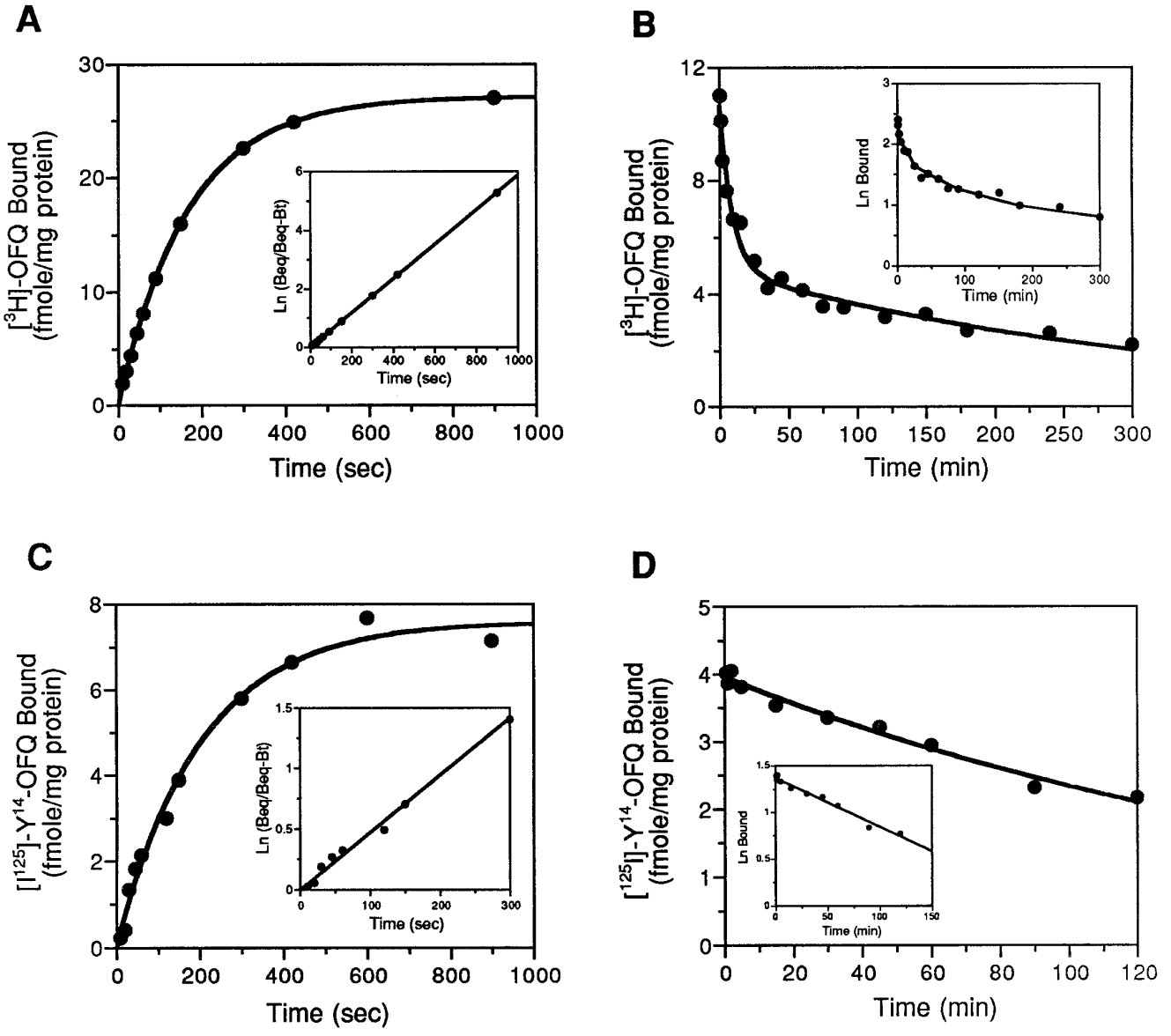

Fig. 1. Kinetics of ^{125}I-Tyr14-OFQ and [^{3}H]OFQ binding. A and C, Association of [^{3}H]OFQ to 293/OFQ-R membranes (A) and of ^{125}I-Tyr14-OFQ to CHO/OFQ-R membranes (C). 293/OFQ-R membranes were incubated with 0.2 nM [^{3}H]OFQ and CHO/OFQ-R membranes were incubated with 0.1 nM ^{125}I-Tyr14-OFQ at room temperature for varying times before termination of the reaction by filtration as described in Materials and Methods. For both ligands, nonspecific binding was determined in the presence of 50 nM cold OFQ. *Insets*, pseudo-first-order representation of the data. *Beq*, amount radiolabeled OFQ at equilibrium. *Bt*, amount bound at each time point. B and D, Dissociation of [^{3}H]OFQ from 293/OFQ-R membranes (B) and of ^{125}I-Tyr14-OFQ from CHO/OFQ-R membranes (D). 293/OFQ-R membranes were incubated with 0.2 nM [^{3}H]OFQ and CHO/OFQ-R membranes were incubated with 10 pM ^{125}I-Tyr14-OFQ for 30 min at room temperature. Dissociation was initiated by the addition of 50 nM cold OFQ peptide, and the incubations were terminated by filtration at various times as described in Materials and Methods. Nonspecific binding was determined at the start of dissociation (*time 0*) by inclusion of 50 nM cold OFQ during the 30-min labeling incubation. *Insets*, pseudo-first-order representation of the dissociation experiments. All data are from representative experiments done in triplicate and were repeated at least three times with similar results.

respectively. These values compare well with the $t_{1/2}$ values of 3.4 and 80 min obtained using ^{125}I-Tyr14-OFQ, although the proportion of fast and slow dissociating sites (29% and 71%, respectively) differs somewhat (Table 1). High and low affinity equilibrium dissociation constants (K_{dH} and K_{dL}) could then be calculated, yielding for [^{3}H]OFQ a K_{dH} value of 5.73×10^{-12} M and a K_{dL} value of 1.65×10^{-10} M. These values agree very well with the K_{dH} value of 5.95×10^{-12} M and the K_{dL} value of 1.38×10^{-10} M calculated for ^{125}I-Tyr14-OFQ (Table 1).

A distinct advantage of iodinated ligands lies in their intrinsically high specific activity. This allows the study of receptors expressed at low levels that would otherwise not be reliably detected with tritiated ligands. Because of this high specific activity, it was of interest to determine the utility of ^{125}I-Tyr14-OFQ in studying the OFQ-R expressed at low levels. The kinetics of association and dissociation of ^{125}I-Tyr14-OFQ were therefore investigated in CHO cells stably expressing the OFQ-R (CHO/OFQ-R) at ~40-fold lower levels than in the 293/OFQ-R cells. As in the 293/OFQ-R cells, association of 0.1 nM ^{125}I-Tyr14-OFQ was rapid and monophasic, with a $t_{1/2}$ of 158 sec (Table 1 and Fig. 1C).

TABLE 1

Comparison of binding parameters for [^{3}H]OFQ and ^{125}I-Tyr14-OFQ in 293/OFQ-R and CHO/OFQ-R membranes

The dissociation kinetic parameters k_{d1} and k_{d2} were derived from experimental binding data by nonlinear curve fitting as detailed in Materials and Methods. The association rate constant, k_a, for [^{3}H]-OFQ was determined from the slope of a plot of k_{obs} versus [^{3}H]OFQ concentration for association experiments carried out at four concentrations of [^{3}H]OFQ (9). The k_a value derived by this method did not differ from the k_a value calculated using the pseudo-first-order simplification; thus, k_a values for ^{125}I-Tyr14-OFQ binding were determined using pseudo-first-order calculations (9). The equilibrium dissociation constants K_{dH} and K_{dL} were derived from the averaged kinetic values as follows: $K_{dH} = k_{d1}/k_a$ and $K_{dL} = k_{d2}/k_a$. The steady-state values for K_{dH}* and K_{dL}* were obtained by nonlinear curve fitting of saturation binding data as detailed in Materials and Methods. All values represent the mean ± standard error of three or four experiments or mean ± range of two experiments, all carried out in duplicate or triplicate.

	293 OFQ-R		CHO OFQ-R
	[^{3}H]OFQ	^{125}I-Tyr14-OFQ	^{125}I-Tyr14-OFQ
Kinetic parameters			
k_a (M min) ($\times 10^8$)	5.6 ± 1 (n = 4)	14.6 ± 0.1 (n = 3)	26.0 ± 0.3 (n = 3)
k_{d1} (min^{-3}) ($\times 10^{-3}$)	3.2 ± 0.2 (n = 3)	8.7 ± 0.6 (n = 2)	3.6 ± 0.1 (n = 3)
% Slow	45%	71%	
k_{d2} (min^{-1}) ($\times 10^{-3}$)	91.5 ± 2.5 (n = 3)	202 ± 1 (n = 2)	
% Fast	55%	29%	
K_{dH} (pM)	5.73	5.95	1.39
K_{dL} (pM)	165	138	
Equilibrium parameters			
K_{dH}* (pM)	8.4 ± 1.3 (n = 4)	21 ± 4 (n = 3)	54 ± 7 (n = 12)
B_{maxH} (pmol/mg of protein)	1.6 ± 0.25 (n = 4)	1.9 ± 0.7 (n = 3)	0.12 ± 0.02 (n = 12)
% Total	47%	41%	
K_{dL}* (pM)	229 ± 56 (n = 4)	469 ± 64 (n = 3)	
B_{maxL} (pmol/mg of protein)	1.8 ± 0.4 (n = 4)	2.7 ± 0.7 (n = 3)	
% Total	53%	59%	

However, in contrast to the 293/OFQ-R cells, dissociation of ^{125}I-Tyr14-OFQ bound to CHO/OFQ-R membranes was monophasic (Fig. 1D). Nevertheless, the K_d value of 3.603 ± 0.098 (three experiments) $\times 10^{-3}$ min^{-1} and $t_{1/2}$ of 192 min compare favorably with the slow dissociating component observed in 293/OFQ-R cells (Table 1), and the calculated K_d value of 1.3 pM agrees well with the K_{dH} value calculated for ^{125}I-Tyr14-OFQ in the 293/OFQ-R cells (Table 1).

Saturation analysis of ^{125}I-Tyr14-OFQ and [^{3}H]OFQ to the OFQ-R. Preliminary experiments were carried out to investigate the stability of OFQ radioligand binding. These experiments revealed that although binding parameters were stable from 1 through 3 hr, binding was not stable for longer periods. Incubation at elevated temperatures further decreased the stability of the binding preparation. Thus, an incubation time of 1 hr at room temperature was chosen for saturation analysis. In additional experiments, a variety of protease inhibitors were tested for their ability to enhance the stability of binding; only 0.01% bacitracin was found to have a stabilizing effect, and it was routinely included in the binding assays.

Under these experimental conditions, incubation of increasing concentrations of [^{3}H]OFQ with membranes from 293/OFQ-R cells revealed specific, saturable, and high affinity binding. Nonspecific binding represented < 10% of the total binding observed at all concentrations tested. Nonlinear curve fitting of the saturation isotherm revealed the presence of two affinity sites for [^{3}H]OFQ binding (Fig. 2A), with high and low affinity K_d values of 8.44 ± 1.33 (four experiments) and 229 ± 55.8 (four experiments) pM respectively, and B_{max} values of 1.61 ± 0.25 (four experiments) and 1.82 ± 0.37 (four experiments) pmol/mg of protein for the high and low affinity sites, respectively. These affinity values correspond well with the two affinity values determined from kinetic experiments (see Table 1), as do the proportion of high and low affinity sites that were observed. In accordance with detection of two affinity sites, Scatchard transformation of the saturation binding data was curvilinear (Fig. 2A, *inset*).

Saturation analysis of ^{125}I-Tyr14-OFQ binding to 293/OFQ-R cell membranes likewise revealed binding to two specific and saturable affinity sites, as indicated by a curvilinear Scatchard transformation (data not shown). Nonlinear curve fitting yielded K_d values of 20.93 ± 3.73 (three experiments) and 469.63 ± 65.6 (three experiments) pM and corresponding B_{max} values of 1.87 ± 0.73 (three experiments) and 2.66 ± 0.73 (three experiments) pmol/mg of protein (Table 1).

In CHO/OFQ-R cell membranes, ^{125}I-Tyr14-OFQ also exhibited specific and saturable high affinity binding, with a K_d value of 53.86 ± 7.3 pM (12 experiments) and a B_{max} value of 115 ± 16 fmol/mg of protein (12 experiments) (Fig. 2B). In these cells, the saturation isotherm was monophasic, as shown by a linear Scatchard transformation of the data (Fig. 2B, *inset*) and Hill slopes that were not significantly different from 1. Nonspecific binding was ~15% of total binding at 50 pM ^{125}I-Tyr14-OFQ.

Effect of monovalent and divalent cations on OFQ radioligand binding. To determine whether cations are able to modulate the binding of OFQ ligands to the OFQ-R, we investigated the effects of several different cations on [^{3}H]OFQ binding to 293/OFQ-R membranes and compared them with the effects on ^{125}I-Tyr14-OFQ binding to CHO/OFQ-R membranes. The effect of the cation Mg^{2+} was first examined. Decreasing the $MgCl_2$ concentration to 1 mM had no significant effect on either ^{125}I-Tyr14-OFQ or [^{3}H]OFQ binding, as determined by saturation analysis (data not shown). However, saturation binding carried out in 0.125 mM $MgCl_2$ yielded decreased binding levels that could not be appropriately analyzed (data not shown).

Because Na^+ is well known to modulate agonist binding in numerous receptor systems, saturation isotherms for the binding of [^{3}H]OFQ were carried out in the presence and absence of NaCl in standard binding buffer containing 5 mM $MgCl_2$ (Fig. 3). The addition of 5 mM NaCl had a minimal effect on [^{3}H]OFQ binding, reducing the total binding capacity by~10%, with no significant change in the affinity of either binding site or the proportion of the two affinity sites

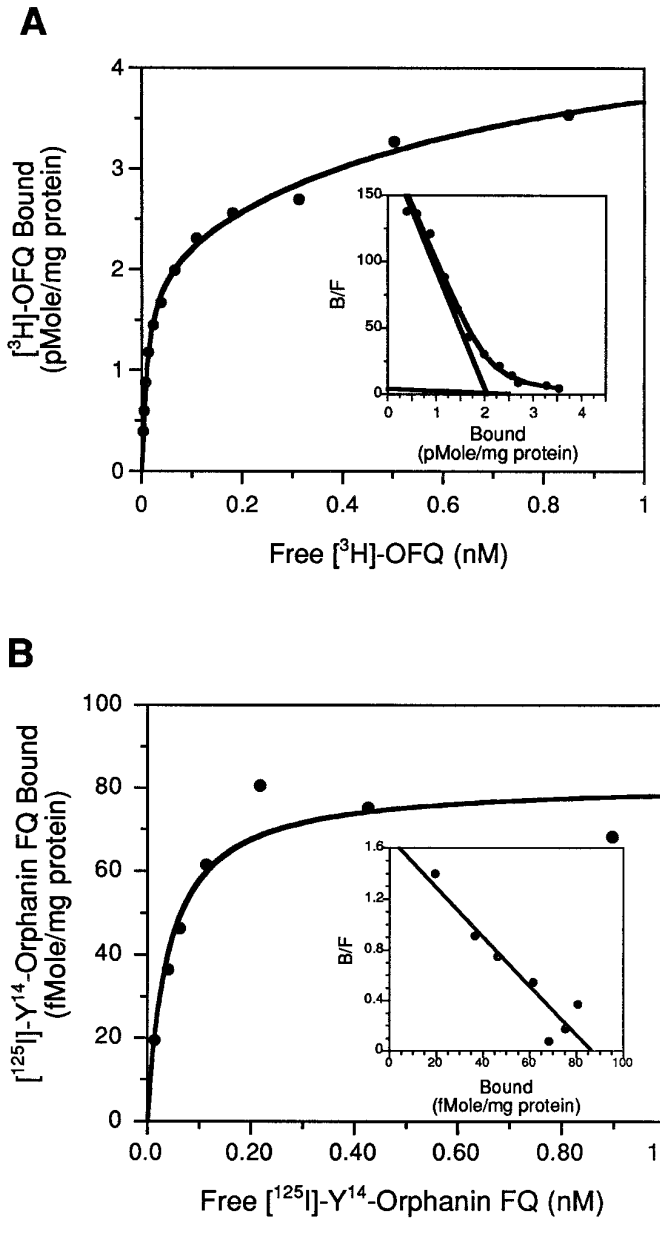

Fig. 2. Saturation binding of [^{3}H]OFQ to 293/OFQ-R membranes and of ^{125}I-Tyr14-OFQ to CHO/OFQ-R membranes. A, Saturation binding of [^{3}H]OFQ binding to 293/OFQ-R membranes. 293/OFQ-R membranes were incubated with increasing concentrations of [^{3}H]OFQ (3- 800 pM) in a final volume of 2 ml for 1 hr at room temperature. Incubations were terminated by rapid filtration as described in Materials and Methods. Nonspecific binding was determined in the presence of 50 nM cold OFQ and was < 10% of total binding at all concentrations tested. Specific binding only is shown for the sake of clarity. *Inset*, Scatchard transformation of specific [^{3}H]OFQ binding. B, Saturation binding of ^{125}I-Tyr14-OFQ to CHO/OFQ-R membranes. CHO/OFQ-R membranes were incubated with increasing concentrations of ^{125}I-Tyr14-OFQ (2- 900 pM) in a final volume of 200 μl for 1 hr at room temperature. Incubations were terminated by rapid filtration as described in Materials and Methods. Nonspecific binding was determined in the presence of 50 nM cold OFQ and was generally ~15% of total binding at 50 nM ^{125}I-Tyr14-OFQ. Specific binding only is shown for the sake of clarity. *Inset*, Scatchard transformation of specific ^{125}I-Tyr14-OFQ binding. Data shown are of representative experiments performed in triplicate.

(data not shown). However, increasing the concentration of Na^+ from 5 to 100 mM reduced specific [^{3}H]OFQ binding with an IC_{50} value of 23 mM (Table 2). The addition of NaCl to 120 mM resulted in the detection of only a single affinity state with a K_d value of 56 ± 1.1 (two experiments) pM and a 68% reduction in binding capacity (Fig. 3). The binding of ^{125}I-Tyr14-OFQ to CHO/OFQ-R membranes was also sensitive to the presence of NaCl. In the presence of 5 mM NaCl, the K_d value was increased to 165 ± 2.0 (three experiments) pM, whereas the B_{max} value was not significantly changed (90 fmol/mg of protein) (data not shown). On increasing the NaCl concentration from 5 to 100 mM, specific binding of 0.1 nM ^{125}I-Tyr14-OFQ was reduced by ≈80%, with an IC_{50} value of 20 mM NaCl (Fig. 4 and Table 2).

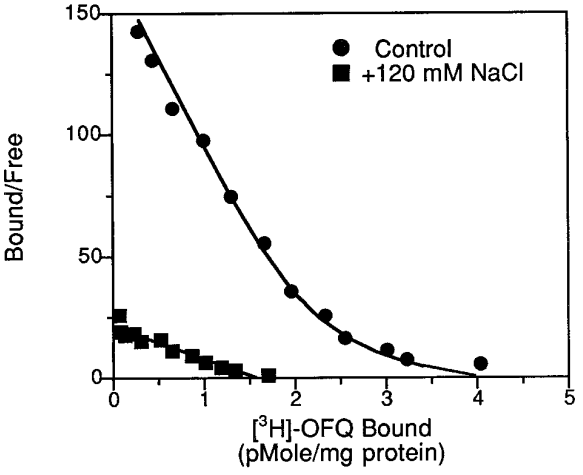

Fig. 3. Effect of sodium on the binding of [^{3}H]OFQ to 293/OFQ-R membranes. Scatchard transformation of specific [^{3}H]OFQ saturation binding to 293/OFQ-R membranes in the absence (●) or presence (■) of 120 mM NaCl. Saturation binding assays were carried out and analyzed as described in Materials and Methods. Data are representative of four independent experiments carried out in duplicate.

TABLE 2

Effect of cations on OFQ radioligand binding

CHO/OFQ-R or 293/OFQ-R membranes prepared in buffer A containing reduced $MgCl_2$ (0.125 mM final concentration) were incubated with 0.1 nM ^{125}-Tyr14-OFQ or 0.2 nM [^{3}H]OFQ, respectively, in the presence of increasing concentrations of various cations as described in Materials and Methods. IC_{50} values were determined by nonlinear curve fitting. Experiments were performed twice in triplicates, and values represent the average ± range.

	IC_{50}	
	^{125}I-Tyr14-OFQ CHO/OFQ-R	[^{3}H]OFQ 293/OFQ-R
	mM	
$CaCl_2$	3.65 ± 0.46	3.0 ± 0.17
$MnCl_2$	2.79 ± 0.30	1.5 ± 0.09
$ZnCl_2$	0.66 ± 0.02	1.0 ± 0.3
NaCl	20.0 ± 4.0	23 ± 5.0

The effects of Ca^{2+}, Mn^{2+}, and Zn^{2+} on the binding of both radioligands was also examined. In these experiments, membranes were diluted in buffer A with a final Mg^{2+} concentration of 0.125 mM; then, the appropriate concentration of the ion investigated was added separately. The addition of $CaCl_2$, $MnCl_2$, and $ZnCl_2$ (0.5- 100 mM) caused reductions in the specific binding of both ligands, with IC_{50} values of 0.6–4 mM when 0.1 nM ^{125}I-Tyr14-OFQ (Fig. 4 and Table 2) or 0.2 nM [^{3}H]OFQ (Table 2) was used. Taken together, these results indicate that binding of both ligands is sensitive to the presence of Na^+, Ca^{2+}, Mn^{2+}, and Zn^{2+}, whereas optimal specific binding is achieved in the presence of 5 mM $MgCl_2$.

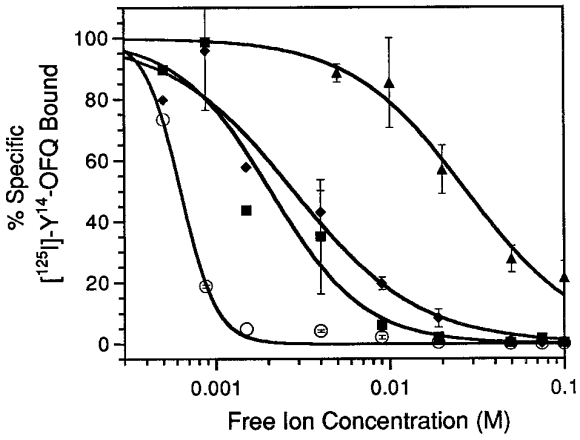

Fig. 4. Inhibition of ^{125}I-Tyr14-OFQ binding to CHO/OFQ-R membranes by various divalent ions. Increasing concentrations of $ZnCl_2$ (○), $CaCl_2$ (♦), $MnCl_2$ (■), or NaCl (▲) were added to CHO/OFQ-R membranes prepared in buffer A containing 0.125 mM $MgCl_2$. Incubations were carried out with 0.1 nM ^{125}I-Tyr14-OFQ for 1 hr at room temperature before termination of the assay by filtration as described in Materials and Methods. Nonspecific binding was estimated using 50 nM cold OFQ. Specific ^{125}I-Tyr14-OFQ binding in the absence of added ions (other than $MgCl_2$) was ~12,600 dpm, whereas nonspecific binding was ~2200 dpm. Values represent the percentage of specific binding in the absence of added ions and are mean ± standard error of three independent experiments performed in triplicate.

Effect of guanine nucleotides on the binding of OFQ ligands. Coupling of the OFQ-R with G proteins was explored by evaluating the effect of guanine nucleotides on [^{3}H]OFQ and ^{125}I-Tyr14-OFQ binding. The addition of 200 μM of the nonhydrolyzable GTP analog Gpp(NH)p to 293/OFQ-R membranes affected the binding of [^{3}H]OFQ by shifting the proportion of high to low affinity sites from 47% high/53% low to 15% high/85% low (Fig. 5A). There was no significant change in the total binding capacity or in the K_d value of either affinity site. In CHO/OFQ-R membranes, the addition of 200 μM Gpp(NH)p reduced the affinity of ^{125}I-Tyr14-OFQ binding by 43%, from 57 to 134 pM, with little change in the B_{max} value (94–83 fmol/mg of protein) (Fig. 5B). Under these conditions, nonlinear curve fitting revealed the presence of only a single class of binding sites, as was observed in the absence of Gpp(NH)p.

The specificity of the guanine nucleotide effect was investigated by comparing the ability of several nucleotides to reduce the binding of [^{3}H]OFQ (40 pM). The addition of increasing concentrations (10^{-10} to 10^{-3} M) of Gpp(NH)p, GTP, and GDP resulted in ≈50–60% reductions in specific [^{3}H]OFQ binding, with 50% of the effect achieved at ~1 μM (Fig. 6). The nucleotides GMP, ATP, and AMP had no effect on [^{3}H]OFQ binding.

^{125}I-Tyr14-OFQ binding in rat brain and peripheral tissue membranes. Because the mRNA for OFQ-R has been shown to be widely distributed in the brain (1, 2), we attempted to determine whether ^{125}I-Tyr14-OFQ could be used to detect OFQ-R binding activity in membranes prepared from different brain regions. As shown in Fig. 7, specific, saturable ^{125}I-Tyr14-OFQ binding could be detected in membranes prepared from cerebral cortex. Analysis of the saturation isotherm yielded a K_d value of 65.9 ± 6.8 pM (three experiments), which is in good agreement with the value observed in CHO/OFQ-R cells. Next, we evaluated the level

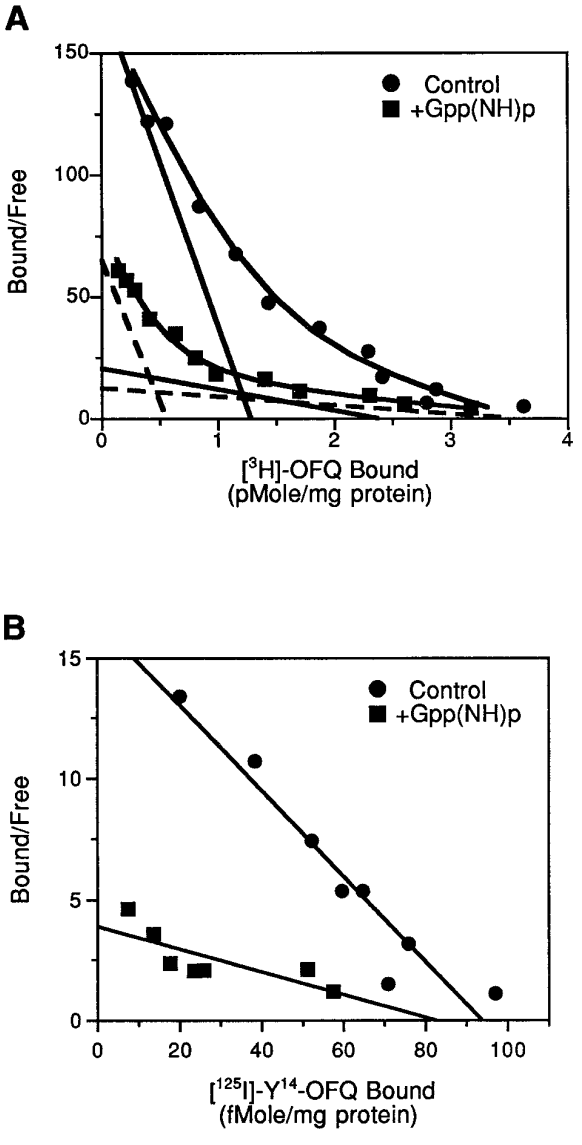

Fig. 5. Effect of Gpp(NH)p on the binding of [^{3}H]OFQ to 293/OFQ-R membranes and of ^{125}I-Tyr14-OFQ to CHO/OFQ-R membranes. A, Scatchard transformation of [^{3}H]OFQ saturation binding to 293/OFQ-R membranes in the absence (●) or presence (■) of 200 μM of Gpp(NH)p. Nonlinear curve fitting confirmed the presence of two affinity states under both conditions. *Solid lines*, the two calculated affinity sites for the control condition. *Dashed lines*, the two calculated affinity sites for the +Gpp(NH)p condition. B, Scatchard transformation of ^{125}I-Tyr14-OFQ saturation binding to CHO/OFQ-R membranes in the absence (●) or presence (■) of 200 μM of Gpp(NH)p. Nonlinear curve fitting indicated the presence of a single affinity state under both conditions. For both A and B, membranes were incubated with increasing amounts of radioligand in the presence or absence of Gpp(NH)p for 1 hr at room temperature as described in Materials and Methods. Data are representative of two or three experiments performed in triplicate.

of OFQ-R expressed in different rat brain regions. Table 3 indicates that highest expression level of OFQ-R was detected in the hypothalamus, followed by the hippocampus, thalamus, and cortex (507, 489, 249, and 226 fmol/mg of protein, respectively). Other brain regions, including the striatum, cerebellum, and midbrain, contained modest-to-low levels of OFQ-R with no detectable binding in the pituitary. Again, analysis by nonlinear curve fitting revealed only a single class of binding sites in all regions examined, with Hill

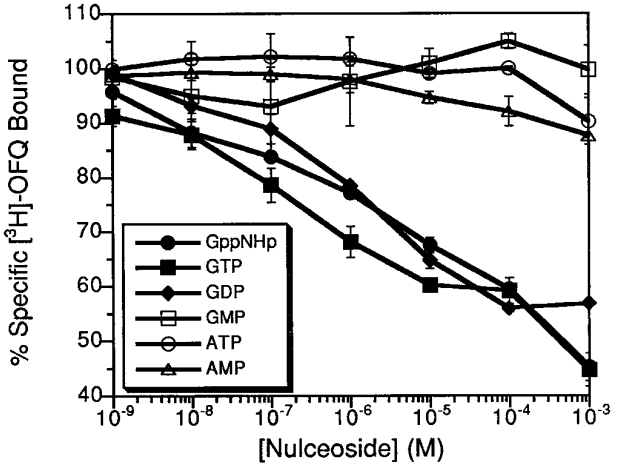

Fig. 6. Effect of nucleotides on [^{3}H]OFQ binding to 293/OFQ-R membranes. Membranes from 293/OFQ-R cells were incubated with 40 pM [^{3}H]OFQ and increasing concentrations of the indicated nucleotides for 1 hr at room temperature before filtration as described in Materials and Methods. Data represent the percentage of specific [^{3}H]OFQ bound in the absence of added nucleotide and are mean ± standard error of three independent experiments performed in duplicate.

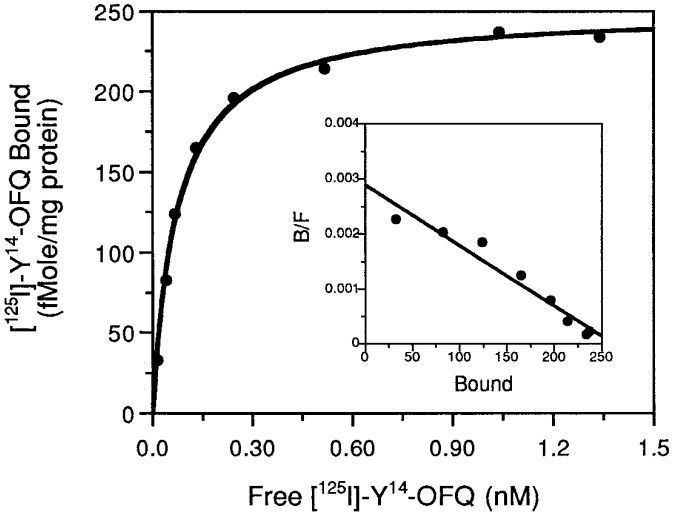

Fig. 7. Saturation binding of ^{125}I-Tyr14-OFQ binding to rat cerebral cortex membranes. Specific binding of increasing concentrations of ^{125}I-Tyr14-OFQ (23 pM to 2 nM) to membranes from rat cerebral cortex. Nonspecific binding was defined with 50 nM OFQ as described in Materials and Methods. Total and nonspecific binding data are not shown for the sake of clarity. *Inset*, Scatchard transformation of specific ^{125}I-Tyr14-OFQ binding. Data are representative of three experiments performed in triplicate.

coefficients close to unity. Interestingly, the apparent affinity of ^{125}I-Tyr14-OFQ in all brain regions was in close agreement with the K_d value for CHO/OFQ-R cells (Table 3). No specific binding of ^{125}I-Tyr14-OFQ was detected in peripheral tissues, including the spleen, thymus, kidney, intestine, heart, lung, and skeletal muscle.

To verify that ^{125}I-Tyr14-OFQ binding in cortical membranes represents specific interaction with the native OFQ-R, we examined the ability of several alanine-substituted OFQ analogs to inhibit ^{125}I-Tyr14-OFQ binding to cortex membranes. We have previously shown that alanine substitution at positions 1, 4, and 8 in the OFQ molecule (Ala1-OFQ, Ala4-OFQ, Ala8-OFQ, respectively) negatively affects

TABLE 3

Distribution of ^{125}I-Tyr14-OFQ binding in different regions of rat brain

Saturation binding of ^{125}I-Tyr14-OFQ to membranes prepared from various rat brain regions was carried out as indicated in Material and Methods. K_d and B_{max} values were determined by nonlinear curve fitting to the saturation binding data. The results are given as average ± standard error calculated from three independent experiments in triplicate.

Brain region	B_{max}	K_d
	fmol/mg of protein	*pM*
Hypothalamus	507 ± 23	49.8 ± 2.2
Hippocampus	489 ± 34	45.1 ± 15.7
Thalamus	249 ± 7	49.0 ± 5.0
Cortex	226 ± 15	65.9 ± 6.8
Striatum	121 ± 13	70.6 ± 12.4
Midbrain	72 ± 25	42.3 ± 6.8
Cerebellum	85 ± 4	59.0 ± 4.4

biological activity as measured by the inability of these analogs to inhibit forskolin-stimulated cAMP accumulation in CHO/OFQ-R cells and by their reduced binding affinity compared with native OFQ (5). These analogs were thus used as pharmacological agents to further characterize the site of ^{125}I-Tyr14-OFQ binding in cortical membranes. Displacement of ^{125}I-Tyr14-OFQ binding in cortex membranes by these substituted OFQ analogs was in agreement with K_i values determined in membranes from CHO/OFQ-R cells (Table 4), with the rank order of potency being OFQ > Ala1-OFQ = Ala8-OFQ > Ala4-OFQ. These results indicate that ^{125}I-Tyr14-OFQ binds to a site in cortex membranes that is phamacologically indistinguishable from the cloned OFQ-R expressed in CHO cells.

Discussion

In the present study, we characterized the interaction of two peptidergic radioligands with the receptor for a novel neuropeptide, OFQ. The results of this study revealed that both the tritiated, native OFQ ([^{3}H]OFQ) and the radioiodinated, Tyr14-substituted analog (^{125}I-Tyr14-OFQ) interacted with the OFQ-R in a virtually identical manner. These radioligands met the criteria of saturability, reversibility, and low nonspecific binding that are necessary for useful radioligands. In addition, the binding of these radioligands was found to be modulated by environmental factors known to modulate agonist binding in other receptor systems.

In our first studies of [^{3}H]OFQ and ^{125}I-Tyr14-OFQ bind-

TABLE 4

Inhibition of ^{125}I-Tyr14-OFQ binding by OFQ and alanine-substituted OFQ analogs in CHQ OFQ-R and rat cerebral cortex membranes

Membranes prepared from rat cerebral cortex were incubated with 100 pM ^{125}I-Tyr14-OFQ and increasing concentrations of the indicated peptides. Data were analyzed and K_i values calculated with LIGAND. Values determined in CHO OFQ-R membranes are taken from Ref. 5. Results are the mean ± standard error of three independent determinations, each performed in triplicate.

Peptide	K_i	
CHO/OFQ-R	Rat cerebral cortex	
	nM	
OFQ	0.196 ± 0.21	0.10 ± 0.04
[Ala1]-OFQ	20.30 ± 6.8	13.30 ± 1.19
[Ala4]-OFQ	36.9 ± 1.6	26.06 ± 3.09
[Ala8]-OFQ	18.1 ± 6.9	16.13 ± 1.07

ing, we examined the kinetics of association and dissociation from the OFQ-R expressed in CHO or HEK 293 cells. Both radioligands associated rapidly, in a monophasic manner, with the OFQ-R, with $t_{1/2}$ values of 201 and 158 sec for 0.2 nM [^{3}H]OFQ and 0.1 nM ^{125}I-Tyr14-OFQ, respectively. The binding of both ligands was stable for 60 min at room temperature and was blocked by an excess of OFQ peptide. Dissociation of both [^{3}H]OFQ and ^{125}I-Tyr14-OFQ from OFQ-R expressed in HEK 293 cells occurred in a distinctly biphasic manner. In contrast, dissociation of ^{125}I-Tyr14-OFQ from OFQ-R expressed in CHO cells occurred in a monophasic manner, with a relatively long $t_{1/2}$ value of 3.2 hr. Comparison of the slow components of dissociation from 293/OFQ-R with dissociation of ^{125}I-Tyr14-OFQ from CHO/OFQ-R suggested that both ligands recognize the same high affinity state of the receptor in both cell lines but that the low affinity state is not present in the CHO cell line. Such a result may be due to the difference in expression level of OFQ-R between the two cell lines and suggests that in the CHO cell line, the OFQ-R may be very tightly coupled to G proteins.

In accord with the clear presence of two affinity states for agonist binding to the OFQ-R in HEK 293 cells, as revealed by kinetic experiments, two affinity states were likewise observed in saturation binding experiments. The affinities for these sites, calculated by nonlinear curve fitting of the saturation isotherm, agreed well with the kinetically determined values for both the high and low affinity sites for both radioligands, indicating that under the conditions used for saturation binding, both radiolabeled OFQ ligands bind to two affinity states of the OFQ-R according to the law of mass action.

The presence of two affinity states for agonist binding is common in G protein-coupled receptors and is often taken to indicate a functional interaction with signal-transducing G proteins. That this is the case for the OFQ-R is demonstrated by the effect of the nonhydrolyzable guanine nucleotide analog Gpp(NH)p on radiolabeled OFQ binding. In the 293/OFQ-R cells, the addition of Gpp(NH)p resulted in a shift in the proportion of high to low affinity states of the OFQ-R. In the absence of guanine nucleotides, ~50% of the OFQ-R is in the high affinity state, presumably coupled to G proteins. In the presence of Gpp(NH)p, the percentage of high affinity sites is reduced to 15%, whereas the total number of binding sites remains virtually the same. This indicates that the bulk of the receptor has been shifted to the low affinity state, which is considered to be uncoupled from G proteins. In CHO/OFQ-R cells, 100% of the receptor appears to be in the high affinity, G protein-coupled state in the absence of guanine nucleotide, as demonstrated by monophasic kinetic and saturation binding analysis. The addition of Gpp(NH)p to membranes from these cells resulted in virtually complete conversion of receptor sites from high affinity to low affinity. That this effect is mediated by G proteins is indicated by the finding that only guanine, not adenine, nucleotides are able to modulate OFQ radioligand binding.

For G protein-coupled receptors, ligand binding, and agonist ligand binding in particular, is often also modulated by the presence of ions such as Na^+ and Mg^{2+}. In general, Na^+ seems to reduce the affinity of agonist binding, whereas antagonist binding is less affected (10). In contrast, Mg^{2+} tends to promote high affinity agonist binding (Refs. 10–21 and references therein). In the case of the OFQ-R, and independent of the radioligand used, Mg^{2+} concentrations of 1–5 mM were required to demonstrate high affinity binding. Surprisingly, Mg^{2+} concentrations of < 1 mM did not give rise to a low affinity uncoupled state of the receptor as predicted but instead yielded an unusual binding mode, suggesting that alternative sites of action for Mg^{2+} may need to be considered for the OFQ-R (11).

On the other hand, specific binding of radioligands to the OFQ-R was decreased by increasing concentrations of Na^+ with an IC_{50} value of 20 mM. Although the site for Na^+ modulation of ligand binding has been proposed to be a conserved aspartic acid residue in transmembrane region 2 for a wide variety of G protein-coupled receptors, including the rat OFQ-R, the precise molecular mechanism remains to be elucidated (22).

Other divalent cations were also found to modulate the binding of both OFQ radioligands. The addition of Mn^{2+} and Ca^{2+} to the binding buffer resulted in inhibition of specific binding; this is surprising considering that Mn^{2+} and Ca^{2+} have been reported to improve agonist and antagonist binding to opiate receptors (9). It is also interesting that neither of these cations is able to substitute for the Mg^{2+} required for high affinity binding of the OFQ radioligands. At the present time, we are unable to account for these discrepancies because our knowledge on the mechanism of action of the cations is limited. In contrast, the inhibition of OFQ binding by Zn^{2+} agrees well with a previous finding regarding the effect of this cation on enkephalin binding to opiate receptors (23).

Having shown that ^{125}I-Tyr14-OFQ is a high specific activity agonist and a valid ligand for heterologously expressed OFQ-Rs, we used it to quantify OFQ binding sites in different tissues. The highest levels of ^{125}I-Tyr14-OFQ binding were found in the hypothalamus and hippocampus, followed by the thalamus and cerebral cortex. Lower levels of binding were observed in the striatum, midbrain, and cerebellum. In all regions where specific binding was observed, ^{125}I-Tyr14-OFQ bound to a single class of saturable, high affinity sites, with K_d values that were not significantly different from the those for OFQ-R expressed in CHO cells. Interestingly, saturation binding analysis revealed that in general, ^{125}I-Tyr14-OFQ binding correlated very well with the abundance and distribution of mRNA for the OFQ-R previously reported by several groups (1, 2, 24).

We chose to further characterize the properties of ^{125}I-Tyr14-OFQ binding in brain by examining the ability of several OFQ peptide analogs to displace its binding from cortical membranes. For all peptides tested, both the affinity and rank order of inhibition of ^{125}I-Tyr14-OFQ binding were similar to those seen in membranes from CHO cell membranes. These results indicate that in brain membranes, ^{125}I-Tyr14-OFQ labels a receptor site with pharmacological properties identical to those exhibited by the OFQ-R heterologously expressed in CHO cell membranes.

The results presented here demonstrate that the radioligands ^{125}I-Tyr14-OFQ and [^{3}H]OFQ are high specific activity agonists that can be used for analyzing the properties and functions of the OFQ-R in transfected cells and native tissue preparations. Both ligands behave in a nearly identical manner; however, each ligand offers distinct advantages. ^{125}I-Tyr14-OFQ, with its very high specific activity, will be useful for studies of the OFQ-R in native tissues in which expression levels may be relatively low. [^{3}H]OFQ, on the other

hand, has the advantage of being identical to the native peptide and thus is likely to be a preferable ligand for detailed studies of the interactions between the OFQ peptide and its receptor, as well as for pharmacological characterizations of the OFQ-R. Given their respective advantages, the use of these radioligands will promote an enhanced understanding of the biology of this novel neuropeptide system at the molecular level.

Acknowledgments

We thank Dr. Andrew J. Sleight for advice and critical reading of the manuscript.

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