COMPARISON OF [DMT^1]DALDA AND DAMGO IN BINDING AND G PROTEIN ACTIVATION AT MU, DELTA AND KAPPA OPIOID RECEPTORS

Guo-Min Zhao, Xuanxuan Qian, Peter W. Schiller and Hazel H. Szeto

Department of Pharmacology, Joan and Sanford I. Weill Medical College of Cornell University, New York, N.Y., 10021, USA (G.-M.Z., X.Q., H.H.S.) and Laboratory of Chemical Biology and Peptide Research, Clinical Research Institute of Montreal, Montreal, Quebec, Canada H2W 1R7 (P.W.S.).
Running Title: [Dmt^1]DALDA and DAMGO binding and G protein activation

Corresponding author: Hazel H. Szeto, M.D., Ph.D.
Department of Pharmacology
Weill Medical College of Cornell University
1300 York Avenue
New York, N.Y. 10021
Tel: 212-746-6232; Fax: 212-746-8835
Email: hhszeto@med.cornell.edu

Number of text pages: 15
Number of tables: 5
Number of figures: 5
Number of references: 22
Number of words in Abstract: 246
Number of words in Introduction: 458
Number of words in Discussion: 1024

Abbreviations:
A_2pr, α,β-diaminopropionic acid; [Dmt^1]DALDA, H-Dmt-D-Arg-Phe-Lys-NH_2; Dmt = 2',6'-dimethyltyrosine; [^{35}S]GTP\_γS, guanosine 5'-O-(3-[^{35}S]thiotriphosphate); hMOR, cloned human μ opioid receptor; hDOR, cloned human δ opioid receptor; hKOR, cloned human κ opioid receptor; DAMGO, H-Tyr-D-Ala-Gly-NMePhe-Gly-ol; DPDPE, H-Tyr-D-Pen-Gly-Phe-D-Pen; DSLET, H-Tyr-D-Ser-Gly-Phe-Leu-Thr-OH; U50,488H, [trans-(±)-3,4-dichloro-N-methyl-[2-(1-pyridinyl)-cyclohexyl] benzeneacetamide; U69,593, (5α,7α,8β)-(+-)N-methyl-N-(7-[1-pyrrolidinyl]-1-oxaspiro[4.5]dec-8-yl)-benzeneacetamide;
Abstract

[(Dmt\(^1\))DALDA (H-Dmt-D-Arg-Phe-Lys-NH\(_2\); Dmt = 2',6'-dimethyltyrosine) binds with high affinity and selectivity to the \(\mu\) opioid receptor and is a surprisingly potent and long-acting analgesic, especially after intrathecal administration. In an attempt to better understand the unique pharmacological profile of [(Dmt\(^1\))DALDA, we have prepared \([3H][(Dmt\(^1\))DALDA and compared its binding properties with that of \([3H]\)DAMGO. Kinetic studies revealed rapid association of \([3H][(Dmt\(^1\))DALDA when incubated with mouse brain membranes \((K_{\text{a}} = 0.155 \text{ nM}^{-1} \text{ min}^{-1})\). Dissociation of \([3H][(Dmt\(^1\))DALDA was also rapid \((K_{\text{d}} = 0.032 \text{ min}^{-1})\) and indicated binding to a single site. \([3H][(Dmt\(^1\))DALDA binds with very high affinity to hMOR \((K_d = 0.199 \text{ nM})\), and \(K_d\) and Bmax were reduced by sodium but not Gpp(NH)p. Similar \(K_d\) values were obtained in brain and spinal cord tissues and SH-SY5Y cells. The hMOR:hDOR selectivity of [(Dmt\(^1\))DALDA (~10,000) is 8-fold higher than DAMGO. However, [(Dmt\(^1\))DALDA is less selective than DAMGO against hKOR (26-fold versus 180-fold). The \(K_i\) values for a number of opioid ligands were generally higher when determined by competitive displacement binding against \([3H][(Dmt\(^1\))DALDA compared to \([3H]\)DAMGO, with the exception of Dmt\(^1\)-substituted peptide analogs. All Dmt\(^1\) analogs showed much higher affinity for the \(\mu\) receptor than corresponding Tyr\(^1\) analogs. \([35S]\)GTP\(\gamma\)S binding showed that [(Dmt\(^1\))DALDA and DAMGO are full agonists at hMOR and hDOR, but are only partial agonists at hKOR. The very high affinity and selectivity of \([3H][(Dmt\(^1\))DALDA for the \(\mu\) receptor, together with its very low non-specific binding (10-15%) and metabolic stability, make \([3H][(Dmt\(^1\))DALDA an ideal radioligand for labeling \(\mu\) receptors.
Opioid receptors belong to the superfamily of guanine nucleotide binding protein-coupled receptors. Early pharmacological and biochemical studies led to the proposal of three major subtypes of opioid receptors (µ, δ and κ), which were subsequently confirmed by molecular cloning efforts (Snyder and Pasternak, 2003). The availability of the cloned receptors (MOR, DOR and KOR) allows studies of individual receptor subtypes with regard to receptor signaling pathways and pharmacological profiles. However, ligands with high selectivity for the individual receptor subtypes are necessary for the study of their functional roles in biological tissues or animals, and as therapeutic agents for clinical use. In particular, highly selective radioligands are invaluable for quantifying receptor population in biological tissues and for characterization of novel ligands in competitive displacement binding assays.

\[\text{[Dmt}^1\text{]}\text{DALDA} (\text{H-Dmt-D-Arg-Phe-Lys-NH}_2; \text{where Dmt} = 2',6'\text{-dimethyltyrosine}) is a new dermorphin analog that has very high affinity and selectivity for the \(\mu\) receptor (Schiller et al., 2000). In competitive displacement binding studies against \(\left[^3\text{H}\right]\text{DAMGO} (\text{H-Tyr-D-Ala-Gly-NMePhe-Gly-ol}) in mouse brain membranes, the Ki for \([\text{Dmt}^1]\text{DALDA} was found to be 0.143 \pm 0.015 \text{ nM at the } \mu \text{ receptor. By comparing its Ki against } [^3\text{H}]\text{DAMGO binding and } [^3\text{H}]\text{DSLET (H-Tyr-D-Ser-Gly-Phe-Leu-Thr-OH)} binding, the } \mu:\delta \text{ selectivity of } [\text{Dmt}^1]\text{DALDA was determined to be 14,700. [Dmt}^1\text{]}\text{DALDA also displayed good } \mu:\kappa \text{ selectivity in displacement binding against } [^3\text{H}]\text{U69,593, with Ki}^\kappa/\text{Ki}^\mu = 156. \text{ In comparison, the affinity of DAMGO for the } \mu \text{ opioid receptor was reported to be } \sim 1.2 \text{ nM, with } \mu:\delta \text{ selectivity of only 1050 (Schiller et al. 2000). [Dmt}^1\text{]}\text{DALDA behaved as a full agonist in the guinea pig ileum assay (Schiller et al. 2000).}

\[\text{[Dmt}^1\text{]}\text{DALDA is a highly potent and long-acting analgesic after both spinal or supraspinal administration, although it is more potent when given intrathecally (Neilan et al.,}

\[\text{2000).}

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JPET/2003/054775-R2

2001; Shimoyama et al., 2001; Zhao et al., 2002; Riba et al., 2002). The potency of [Dmt\(^1\)]DALDA in the spinal cord (1000-3000 times that of morphine) is substantially higher than would be suggested by its affinity at the \(\mu\) receptor (only 7-fold higher than morphine). The extraordinary potency of [Dmt\(^1\)]DALDA in the spinal cord, and the inability of naloxonazine (\(\mu_1\) antagonist) to inhibit supraspinal [Dmt\(^1\)]DALDA, led to the suggestion that [Dmt\(^1\)]DALDA and morphine may act at different \(\mu\) receptors (Neilan et al. 2001).

In an attempt to better understand the unique pharmacological profile of [Dmt\(^1\)]DALDA, we have prepared \(^3\)H[Dmt\(^1\)]DALDA and compared its binding properties with that of \(^3\)H]DAMGO. We have also compared the binding characteristics of [Dmt\(^1\)]DALDA in membrane preparations expressing cloned human opioid receptors (hMOR, hDOR and hKOR), as well as in membranes prepared from SH-SY5Y cells (human neuroblastoma cell line) and brain and spinal cord tissues from mice and rats. Finally, we have compared the potency and intrinsic activity of [Dmt\(^1\)]DALDA and DAMGO in activation of G proteins as measured by \(^35\)S]GTP\(\gamma\)S binding.
Materials and Methods

Drugs and Chemicals. [Dmt\textsuperscript{1}]DALDA (H-Dmt-D-Arg-Phe-Lys-NH\textsubscript{2}; Dmt = 2',6'-dimethyltyrosine) was synthesized according to methods described previously (Schiller et al., 1989; Schiller et al. 2000). DAMGO (H-Tyr-D-Ala-Gly-NMePhe-Gly-ol); DPDPE (H-Tyr-D-Phe-Gly-Phe-D-Pen), \[^{3}H\]DPDPE (42 Ci/mmol, 0.5mCi/ml), [D-Ala\textsuperscript{2}]deltorphin II (H-Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH\textsubscript{2}) and U50,488H ([trans-(±)-3,4-dichloro-N-methyl-[2-(1-pyrolidinyl)-cyclohexyl] benzeneacetamide) were supplied by the National Institute on Drug Abuse (Rockville, Maryland). \[^{3}H\]DAMGO (50 Ci/mmol, 1.0 mCi/ml), \[^{3}H\]U69593 (5\(\alpha\),7\(\alpha\),8\(\alpha\))-(-)-N-methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl] benzeneacetamide; 59.0 Ci/mmol, 1.0mCi/ml) and \[^{35}S\]GTP\(\gamma\)S (1000-1200 Ci/mmol, 1.0 mCi/ml) was purchased from Amersham Pharmacia Biotech Inc. (Piscataway, NJ). All other chemicals were obtained from Sigma Inc. (St. Louis, MO).

Preparation of \[^{3}H\][Dmt\textsuperscript{1}]DALDA. For the preparation of [Dmt\textsuperscript{1}]DALDA in tritiated form, a precursor peptide containing 2',6'-dimethyl-3',5'-diiodotyrosine [Tyr(2',6'-Me\textsubscript{2},3',5'-I\textsubscript{2})] needed to be synthesized. Fmoc-Dmt-OH was iodinated by treatment with I\textsubscript{2} in the usual manner to yield Fmoc-Tyr(2',6'-Me\textsubscript{2},3',5'-I\textsubscript{2})-OH. This protected amino acid was then used in the solid phase synthesis of H-Tyr(2',6'-Me\textsubscript{2},3',5'-I\textsubscript{2})-D-Arg-Phe-Lys-NH\textsubscript{2} according to a protocol published elsewhere (Schiller et al. 2000). The peptide was purified by preparative reversed-phase chromatography and its structure was confirmed by FAB mass spectrometry. Catalytic tritiation of this precursor peptide was performed at the Institute of Isotopes, Budapest, Hungary, resulting in a product with a specific radioactivity of 47.18 Ci/mmol.

Tissue Preparation. Male CD-1 mice (25-30g) and Sprague-Dawley rats (250-300 g) were purchased from Charles River laboratories (Wilmington, MA). All studies were conducted in
accordance with guidelines approved by the Institution for the Care and Use of Animals at Weill Medical College of Cornell University. Mice and rats were decapitated, and brains and spinal cords were removed and stored at –80°C until being used. On the day of experiment, tissue was thawed in 30 volume of ice-cold 50 mM Tris-HCl buffer (0.5 mM EDTA, pH7.4), homogenized for 10 s and centrifuged at 40,000 g for 20 min at 4°C. After this process was repeated a second time, the pellet was re-suspended in Tris-HCl buffer (pH7.4) at a final concentration of 1.5-2.0 mg protein/ml. Protein concentrations were determined by the Bradford procedure (Bio-Rad, Hercules, CA).

**Preparation of Cell Membranes.** Membranes prepared from either CHO-K1 cells transfected with hMOR or hDOR, or HEK293 cells transfected with hKOR were purchased from Perkins Elmer Life Sciences (Boston, MA). SH-SY5Y cells were cultured in DMEM plus 10% FBS, 100 units/ml penicillin, 100 µg/ml streptomycin, and harvested after growing for 4 days to 95% confluence, centrifuged at 500 g for 5 min, and stored in –80°C. On the day of the experiment, cells were homogenized in Tris-HCl buffer (0.5mM EDTA, pH7.4) and centrifuged at 40,000 g for 20 min at 4°C. The pellets were re-suspended in Tris-HCl buffer (pH7.4) at a final concentration of 0.6 mg/ml. Protein concentrations were determined by the Bradford procedure (Bio-Rad, Hercules, CA).

**Radioligand Binding Assay.** Binding assays were performed in 50 mM Tris-HCl buffer (pH 7.4) in a final volume of 0.6 – 1.2 ml. The amount of protein added varied from 12-15 µg for membranes derived from transfected CHO or HEK cells to 300-400 µg in the case of brain or spinal cord membranes. Free radioligand was separated from bound radioligand by rapid filtration through GF/B filters pre-immersed for 45 min in 0.2% PEI solution (Brandel, Gaithersberg, MD). Filters were washed 3 times with 3 ml of Tris-HCl buffer. Radioactivity was
determined by liquid scintillation counting. All binding experiments were carried out in triplicate, and the results represent mean ± S.E. from 4-6 experiments.

**Saturation binding.** Aliquots of membrane homogenates were incubated with $[^3\text{H}][\text{Dmt}^1]\text{DALDA}$ or $[^3\text{H}]\text{DAMGO}$ for 60 min at 25°C and 37°C, respectively. Nonspecific binding was assessed by including 0.1µM unlabeled [Dmt$^1$]DALDA or 10 µM naloxone. Equilibrium dissociation constant ($K_d$) and receptor number ($B_{max}$) were determined using a one-site binding equation and nonlinear regression (Graphpad™, San Diego, CA).

**Binding kinetics.** The association rate constant for $[^3\text{H}][\text{Dmt}^1]\text{DALDA}$ binding was determined by incubating 0.20 nM $[^3\text{H}][\text{Dmt}^1]\text{DALDA}$ with mouse brain membranes at 25°C for various times up to 60 min. The observed rate constant ($K_{ob}$) was determined by fitting the specific binding data to the mono-exponential equation $Y = Y_{max} \times (1 - \exp(-K_{ob}t))$, where $t$ is the time from onset of association, $Y_{max}$ is the maximum specific binding at this concentration. Dissociation of the binding of $[^3\text{H}][\text{Dmt}^1]\text{DALDA}$ to mouse brain membranes was achieved by the addition of high concentrations of unlabelled [Dmt$^1$]DALDA after the 60 min incubation, and the reaction mixture stopped at various times by rapid filtration. The dissociation rate constant ($K_{-1}$) was calculated from exponential decay analysis: $Y = Y_{max} \times \exp(-K_{-1}t)$. The association rate constant ($K_{+1}$) was determined from $K_{+1} = (K_{ob} - K_{-1}) / [L]$, where $[L]$ is the radioligand concentration.

**Competitive displacement binding.** Binding affinities of other opioid ligands to hMOR was determined by competitive displacement binding with graded concentrations of unlabeled ligand incubated with 0.10 nM $[^3\text{H}][\text{Dmt}^1]\text{DALDA}$ for 60 min at 25°C. Non-specific binding was determined using 0.1 µM [Dmt$^1$]DALDA. The selectivity of [Dmt$^1$]DALDA and DAMGO for µ, δ and κ receptors was determined with the use of competitive displacement binding assays.
carried out with membranes expressing hMOR, hDOR or hKOR, respectively. hMOR membranes were incubated with 0.100 nM $[^3H][Dmt^1]$DALDA and graded concentrations of unlabeled $[Dmt^1]$DALDA for 60 min at 25°C, and non-specific binding determined using 0.1 μM $[Dmt^1]$DALDA. hDOR membranes were incubated with 2 nM $[^3H]$DPDPE and graded concentrations of $[Dmt^1]$DALDA for 120 min at 25°C, and non-specific binding determined using 2 μM unlabeled DPDPE. hKOR membranes were incubated with 0.8 nM $[^3H]$U69,593 and graded concentrations of unlabeled $[Dmt^1]$DALDA for 80 min at 25°C, and non-specific binding determined with 10 μM naloxone. For all competitive binding assays, IC$_{50}$ was determined from the displacement curves using a one-site model and nonlinear regression (Graphpad™, San Diego, CA). Ki values were calculated from the obtained IC$_{50}$ values by means of the Cheng and Prusoff equation, $K_d = IC_{50}/(1+L/K_d)$, where L and $K_d$ are the concentration and affinity of the radiolabeled ligand in the assay (Cheng and Prusoff, 1973).

$[^3S]$GTPγS Binding Assay. The potency and intrinsic activity of $[Dmt^1]$DALDA and DAMGO at hMOR, hDOR and hKOR were determined using $[^3S]$GTPγS binding. Aliquots of membrane homogenates (6 - 10 μg protein) were incubated with 50 pM $[^3S]$GTPγS and 30 μM GDP in 1 ml Tris buffer (50 mM Tris-HCl, 100 mM NaCl, 5 mM MgCl$_2$, 1 mM DTT, 1 mM EDTA, 0.1%BSA, pH 7.4) in the presence of varying concentrations of $[Dmt^1]$DALDA or DAMGO for 60 min at 30°C. For DOR and KOR membranes, the concentration of GDP was 60 μM and 10 μM, respectively. Nonspecific binding was determined using 10 μM unlabelled GTPγS. Free radioligand was separated from bound radioligand by rapid filtration as described above. Potency (EC$_{50}$) and intrinsic activity (Emax) were determined from dose-response curves analyzed using nonlinear regression (Graphpad™, San Diego, CA). Full agonist activity at the
three receptors were determined using DAMGO for hMOR, deltorphin II at hDOR, and U50,488H at hKOR.
Results

Saturation Binding of \([^{3}\text{H}][\text{Dmt}^{1}]\text{DALDA}\). Saturation binding of \([^{3}\text{H}][\text{Dmt}^{1}]\text{DALDA}\) was carried out using membranes prepared from cells transfected with hMOR (CHO-K1/hMOR). Specific binding of \([^{3}\text{H}][\text{Dmt}^{1}]\text{DALDA}\) was saturable and of high affinity (Fig. 1). The extent of non-specific binding was similar whether it was determined using excess unlabelled [Dmt\textsuperscript{1}]DALDA or naloxone, and was only 10% - 15% of total binding at radioligand concentrations near \(K_d\) value. The \(K_d\) and \(B_{max}\) were determined to be 0.199 ± 0.019 nM, and 926 ± 55 fmol/mg protein, respectively (n=6).

Saturation binding with \([^{3}\text{H}][\text{Dmt}^{1}]\text{DALDA}\) was also carried out using membranes prepared from SH-SY5Y cells, rat brain and spinal cord, and mice brain and spinal cord (Fig. 1). A one-site binding model provided good fit for all binding curves. The \(K_d\) and \(B_{max}\) values for these various membranes are summarized in Table 1. The \(K_d\) for \([^{3}\text{H}][\text{Dmt}^{1}]\text{DALDA}\) was comparable in all membrane preparations and was ~ 0.10 – 0.20 nM. Receptor density was 6-9 fold lower in brain and spinal cord compared to the receptor number expressed on CHO cells. The number of binding sites was very low in undifferentiated SH-SY5Y cells.

Comparison of \([^{3}\text{H}][\text{Dmt}^{1}]\text{DALDA}\) and \([^{3}\text{H}]\text{DAMGO}\) binding. The binding of \([^{3}\text{H}][\text{Dmt}^{1}]\text{DALDA}\) was compared to the binding of \([^{3}\text{H}]\text{DAMGO}\) using membranes prepared from hMOR and mouse brain. Non-specific binding of \([^{3}\text{H}]\text{DAMGO}\) (~10%) was similar to that of \([^{3}\text{H}][\text{Dmt}^{1}]\text{DALDA}\). The affinity of \([^{3}\text{H}]\text{DAMGO}\) for hMOR was 26-fold lower compared to \([^{3}\text{H}][\text{Dmt}^{1}]\text{DALDA}\) (Kd = 3.10 ± 0.53 nM), while \(B_{max}\) was a little lower at 799 ± 47 fmol/mg protein (n=5). With mouse brain membranes, the \(K_d\) for \([^{3}\text{H}][\text{Dmt}^{1}]\text{DALDA}\) and \([^{3}\text{H}]\text{DAMGO}\) were estimated to be 0.123 ± 0.012 nM (n=5) and 1.59 ± 0.14 nM (n=4), respectively. The
receptor density (Bmax) determined with [3H][Dmt1]DALDA (130 ± 9 fmol/mg protein) was not different from that determined using [3H]DAMGO (115 ± 3 fmol/mg protein).

**Effects of sodium and Gpp(NH)p on [3H][Dmt1]DALDA binding.** Both Kd and Bmax of [3H][Dmt1]DALDA binding to mouse brain membranes were significantly affected by the addition of NaCl (100 mM) and Gpp(NH)p (10 µM) (Table 2). However, addition of Gpp(NH)p alone, even at 100 µM, had no effect on either Kd or Bmax, while the addition of NaCl alone resulted in almost 50% decrease in Bmax and 2-3 fold increase in Kd. When the binding assay was carried out under conditions used for [35S]GTPγS binding (100 mM and 30 µM GDP), Kd and Bmax were 0.926 ± 0.17 nM and 940 ± 66 fmol/mg protein, respectively (n=6).

**Kinetics of [3H][Dmt1]DALDA binding.** Kinetic studies revealed rapid association and dissociation of [3H][Dmt1]DALDA when incubated with mouse brain membrane homogenates. The rate of binding of [3H][Dmt1]DALDA was concentration-dependent and specific binding of [3H][Dmt1]DALDA reached steady state after 50 min of incubation at concentration of 0.20 nM (Fig. 3A). Dissociation of specifically bound radioligand was initiated by the addition of 10 µM unlabelled [Dmt1]DALDA. Nonlinear regression of the dissociation curve showed that it was best fit with a monoexponential equation and the dissociation half-life was estimated to be ~21 min). The association rate constant (K+1) and dissociation rate constant (K−1) were determined to be 0.1551 ± 0.0402 nM−1 min−1 (n=6) and 0.0320 ± 0.0074 min−1, respectively (n=6). The equilibrium dissociation constant (Kd) was calculated from these values to be 0.206 nM.

**Competitive displacement binding.** [3H][Dmt1]DALDA binding to mouse brain membranes was completely displaced by increasing concentrations of the opioid ligands shown in Table 3. The Ki values, and the corresponding Ki values determined by displacement of [3H]DAMGO binding are also summarized in Table 3 (Schiller et al. 2000). In general, there was excellent
correlation between Ki([3H][Dmt^1]DALDA) and Ki ([3H]DAMGO) for those peptide analogs with [Dmt^1]. However, Ki([3H][Dmt^1]DALDA) were higher than Ki([3H]DAMGO) for all other ligands. This discrepancy resulted in significantly different relative affinities when the peptide ligands were compared to morphine. The relative affinity of [Dmt^1]DALDA was 7-times greater than morphine when determined using Ki([3H]DAMGO) but 35-fold when determined using Ki([3H][Dmt^1]DALDA). Substitution of Tyr^1 with Dmt^1 increased affinity of these dermorphin (1-4)-tetrapeptide analogs 10-20 fold, with all [Dmt^1] analogs having very high affinity binding (Ki ~ 0.15 nM).

**Binding of [Dmt^1]DALDA and DAMGO to MOR, DOR and KOR membranes.** The selectivity of [Dmt^1]DALDA and DAMGO for µ, δ and κ receptors were determined using hMOR, hDOR or hKOR membranes. Fig. 4A illustrates the competitive displacement curves for [Dmt^1]DALDA against [3H][Dmt^1]DALDA, [3H]DPDPE and [3H]U69,593 binding to hMOR, hDOR and hKOR membranes, respectively. Fig. 4B shows the corresponding competitive displacement curves for DAMGO. The K_d for [3H][Dmt^1]DALDA, [3H]DPDPE and [3H]U69,593 in hMOR, hDOR and hKOR were 0.199 ± 0.019 nM (n=6), 1.95 ± 0.20 nM (n=4) and 0.694 ± 0.08 nM (n=3), respectively. The Ki for [Dmt^1]DALDA and DAMGO at the three receptors are summarized in Table 4. The µ:δ selectivity was almost 10-fold higher for [Dmt^1]DALDA than DAMGO, whereas µ:κ selectivity was higher for DAMGO.

**Effects of [Dmt^1]DALDA in stimulation of GTPγS binding.** The potency and intrinsic activity of [Dmt^1]DALDA and DAMGO at hMOR, hDOR and hKOR were assessed by their ability to stimulate [35S]GTPγS binding and compared to full agonists (DAMGO for hMOR, deltorphin II for hDOR, and U50,488H for hKOR). Fig. 5 illustrates the dose-response curves
for [Dmt$^1$]DALDA and DAMGO in hMOR, hDOR and hKOR membranes. [Dmt$^1$]DALDA and DAMGO acted as agonist at all three receptors. The results are summarized in Table 5.

The EC$_{50}$ for [Dmt$^1$]DALDA at hMOR was 12-fold lower than that of DAMGO. The Emax for [Dmt$^1$]DALDA (335.3%) was not significantly different from the maximal effect elicited by DAMGO (362.3%). The maximal effect elicited by [Dmt$^1$]DALDA (158 ± 7.5%) was also comparable to that elicited by DAMGO (151.0 ± 5.3%) when $[^{35}\text{S}]$GTP$_{\gamma}$S binding was carried out using mouse brain membranes. At hDOR, both [Dmt$^1$]DALDA and DAMGO were able to elicit a maximal response that was larger than that elicited by deltorphin II. By comparing the potency of [Dmt$^1$]DALDA and DAMGO at hMOR and hDOR, their MOR:DOR selectivity was determined to be 118 and 7, respectively. In contrast, both [Dmt$^1$]DALDA and DAMGO were only partial agonists at KOR, with Emax being 56.1% and 79.3%, respectively, compared to U50,488H. The MOR:KOR selectivity was 29 for [Dmt$^1$]DALDA and 37 for DAMGO.
Discussion

The affinity of [Dmt$^1$]DALDA for $\mu$, $\delta$, and $\kappa$ receptors was originally ascertained by displacement of [${}^3$H]DAMGO and [${}^3$H]DSLET binding from rat brain membranes, and displacement of [${}^3$H]U69,593 binding from guinea pig brain membranes, respectively (Schiller et al. 2000). However, brain tissues express multiple subtypes of opioid receptors and these radioligands lack specificity. The availability of [${}^3$H][Dmt$^1$]DALDA and cells transfected with hMOR, hDOR or hKOR allowed us to fully characterize the binding and G protein activation of [Dmt$^1$]DALDA at $\mu$, $\delta$, and $\kappa$ receptors.

Kinetic studies revealed rapid association of [${}^3$H][Dmt$^1$]DALDA when incubated with mouse brain membranes, with $K_{+1}$ of 0.155 nM$^{-1}$ min$^{-1}$. This is a bit faster than the association constant reported for [${}^3$H]diprenorphine (0.116 nM$^{-1}$ min$^{-1}$) (Ott et al., 1986), [${}^3$H]TAPP (H-Tyr-D-Ala-Phe-Phe-NH$_2$) (0.117 nM$^{-1}$ min$^{-1}$) (Spetea et al., 1998), and [${}^3$H]DAMGO (0.0846 nM$^{-1}$ min$^{-1}$) (Zajac and Roques, 1985). Dissociation of [${}^3$H][Dmt$^1$]DALDA was also rapid and binding declined in a monoexponential manner ($K_{-1} = 0.0320$ min$^{-1}$; $t_{1/2} = 21.7$ min), suggesting that [${}^3$H][Dmt$^1$]DALDA was binding to a single site. This is in contrast to previous reports suggesting two different sites for [${}^3$H]diprenorphine (Ott et al. 1986) and [${}^3$H]DAMGO (Brown and Pasternak, 1998).

Equilibrium binding studies showed that [${}^3$H][Dmt$^1$]DALDA binds with very high affinity to hMOR with $K_d$ of 0.199 nM, and this is in agreement with the $K_d$ calculated from the kinetic studies using mouse brain membranes ($K_d = K_{+1}/K_{-1} = 0.206$ nM), as well as the Ki determined against [${}^3$H]DAMGO binding (0.143 nM) (Schiller et al. 2000). Receptor numbers are very high in these membranes, and the Bmax determined with [${}^3$H][Dmt$^1$]DALDA (926 fmol/mg protein) was similar to the Bmax obtained with [${}^3$H]diprenorphine (1080 fmol/mg protein).
protein) in these membranes (provided by the manufacturer). The relative affinity of
$[^3]H[Dmt^1]DALDA$ for hMOR and hDOR ($\sim$10,000) is similar to the earlier value of 14,700
determined by displacement binding using mouse brain membranes (Schiller et al. 2000),
making $[Dmt^1]DALDA$ 8-fold more selective than DAMGO. However, $[^3]H[Dmt^1]DALDA$
also binds hKOR with nanomolar affinity, thus resulting in a $\mu:\kappa$ selectivity (26) that is lower
than previously determined by displacement binding (156) (Schiller et al. 2000), and is lower
compared to DAMGO (180).

The high affinity and selectivity of $[^3]H[Dmt^1]DALDA$ for the $\mu$ receptor, together with
its low non-specific binding, makes it an ideal radioligand for labeling $\mu$ receptors in biological
tissues. The $K_d$ for $[^3]H[Dmt^1]DALDA$ obtained from equilibrium binding studies using brain
and spinal cord tissues from mice and rats were the same as that determined with hMOR. The
low non-specific binding of $[^3]H[Dmt^1]DALDA$ made it particularly good for quantifying low
receptor numbers, such as that expressed on SH-SY5Y cells.

We have compared the use of $[^3]H[Dmt^1]DALDA$ with $[^3]H[DAMGO$ for evaluating the
affinity of other opioid ligands in binding to mouse brain membranes. The $K_i$ determined
against $[^3]H[Dmt^1]DALDA$ were generally higher than the $K_i$ determined against $[^3]H[DAMGO$
binding, with the exception of peptide analogs that had a Dmt$^1$ substitution for Tyr$^1$. These data
suggest that the Dmt$^1$ analogs may bind to a different site and the other ligands do not express
the same affinity for this site. All of the Dmt$^1$ peptide analogs showed much higher affinity for
the $\mu$ receptor compared to the corresponding Tyr$^1$ analogs. The substitution of Dmt$^1$ for Tyr$^1$
has been shown to consistently increase the binding affinity towards both $\mu$ and $\delta$ receptors but
the mechanism remains uncertain (Hansen, Jr. et al., 1992; Sasaki et al., 1999; Schiller et al.
2000; Harrison et al., 2003).
Sodium and guanine nucleotides are known to diminish the binding of opioid agonists but not antagonists (Pert et al., 1973). Sodium has been reported to decrease the binding affinity of \[^{3}H\]DAMGO, and either increase or had no change on Bmax (Werling et al., 1986; Bolger et al., 1987; Krumins et al., 1993; Brown and Pasternak, 1998). The presence of Gpp(NH)p also decreased binding affinity of \[^{3}H\]DAMGO but had little effect on Bmax (Werling et al. 1986; Bolger et al. 1987; Krumins et al. 1993; Brown and Pasternak, 1998). As expected, Kd was increased 3-fold when \[^{3}H\][Dmt\(^{1}\)]DALDA binding was carried out in the presence of sodium and guanine nucleotides. Surprisingly, the reduced affinity in \[^{3}H\][Dmt\(^{1}\)]DALDA binding was entirely due to the presence of sodium whereas Gpp(NH)p itself had no effect. Although guanine nucleotides inhibit the binding of most opioid agonists, the binding of etorphine was also not affected by GTP (Childers and Snyder, 1980). The lack of effect of Gpp(NH)p suggests that \[^{3}H\][Dmt\(^{1}\)]DALDA binds to both the G-protein-coupled and uncoupled receptor with similar affinity. This may account for the very high \textit{in vivo} analgesic potency of \[Dmt^{1}\]DALDA compared to morphine.

Our results also show that \[Dmt^{1}\]DALDA is an agonist at all three receptor subtypes. \[^{35}S\]GTP\(\gamma\)S binding revealed that \[Dmt^{1}\]DALDA is a full agonist at hMOR and hDOR, although its potency is 118-fold higher at hMOR. In contrast, DAMGO only showed 7-fold selectivity for hMOR versus hDOR. It is interesting that \[Dmt^{1}\]DALDA showed significantly higher MOR:DOR selectivity in receptor binding assays compared to GTP\(\gamma\)S binding assays (9,707 versus 118). This discrepancy can not be explained by the different conditions used for the two assays because the Kd of \[^{3}H\][Dmt\(^{1}\)]DALDA was only increased slightly in the presence of 100 mM NaCl and 30 \(\mu\)M GDP. It is possible, however, that the coupling efficiency of DOR is much higher than MOR. This possibility is supported by the fact that the MOR:DOR selectivity of
DAMGO was also much greater in the radioligand binding assay compared to $[^3\text{S}]\text{GTP} \gamma \text{S}$ binding (1,212 versus 7). Both $[\text{Dmt}^1]\text{DALDA}$ and DAMGO were partial agonists at hKOR, but the Emax for $[\text{Dmt}^1]\text{DALDA}$ (56%) was lower compared to DAMGO (79%). Thus the efficacy of $[\text{Dmt}^1]\text{DALDA}$ at the $\kappa$ receptor is limited despite its high binding affinity.

In summary, $[^3\text{H}][\text{Dmt}^1]\text{DALDA}$ appears to be a superior radioligand for labeling $\mu$ receptors in biological tissues. Its advantages include high affinity and high selectivity, low non-specific binding and metabolic stability (Szeto et al., 2001). In addition, $[\text{Dmt}^1]\text{DALDA}$ is a potent and long-acting analgesic after intrathecal and subcutaneous administration (Neilan et al. 2001; Shimoyama et al. 2001; Zhao et al. 2002; Riba et al. 2002), and can protect the heart against ischemia-reperfusion injury (Wu et al., 2002).
References


Cheng YC and Prusoff WH (1973) Relationship between the inhibition constant ($K_I$) and the concentration of inhibitor which causes 50% inhibition ($I_{50}$) of an enzymatic reaction. *Biochem Pharmacol* **22**:3099-3102.


Footnotes

This work was supported, in part, by a multi-center program project grant (DA08924) from the National Institute on Drug Abuse.
TABLE 1

COMPARISON OF BINDING PARAMETERS FOR $[^3H][\text{DMT}^1]$DALDA

<table>
<thead>
<tr>
<th></th>
<th>$K_d$ (nM)</th>
<th>$B_{\text{max}}$ (fmol/mg protein)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO-K1/hMOR</td>
<td>0.199 ± 0.019</td>
<td>926 ± 55</td>
<td>6</td>
</tr>
<tr>
<td>SH-SY5Y cells</td>
<td>0.118 ± 0.014</td>
<td>77 ± 10</td>
<td>3</td>
</tr>
<tr>
<td>Mouse brain</td>
<td>0.123 ± 0.012</td>
<td>130 ± 9</td>
<td>5</td>
</tr>
<tr>
<td>Mouse spinal cord</td>
<td>0.126 ± 0.008</td>
<td>125 ± 5</td>
<td>4</td>
</tr>
<tr>
<td>Rat brain</td>
<td>0.104 ± 0.018</td>
<td>160 ± 8</td>
<td>5</td>
</tr>
<tr>
<td>Rat spinal cord</td>
<td>0.104 ± 0.014</td>
<td>145 ± 8</td>
<td>5</td>
</tr>
</tbody>
</table>

Saturation binding was carried with $[^3H][\text{DMT}^1]$DALDA using membranes prepared from CHO cells transfected with hMOR (CHO-K1/hMOR), SH-SY5Y cells that natively expresses $\mu$ and $\delta$ receptors, and brain and spinal cord tissues from rats (Sprague-Dawley) and mice (CD-1). Membranes were incubated with $[^3H][\text{DMT}^1]$DALDA (0.010 – 2.000 nM) for 60 min at 25°C. Non-specific binding was assessed by including 0.1 µM unlabelled [DMT$^1$]DALDA or 10 µM naloxone. $K_d$ and $B_{\text{max}}$ were determined by fitting the data to a one-site binding model using nonlinear regression. Data are presented as mean ± SEM; n = number of independent determinations.
TABLE 2


<table>
<thead>
<tr>
<th>Condition</th>
<th>Bmax (fmol/mg)</th>
<th>$K_d$ (nM)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl buffer</td>
<td>923 ± 38</td>
<td>0.199 ± 0.019</td>
<td>6</td>
</tr>
<tr>
<td>+ NaCl (100 mM)/Gpp(NH)p (10 µM)</td>
<td>691 ± 36*</td>
<td>0.430 ± 0.045*</td>
<td>3</td>
</tr>
<tr>
<td>+ Gpp(NH)p (0.1 µM)</td>
<td>902 ± 24</td>
<td>0.182 ± 0.016</td>
<td>4</td>
</tr>
<tr>
<td>+ Gpp(NH)p (1.0 µM)</td>
<td>798 ± 51</td>
<td>0.209 ± 0.030</td>
<td>4</td>
</tr>
<tr>
<td>+ Gpp(NH)p (10 µM)</td>
<td>1075 ± 74</td>
<td>0.176 ± 0.010</td>
<td>4</td>
</tr>
<tr>
<td>+ Gpp(NH)p (100 µM)</td>
<td>911 ± 27</td>
<td>0.233 ± 0.017</td>
<td>5</td>
</tr>
<tr>
<td>+ NaCl (100 mM)</td>
<td>553 ± 28*</td>
<td>0.432 ± 0.024*</td>
<td>3</td>
</tr>
</tbody>
</table>

Membranes prepared from CHO-K1 cells expressing hMOR were incubated with $[^3]$H[Dmt]$^1$DALDA (0.010 – 2.000 nM) for 60 min at 25°C in the presence or absence of NaCl (100 µM) and Gpp(NH)p (0.1 – 100 µM). Non-specific binding was assessed by including unlabelled [Dmt]$^1$DALDA or naloxone. $K_d$ and Bmax were determined by fitting the data to a one-site binding model using nonlinear regression. Data are presented as mean ± SEM; n = number of independent determinations; P < 0.05 compared to Tris-HCl buffer (t-test).
TABLE 3

COMPARISON OF BINDING AFFINITIES DETERMINED FROM COMPETITIVE DISPLACEMENT BINDING AGAINST [\(^3\)H\]][Dmt\(^1\)]DALDA AND [\(^3\)H\]DAMGO IN MOUSE BRAIN MEMBRANES

<table>
<thead>
<tr>
<th></th>
<th>(^3)H[Dmt(^1)]DALDA</th>
<th>Rel affinity(^b)</th>
<th>(^3)H[DAMGO](^a)</th>
<th>Rel affinity(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphine</td>
<td>5.64 ± 0.24</td>
<td>1.0</td>
<td>1.00 ± 0.04</td>
<td>1.0</td>
</tr>
<tr>
<td>DAMGO</td>
<td>2.83 ± 0.14</td>
<td>2.0</td>
<td>1.22 ± 0.12</td>
<td>0.8</td>
</tr>
<tr>
<td>DALDA</td>
<td>12.9 ± 1.0</td>
<td>0.4</td>
<td>1.69 ± 0.25</td>
<td>0.6</td>
</tr>
<tr>
<td>[Dmt(^1)]DALDA</td>
<td>0.163 ± 0.011</td>
<td>34.6</td>
<td>0.143 ± 0.015</td>
<td>7.0</td>
</tr>
<tr>
<td>Tyr-D-Ala-Phe-Phe-NH(_2)</td>
<td>3.44 ± 0.50</td>
<td>1.6</td>
<td>1.53 ± 0.59</td>
<td>0.7</td>
</tr>
<tr>
<td>Dmt-D-Ala-Phe-Phe-NH(_2)</td>
<td>0.174 ± 0.028</td>
<td>32.4</td>
<td>0.173 ± 0.027</td>
<td>5.8</td>
</tr>
<tr>
<td>Tyr-D-Arg-Phe-Orn-NH(_2)</td>
<td>10.38 ± 0.09</td>
<td>0.5</td>
<td>1.2 ± 0.26</td>
<td>0.8</td>
</tr>
<tr>
<td>Dmt-D-Arg-Phe-Orn-NH(_2)</td>
<td>0.161 ± 0.028</td>
<td>35.0</td>
<td>0.151 ± 0.018</td>
<td>6.6</td>
</tr>
<tr>
<td>Tyr-D-Arg-Phe-A(_2)pr-NH(_2)</td>
<td>12.73 ± 0.09</td>
<td>0.4</td>
<td>2.55 ± 0.27</td>
<td>0.6</td>
</tr>
<tr>
<td>Dmt-D-Arg-Phe-A(_2)pr-NH(_2)</td>
<td>0.145 ± 0.021</td>
<td>38.9</td>
<td>0.0636 ± 0.0110</td>
<td>15.7</td>
</tr>
</tbody>
</table>

Competitive displacement binding by various opioid ligands was carried out by incubating mouse brain membranes with 0.100 – 0.150 nM [\(^3\)H][Dmt\(^1\)]DALDA in the presence of increasing concentrations of unlabeled opioid ligands for 60 min at 25°C. Ki values were calculated from the obtained IC\(_{50}\) values by the Cheng and Prusoff equation. The Kd for [\(^3\)H][Dmt\(^1\)]DALDA was 0.156 nM. All values represent the mean ± SEM of 4-6 experiments. \(^a\) Data published previously (Schiller et al. 2000). \(^b\) Rel affinity = affinity relative to morphine.
TABLE 4

RELATIVE SELECTIVITY OF [DMT1]DALDA AND DAMGO BINDING TO hMOR, hDOR AND hKOR

<table>
<thead>
<tr>
<th>Receptor</th>
<th>[DMT1]DALDA</th>
<th></th>
<th>DAMGO</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ki (nM)</td>
<td>Selectivity ‡</td>
<td>Ki (nM)</td>
<td>Selectivity ‡</td>
</tr>
<tr>
<td>hMOR</td>
<td>0.172 ± 0.026</td>
<td>-</td>
<td>1.180 ± 0.120</td>
<td></td>
</tr>
<tr>
<td>hDOR</td>
<td>1670 ± 40</td>
<td>9,707</td>
<td>1430 ± 20</td>
<td>1,212</td>
</tr>
<tr>
<td>hKOR</td>
<td>4.4 ± 1.7</td>
<td>26</td>
<td>213 ± 28</td>
<td>180</td>
</tr>
</tbody>
</table>

The binding affinity of [DMT1]DALDA and DAMGO to hMOR, hDOR and hKOR was determined by competitive displacement binding of [3H][DMT1]DALDA in hMOR membranes, [3H]DPDPE in hDOR membranes and [3H]U69,593 in hKOR membranes. Ki values were calculated from the obtained IC50 values by the Cheng and Prusoff equation. The Kd for [3H][DMT1]DALDA, [3H]DPDPE and [3H]U69,593 in hMOR, hDOR and hKOR were 0.199 ± 0.019 nM, 1.95 ± 0.20 nM and 0.694 ± 0.08 nM, respectively. All values represent the mean ± SEM of 3-6 experiments. ‡ Selectivity of [DMT1]DALDA and DAMGO for hMOR versus hDOR and KOR was calculated as Ki(hDOR)/Ki(hMOR) and Ki(hKOR)/Ki(hMOR), respectively.
TABLE 5

RELATIVE SELECTIVITY OF [Dmt¹]DALDA AND DAMGO IN [³⁵S]GTPγS BINDING IN hMOR, hDOR AND hKOR

<table>
<thead>
<tr>
<th>Receptor</th>
<th>[Dmt¹]DALDA</th>
<th></th>
<th></th>
<th>DAMGO</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC₅₀ (nM)</td>
<td>Emax ‡</td>
<td>EC₅₀ (nM)</td>
<td>Emax ‡</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hMOR</td>
<td>17.2 (8.8 – 33.5)</td>
<td>91.9%</td>
<td>208 (94-459)</td>
<td>100%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hDOR</td>
<td>2003 (1240-3238)</td>
<td>114.3%</td>
<td>1241 (567-2712)</td>
<td>116.4%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hKOR</td>
<td>500 (364-689)</td>
<td>56.1%</td>
<td>7733 (6034-9911)</td>
<td>79.3%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Membranes were incubated with 50 pM [³⁵S]GTPγS in the presence of varying concentrations of [Dmt¹]DALDA or DAMGO for 60 min at 30°C. The concentration of GDP was 30 µM for hMOR, 60 µM for hDOR, and 10 µM for hKOR. Nonspecific binding was determined using 10 µM unlabeled GTPγS. ‡ Maximal effect is presented as percent compared to a full agonist. Full agonist activity was determined using DAMGO for hMOR, deltorphin II for hDOR, and U50,488H for hKOR. Data are presented with 95% confidence intervals from 4-5 experiments.
Figure Legends

**Fig. 1** Representative binding curves for [³H][Dmt¹]DALDA carried out using membranes prepared from CHO-K1 cells stably transfected with hMOR, SH-SY5Y cells, rat brain or rat spinal cord. Aliquots of membrane homogenates were incubated with [³H][Dmt¹]DALDA (0.010-2.000 nM) for 60 min at 25°C. Nonspecific binding was assessed by including 0.1µM unlabeled [Dmt¹]DALDA or 10 µM naloxone. Kd and Bmax for the various membrane preparations are summarized in Table 1.

**Fig. 2** Saturation binding curves for [³H][Dmt¹]DALDA (A) and [³H][DAMGO (B) using mouse brain membranes. Aliquots of membrane homogenates were incubated with [³H][Dmt¹]DALDA or [³H]DAMGO for 60 min at 25°C or 37°C, respectively. Nonspecific binding was assessed by including 0.1µM unlabeled [Dmt¹]DALDA for [³H][Dmt¹]DALDA or 10 µM naloxone for [³H]DAMGO. The Kd for [³H][Dmt¹]DALDA and [³H]DAMGO were estimated to be 0.123 ± 0.012 nM (n=6) and 1.59 ± 0.14 nM (n=4), respectively. Bmax was 130 ± 9 fmol/mg protein with [³H][Dmt¹]DALDA and 115 ± 3 fmol/mg protein with [³H]DAMGO.

**Fig. 3** Association and dissociation of [³H][Dmt¹]DALDA binding in mouse brain membranes. A. Association of [³H][Dmt¹]DALDA was determined by incubating [³H][Dmt¹]DALDA with mouse brain membranes at 25°C for various times up to 60 min. B. Dissociation of the binding of [³H][Dmt¹]DALDA to mouse brain membranes was initiated by the addition of 0.2 µM of unlabelled [Dmt¹]DALDA after the 60 min incubation. The association rate constant (K₊₁) and dissociation rate constant (K₋₁) were determined to be 0.1551 ± 0.0402 nM⁻¹ min⁻¹ (n=6) and 0.0320 ± 0.0074 min⁻¹, respectively (n=6).
Fig. 4  Competitive displacement binding curves for [Dmt¹]DALDA and DAMGO in hMOR, hDOR and hKOR membranes. A. Competitive displacement curves for [Dmt¹]DALDA against [³H][Dmt¹]DALDA, [³H]DPDPE and [³H]U69,593 binding to hMOR, hDOR and hKOR membranes, respectively. B. Corresponding competitive displacement curves for DAMGO. The Kᵩ for [³H][Dmt¹]DALDA, [³H]DPDPE and [³H]U69,593 in hMOR, hDOR and hKOR were 0.199 ± 0.019 nM (n=6), 1.95 ± 0.20 nM (n=4) and 0.694 ± 0.08 nM (n=3), respectively. The Ki for [Dmt¹]DALDA and DAMGO at the three receptors are summarized in Table 4.

Fig. 5  Stimulation of [³⁵S]GTPγS binding by [Dmt¹]DALDA and DAMGO to membranes prepared from CHO-K1 cells transfected with hMOR or hDOR, and HEK293 cells transfected with hKOR. Membranes were incubated with 50 pM [³⁵S]GTPγS and GDP in the presence of varying concentrations of [Dmt¹]DALDA or DAMGO for 60 min at 30°C. The concentration of GDP was 30 µM for hMOR, 60 µM for hDOR and 10 µM for hKOR. Nonspecific binding was determined using 10 µM unlabeled GTPγS. Full agonist activity at the three receptors was determined using DAMGO for hMOR, deltorphin II (DELT) for hDOR, and U50,488H for hKOR. Data are presented with 95% confidence intervals from 4-5 experiments. Basal [³⁵S]GTPγS binding for hMOR, hDOR and hKOR membranes was 0.30 – 0.45, 0.40 – 0.55 and 0.8 – 1.2 fmol/10 µg protein, respectively.
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A. 

Specific binding (fmol/mg protein) vs. Time (min)

B. 

Specific binding (fmol/mg protein) vs. Time after dissociation (min)

- 200 pM
- 490 pM
A. 

[Graph showing specific binding (% control) vs. [Dmtr\textsuperscript{1}]DALDA (log M).]

B. 

[Graph showing specific binding (% control) vs. DAMGO (log M).]