Available online at www.sciencedirect.com
ScienceDirect
Bioorganic & Medicinal Chemistry 16 (2008) 4341–4346
Bioorganic & Medicinal Chemistry

Internalisation of the μ-opioid receptor by endomorphin-1 and leu-enkephalin is dependant on aromatic amino acid residues

Mark P. Del Borgo, Joanne T. Blanchfield and Istvan Toth*

School of Molecular and Microbial Sciences, University of Queensland, St. Lucia, 4072 Qld, Australia

Received 2 January 2008; revised 21 February 2008; accepted 22 February 2008
Available online 4 March 2008

Abstract—The opioid receptor system in the central nervous system controls a number of physiological processes, most notably pain. However, most opioids currently available have a variety of side-effects as well as exhibiting tolerance. Tolerance is most likely to be a complex phenomenon, however, the role of receptor internalisation is thought to play a crucial role. In this study, we examined the role of aromaticity in ligand-mediated receptor internalisation of the μ-opioid receptor (MOPR). These studies show that the amount of receptor internalisation may be dependant on the amphiphilicity of the ligand. Specifically, deletion of the C-terminus aromatic residues of endomorphin 1, particularly tryptophan reduces receptor-mediated internalisation whilst the addition of tryptophan within the enkephalin sequence increases receptor internalisation and decreases tolerance.

1. Introduction

μ-Opioid receptors (MOPR's) are similar to many other G protein-coupled receptors (GPCR) in that they undergo desensitisation within several minutes of stimulation by agonists,[1,2] and continued agonist exposure results in the removal of receptors from the cell surface.[3,4] MOPR phosphorylation is increased as a consequence of agonist exposure, leading to a commonly held assumption that receptor phosphorylation is essential for MOPR desensitisation and internalisation[3,5–7] and subsequent binding of β-arrestins to the phosphorylated receptor, and removal of MOPR from the cell surface via a clathrin-dependent process.[5,6] The enhanced analgesic potency of morphine and diminished analgesic tolerance in β-arrestin2 knockout mice suggest that the capacity of opioid agonists to cause receptor desensitisation and endocytosis is one of the key cellular mechanisms underlying tolerance.[7] Analgesic tolerance is likely to be a complex phenomenon and an understanding of how MOPR's are regulated by opioid agonists will lead to important insights into this phenomenon.

GPCR internalisation is not simply a secondary consequence of receptor activation by agonists but can be affected differentially by different ligands independently of their efficacy of coupling to G proteins, and for some receptors even apparent antagonists can induce receptor internalisation.[8,9] MOPR agonists appear to have differential capacities to promote receptor internalisation. Etorphine, methadone, endomorphin-1 and DAMGO produce significant MOPR endocytosis, whereas others, most notably morphine, are much less effective.[5,6,10] The relationship between the coupling efficiency of MOPR agonists to stimulate G proteins and induce receptor internalisation is controversial. Some studies have reported that morphine and other agonists such as DAMGO and methadone have similar signalling efficacies but that morphine has a much lower efficacy to induce receptor internalisation.[11,12] Other studies suggest that the capacity of MOPR agonists to induce desensitisation or endocytosis is simply a function of their efficacy to induce activation of G proteins[13,14] and that the partial agonist properties of morphine are the reason for its poor efficacy to induce internalisation. The uncertainty concerning differential efficacy of MOPR agonists for G protein activation, rapid desensitisation and endocytosis has probably arisen for three main reasons. First, most studies of these processes have used heterologous expression systems that greatly overexpress receptors, thus disrupting quantitative determination of relative signalling efficacy as well as the stoichiometry of MOPR/GRK/β-arrestin interactions.[14,18,19] Second, MOPR activation, desensitisation and endocytosis have generally been compared between different cellular

Keywords: Opioid receptors; Endomorphin 1; Leu-enkephalin; Receptor internalisation; Tolerence.
* Corresponding author. Tel.: +61 7 3364892; fax: +61 7 3365 1688; e-mail: i.toth@uq.edu.au

0968-0896/$ - see front matter © 2008 Published by Elsevier Ltd.
doi:10.1016/j.bmc.2008.02.074

expression systems under different conditions.[16,17] This is mostly because cell lines do not generally express effector proteins that can be used as rapid reporters of MOPR activation, whereas receptor internalisation is difficult to study quantitatively in native cells because of relatively low levels of native receptor expression. Finally, the term 'receptor desensitisation' has probably been used to describe different phenomena in different types of studies. In biochemical studies of MOPR function that measure agonist inhibition of cAMP generation or stimulation of GTPγS binding to G proteins, the basic assays take a minimum of 15 min and often longer. Studies of MOPR responses in neurons indicate that fast desensitisation occurs with a time constant of about 3 min,[15] and substantial internalisation is apparent as early as 5–10 min in MOPR-expressing cell lines,[11] meaning that the biochemical studies are starting from a baseline of an at least partially desensitised and/or internalised receptor. Thus, studies to date have not been able to adequately resolve the relationships between MOPR agonist efficacy for activation of G proteins, rapid desensitisation, or internalisation under similar experimental conditions.

In the present study, we have analysed the amount of receptor internalisation over time mediated by the native opioid peptides endomorphin-1, leu-enkephalin and analogues of these to assess which residues, if any, are crucial to MOPR internalisation, whether this is linked to affinity for the receptor and whether internalisation can be increased by the mutation of residues within the ligand which would explain the differential capacities of these ligands to cause receptor internalisation.

2. Results

In order to determine the relationship of aromatic residues and agonist-induced receptor endocytosis the sequence of endomorphin-1 (YPWF-NH2, compound **1**) was mutated to give four new analogues numbered **2**–**5**. Leu-enkephalin and analogues were numbered **6**–**9** and these are shown in Table 1.

Each analogue was synthesised without difficulty and purified to homogeneity by reversed phase HPLC and detailed chemical characterisation by ESI-MS. Peptide **9** is a typical example of all peptides. The principal product was shown to have the expected molecular weight (Fig. 1B) and following purification, analytical reversed phase HPLC of this species gave a single peak (Fig. 1A). After purification, all peptides were obtained in 70–90% overall yield relative to starting crude peptide. A summary of the ESI-MS data for the peptides prepared in this study is provided in Table 1. Following purification, peptides were analysed for their binding affinity to MOPR's. Compounds **2** and **3** retained biological activity of the native peptide **1** despite the mutation of large aromatic residues. However, a drop in affinity of the peptides was observed. Furthermore, a mutation of the proline residue (compound **4**) causes a large drop in affinity (see Table 1). A double mutation in the endomorphin sequence (compound **5**) causes a drop in activity but much less than that seen for compound **4** (Table 1). Compounds **7** and **8** show binding affinities similar to that of the native peptide (**6**) with compound **9** exhibiting an order of magnitude drop in receptor affinity (Table 1).

Table 1. A list of all peptides synthesised showing the observed mass obtained from ESI-MS as well as the K_i of each compound

Compound	Sequence	$[M+H]^+$	K_i (nM)
1	YPWF-NH_2	611.2	1.45 ± 0.2
2	YPW**A**-NH_2	535.7	72.7 ± 2.1
3	YP**A**F-NH_2	495.7	225.9 ± 4.8
4	Y**A**WF-NH_2	585.4	3190 ± 7.4
5	YP**AA**-NH_2	420.5	705 ± 5.1
6	YGGFL-NH_2	555.4	11.4 ± 0.8
7	YGG**W**L-NH_2	594.7	12.1 ± 0.6
8	**Ac**-YGGFL-NH_2	597.8	37.3 ± 0.8
9	YG**W**FL-NH_2	684.7	112.5 ± 3.7

Compounds were then examined for their ability to cause receptor internalisation. Shown in Figure 2 are the internalisation curves for compounds **1**–**5**. Compounds **1** and **4** exhibit a similar trend of receptor internalisation, despite the removal of a residue required for binding in compound **4**. However, compounds **2**, **3** and **5** all show decreased receptor internalisation. Compound **5** shows that removal of both aromatic groups has a synergistic effect on preventing receptor internalisation.

The enkephalin analogues were then examined to determine whether internalisation could be promoted by insertion of aromatic residues. Native leu-enkephalin, **6**, caused approximately 18% of receptors to be internalised. Compounds **7** and **9**, showed an increase in receptor internalisation of 22% and 40%, respectively (see Fig. 3).

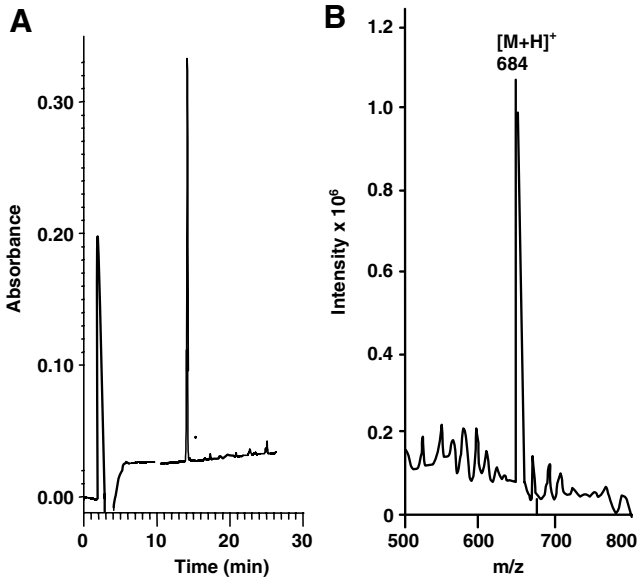

Figure 1. (A) A typical example of an RP-HPLC profile showing a single-peak product and (B) a typical mass spectrum showing the correct molecular weight (compound **9**).

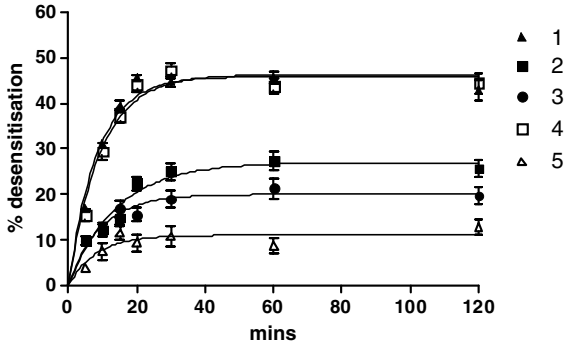

Figure 2. The amount of MOPR internalisation seen in SH-SY5Y cells treated with endomorphin analogues.

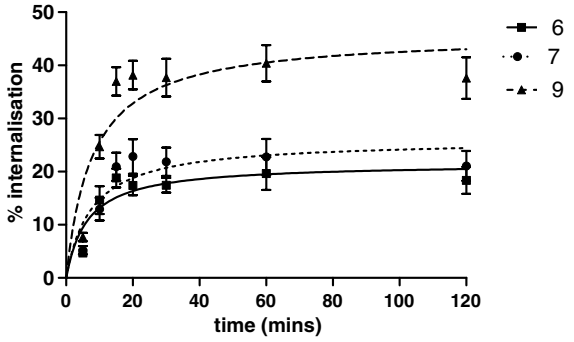

Figure 3. The amount of MOPR internalisation seen in SH-SY5Y cells treated with leu-enkephalin analogues.

Non-specific binding for all compounds was below 3% and was determined by using a large excess of endomorphin-1 (data not shown). The experiment was also carried out in HEK-293T cells without MOPR's to assess non-specific binding, which also proved to be very low. No internalisation was seen in these cells as expected.

Using a cAMP assay, we subsequently performed an in vitro assessment of tolerance formation for compounds **7** and **9**. The IC_{50} for compounds **7** and **9** did not differ significantly, however, following overnight incubation with the respective analogues, compound **7** showed a higher IC_{50}, whereas the IC_{50} value for compound **9** did not alter significantly.

3. Discussion

Receptor internalisation is a regulatory process that modulates the number and functional activity of receptors present in the plasma membrane. Rapid internalisation of μ- or δ-opioid receptors occurs within several minutes of the continued presence of agonist and has been observed in both transfected cell lines[18] and neurons.[23] Once receptors are internalised, they can be resensitised and recycled back to the plasma membrane. Downregulation of receptors also reduces the number of functional receptors on the cell surface. This process occurs over several hours of continued agonist exposure.[4] Although the μ-opioid receptor agonist DAMGO and other peptides are able to induce μ-opioid receptor internalisation, morphine is much less effective.[22,24] This has led to the suggestion that different opioid ligands differ in their ability to induce regulatory endocytosis of μ-opioid receptors independently of their efficacy for other effectors. We have examined in detail the ability of endomorphin-1 to induce internalisation and whether there are residues that are particularly important in this process.

An alanine scan of endomorphin-2 has been previously reported[19] and our results confirmed those findings. Although replacement of the C-terminal residues Trp and Phe caused a slight decrease in binding (compounds **2**, **3** and **5**), endomorphin-1 was still able to bind to MOPRs. However, mutation of the N-terminal Pro residue caused severe impairment in binding (compound **4**). This mimics previous studies that show initial binding and receptor activation to occur at the N-terminus and additional or secondary binding at the C-terminus.[26,27]

Whilst the N-terminus seems crucial for binding, based on our findings, the C-terminus seems to be most important for initiating receptor internalisation. Compounds **2**, **3** and **5**, which have an aromatic residue mutated, lose the ability to cause receptor internalisation. This is in stark contrast to compound **4**, which has a proline mutated for an alanine and has no effect on MOPR internalisation despite having a significantly lower receptor affinity. From these results, we then investigated whether the opioid peptide, leu-enkephalin, would be able to 'regain' the ability to cause MOPR internalisation. Endomorphin-1 and leu-enkephalin share similar sequences (Tyr-Pro-Trp-Phe and Tyr-Gly-Gly-Phe-Leu) but the MOPR internalisation caused from agonism by these two peptides is quite different. Therefore, it was hypothesised that the substitution to tryptophan, increases MOPR internalisation to levels akin to those achieved by endomorphin-1. Compounds **7** and **9** showed an increase in ligand-mediated MOPR internalisation due to the addition of Phe[4] and Gly[3] residues of leu-enkephalin to tryptophan. The role of tryptophan in membrane interactions has been widely described and has been shown to be essential in stabilising proteins within membranes[20] as well as playing an important role in membrane interaction with transmembrane receptors.[21]

To assess whether increased internalisation had a functional significance, we tested the ability of the leu-enkephalin analogues to form tolerance in vitro. For instance, continuous treatment of SH-SY5Y cells with morphine (10 μM) or DAMGO (10 μM) for 24–48 h has been shown to lead to a decreased ability to inhibit cAMP accumulation.[22–24] In our study, SH-SY5Y cells were treated with analogues **7** and **9** as well as control peptide **6** for 24 h and then washed with a mild acid to strip the membrane off the ligand. These cells were subsequently stimulated with forskolin and treated with compounds **6**, **7** and **9**. In the case of leu-enkephalin (**6**) the IC_{50} value increased significantly after incubation of the cells with the compound for 24 h. This indicated that the cells had developed tolerance to leu-enkephalin.

Table 2. The IC_{50} values of compounds **6**, **7** and **9** to cause inhibition of cAMP in SH-SY5Y cells with and without pre-exposure to excess ligand

Compound	Sequence	IC_{50}[a] (nM)	IC_{50}[b] (nM)
6	YGGFL-NH_2	26.1 ± 2.4	57.8 ± 3.3[c]
7	YGG**W**L-NH_2	27.5 ± 2.7	49.2 ± 4.1[c]
9	YG**W**FL-NH_2	126.2 ± 3.3	132.4 ± 2.1

[a] Pre-treament for 24 h with DMEM medium.
[b] Pre-treament for 24 h with 20-fold excess of respective ligand.
[c] $p < 0.05$ (one-way ANOVA, post hoc Newman Kuels).

Compound **7** exhibited a smaller increase in IC_{50} value indicating less tolerance to the compound while compound **9** did not show a significantly different IC_{50} value indicating that there was little tolerance development to compound **9** and suggests that the increase in receptor internalisation correlates to a decrease in tolerance formation (Table 2). The large difference in efficacy between compounds **7** and **9** also shows a lack in correlation between efficacy and internalisation. There have been a number of recent studies to confirm the correlation between MOPR internalisation and tolerance formation and the varying abilities of different ligands to induce MOPR internalisation.[13,25–27] Our findings confirm these results and also show a ligand-dependant mechanism for MOPR internalisation that is focussed within the aromatic residues at the C-terminus of the opioid peptides. Most recently, a compound with significant aromaticity was also found to internalise DOP to a high degree and subsequently showed a diminished tolerance profile.[28] This would indicate that opioid receptors, in general, may require the feature of aromaticity.

This study shows that aromatic residues, in particular tryptophan, are crucially important in the initiation of MOPR internalisation for μ-opioid ligands and may be important in the development of opioid drugs with improved side-effect and tolerance profiles.

4. Methods and materials

4.1. Materials

N-Fmoc-L-OH amino acids, *N*,*N*-dimethylformamide (DMF), 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluroniumhexafluorophosphate (HBTU), diisopropylethylamine (DIPEA) and HPLC grade piperidine were purchased from Auspep (Melbourne, Australia). Rink Amide MBHA resin was purchased from Merck (San Francisco, USA). Tritium labelled acetic anhydride and cAMP EIA kits were purchased from Amersham Biosciences (Buckinghamshire, UK). All solvents used were of synthesis grade.

4.2. Peptide synthesis

Endomorphin-1, leu-enkephalin and analogues were synthesised on a 0.2 mmol scale using Rink Amide MBHA resin as a solid support. Peptides were synthesised using an HBTU/DIEA activation strategy as previously described.[16] Following completion of the peptide synthesis resin was treated with a cleavage mixture of TFA/H_2O/triisopropylsilane (TIS) (95:2.5:2.5 v/v, 10 mL). After 3 h, resin was removed by filtration and the resulting filtrate was concentrated under a stream of N_2. Once the filtrate was reduced to less than 3 mL, the peptide was precipitated by the addition of diethyl ether. The resulting precipitate was centrifuged and dissolved in a solution of H_2O/CH_3CN/TFA (80:20:0.1) and lyophilised.

4.3. Peptide purification and characterisation

All purification was performed by reverse phase-high performance liquid chromatography (RP-HPLC) on a Shimadzu instrument (Osaka, Japan) using a semi-preparative Vydac C18 column (10 × 250 mm) (Hesperia, USA). The solvents used were: buffer A: 0.1% aqueous TFA; buffer B: 0.1% TFA in acetonitrile/water (90:10). A Vydac C18 column (4.6 × 250 mm, 5 μm pore size) was used for all analytical HPLC at a flow rate of 1.0 mL/min using a linear gradient. Peptides were analysed by a Perkin-Elmer Sciex API 3000 mass spectrometer operating in positive ion electrospray mode (ESI-MS) where the positive ion species was detected.

4.4. Acetylation of peptides

Peptides were acetylated with either labelled or unlabelled acetic anhydride. Peptides were dissolved in a small amount of DMF and 1.1 equiv of acetic anhydride was added and the mixture was stirred overnight. The acetic anhydride was evaporated using a stream of N_2 and the resulting sludge was dissolved in acetonitrile/water (20:80) and lyophilised. This process was repeated until a white powder was obtained.

4.5. Cell culture

The human neuroblastoma cell line SH-SY5Y was cultured in flasks with a mixture of Dulbecco's modified Eagle's medium and Hams F12 solution (DMEM:F12) (Life Technologies, USA) containing 100 U/ml penicillin, 2 mM L-glutamine and 10% foetal calf serum.

4.6. Cell-based binding assays

To measure the total binding of endomorphin-1, enkephalin and various analogues, SH-SY5Y cells were plated with binding buffer (20 mM Hepes, pH 7.5, 2% BSA, 0.1 mg/ml lysine, 1.5 mM $CaCl_2$, 50 mM NaCl, 0.01% NaN_4) in 24-well plates. Media was removed and cells were washed with PBS before pre-incubation in 300 μl of binding buffer. Competition binding studies were performed with 100 μl of 3 nM of ^{3}H-labelled DAMGO in the absence or the presence of increasing concentrations of 100 μl of competitor or in binding buffer at 25 °C for 60 min. A 1 μM excess of unlabelled endomorphin-1 was used to determine non-specific binding. Following incubation, the cells were washed with ice-cold binding buffer three times and recovered from culture plates using 500 μl of 1 M NaOH before transfer to scintillation vials. Liquid scintillation cocktail (Ultima Gold, Packard, Meriden, USA) was added to these vials

for counting in a liquid scintillation analyser (Beckman, San Diego, USA). Data are expressed as means ± SEM of % specific binding of triplicate determinations performed on at least three independent plates of transfected cells. Data were plotted using the one-site competition functions of the PRISM program (Graphpad Inc., San Diego, USA).

4.7. Cell-based assay of endomorphin-induced inhibition of cAMP production

SH-SY5Y cells were plated (100 μl) in a 96-well plate and incubated (usually overnight) until 80% confluent. Once cells were ready for assay, endomorphin-1, enkephalin and analogues were diluted in DMEM culture medium containing 30 μM forskolin to stimulate cAMP production in the cell. Cells were incubated with peptides at 37 °C for 15 min, medium was aspirated and cells were lysed using lysis buffer from a cAMP Biotrak enzyme immunoassay kit (Amersham, Buckinghamshire, UK). An anti-cAMP antibody was added to the lysate, and the mixture incubated at 4 °C for 2 h followed by the addition of a cAMP peroxidase conjugate, and incubated at 4 °C for a further hour. A 3,3′,5,5′-tetramethylbenzidine (TMB) substrate provided by the Biotrak EIA kit was then incubated with cell lysate for 1 h and finally 1 μM sulfuric acid was added to facilitate a colour change, which is proportional to the amount of cAMP captured. A cAMP standard curve from 0 to 3200 fmol was also run concurrently as were concentration–response curves for endomorphin-1 or enkephalin. Plates were then read by a plate reader (Biorad model 3550-UV, USA) at 450 nm and compared back to the cAMP standard curve. All data are compared to the maximum amount of inhibition of cAMP in response to endomorphin-1 or enkephalin and are represented as means ± standard error of three experiments performed in triplicate. Tolerance studies were performed in exactly the same fashion with the exception that cells were pre-incubated with endomorphin-1, enkephalin or analogue for 24 h. Prior to commencement of the assay, cells were washed with pre-warmed PBS and two 40-s washes with 5 mM ice-cold acetic acid in 150 mM NaCl, pH 2.5 to strip all ligand from the membrane and associated receptors and a final wash with PBS. The assay then proceeded as normal. All data are plotted using PRISM program (GraphPad, San Diego, CA).

4.8. Internalisation assay

SH-SY5Y cells were grown in triplicate to confluence in 24-well culture plates. The rate and degree of agonist-induced endocytosis was determined following a previously published procedure.[17] Briefly, cells were washed three times with PBS, covered with 0.9 ml of pre-warmed binding buffer and the plates returned to 37 °C for 30 min. Labelled analogues, in 100 ml of binding buffer, were added to a final concentration 20 times the binding affinity (K_i), and the incubation continued at 37 °C for 2, 10, 30 and 60 min. At each time point, plates were chilled on ice and washed five times with 1.0 ml of ice-cold binding buffer to prevent further internalisation and to remove unbound endomorphin-1 or enkephalin analogues. Bound agonist associated with non-internalised receptors in the plasma membrane was removed by two 40-s washes with 5 mM ice-cold acetic acid in 150 mM NaCl, pH 2.5. These acid washes were retained, and the internalised radioactivity was collected by adding 1.0 ml of 0.2 M NaOH, 0.25% sodium dodecyl sulfate to each well and washing with an additional 0.5 ml of the same solution. Radioactivity in the acid-sensitive and acid-insensitive fractions was measured with a liquid scintillation analyser (Beckman, Fullerton, CA). An index of receptor internalisation was obtained by expressing the acid-insensitive counts as a percentage of the total binding (acid-insensitive plus acid-sensitive) for each well.

References and notes

1. Harris, G. C.; Williams, J. T. *J. Neurosci.* **1991**, *11*, 2574.
2. Osborne, P. B.; Williams, J. T. *Br. J. Pharmacol.* **1995**, *115*, 925.
3. Arden, J. R.; Segredo, V.; Wang, Z.; Lameh, J.; Sadee, W. *J. Neurochem.* **1995**, *65*, 1636.
4. Sternini, C.; Spann, M.; Anton, B.; Keith, D. E., Jr.; Bunnett, N. W.; von Zastrow, M.; Evans, C.; Brecha, N. C. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 9241.
5. Whistler, J. L.; von Zastrow, M. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 9914.
6. Zhang, J.; Ferguson, S. S.; Barak, L. S.; Bodduluri, S. R.; Laporte, S. A.; Law, P. Y.; Caron, M. G. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 7157.
7. Bohn, L. M.; Gainetdinov, R. R.; Lin, F. T.; Lefkowitz, R. J.; Caron, M. G. *Nature* **2000**, *408*, 720.
8. Conchon, S.; Monnot, C.; Teutsch, B.; Corvol, P.; Clauser, E. *FEBS Lett.* **1994**, *349*, 365.
9. Roettger, B. F.; Ghanekar, D.; Rao, R.; Toledo, C.; Yingling, J.; Pinon, D.; Miller, L. J. *Mol. Pharmacol.* **1997**, *51*, 357.
10. Bushell, T.; Endoh, T.; Simen, A. A.; Ren, D.; Bindokas, V. P.; Miller, R. J. *Mol. Pharmacol.* **2002**, *61*, 55.
11. Keith, D. E.; Anton, B.; Murray, S. R.; Zaki, P. A.; Chu, P. C.; Lissin, D. V.; Monteillet-Agius, G.; Stewart, P. L.; Evans, C. J.; von Zastrow, M. *Mol. Pharmacol.* **1998**, *53*, 377.
12. Whistler, J. L.; Chuang, H. H.; Chu, P.; Jan, L. Y.; von Zastrow, M. *Neuron* **1999**, *23*, 737.
13. Alvarez, V. A.; Arttamangkul, S.; Dang, V.; Salem, A.; Whistler, J. L.; Von Zastrow, M.; Grandy, D. K.; Williams, J. T. *J. Neurosci.* **2002**, *22*, 5769.
14. Kovoor, A.; Celver, J. P.; Wu, A.; Chavkin, C. *Mol. Pharmacol.* **1998**, *54*, 704.
15. Fiorillo, C. D.; Williams, J. T. *J. Neurosci.* **1996**, *16*, 1479.
16. Malkinson, J. P.; Falconer, R. A.; Toth, I. *J. Org. Chem.* **2000**, *65*, 5249.
17. Thomas, W. G.; Baker, K. M.; Motel, T. J.; Thekkumkara, T. J. *J. Biol. Chem.* **1995**, *270*, 22153.
18. Horner, K. A.; Zadina, J. E. *Brain Res.* **2004**, *1028*, 121.
19. Okada, Y.; Fujita, Y.; Motoyama, T.; Tsuda, Y.; Yokoi, T.; Li, T.; Sasaki, Y.; Ambo, A.; Jinsmaa, Y.; Bryant, S. D.; Lazarus, L. H. *Bioorg. Med. Chem.* **2003**, *11*, 1983.
20. Becker, M. D.; Greathouse, D. V.; Koeppe, R. E., 2nd; Andersen, O. S. *Biochemistry* **1991**, *30*, 8830.
21. Seelig, A. *Biochemistry* **1992**, *31*, 2897.
22. Carter, B. D.; Medzihradsky, F. *Mol. Pharmacol.* **1993**, *43*, 465.
23. Wang, Z.; Sadee, W. *Eur. J. Pharmacol.* **2000**, *389*, 165.
24. Yu, V. C.; Eiger, S.; Duan, D. S.; Lameh, J.; Sadee, W. *J. Neurochem.* **1990**, *55*, 1390.

25. Daniels, D. J.; Lenard, N. R.; Etienne, C. L.; Law, P. Y.; Roerig, S. C.; Portoghese, P. S. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 19208.
26. Koch, T.; Widera, A.; Bartzsch, K.; Schulz, S.; Brandenburg, L. O.; Wundrack, N.; Beyer, A.; Grecksch, G.; Hollt, V. *Mol. Pharmacol.* **2005**, *67*, 280.
27. Zuo, Z. *Anesth. Analg.* **2005**, *101*, 728.
28. Aguila, B.; Coulbault, L.; Boulouard, M.; Leveille, F.; Davis, A.; Toth, G.; Borsodi, A.; Balboni, G.; Salvadori, S.; Jauzac, P.; Allouche, S. *Br. J. Pharmacol.* **2007**, *152*, 1325.