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Neuropeptide FF (NPFF) Analogs Functionally Antagonize Opioid Activities in $NPFF_2$ Receptor-Transfected SH-SY5Y Neuroblastoma Cells

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ABSTRACT

To elucidate the mechanism of the cellular antiopioid activity of neuropeptide FF (NPFF), we have transfected the SH-SY5Y neuroblastoma cell line, which expresses μ-and δ-opioid receptors, with the human $NPFF_2$ receptor. The selected clone, SH_2-D9, expressed high-affinity $NPFF_2$ receptors in the same range order as μ- and δ-opioid receptors (100–300 fmol/mg of protein). The NPFF analog [D-Tyr^1, (NMe)Phe^3]NPFF (1DMe) did not modify the binding parameters of the μ- and δ-specific agonists [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]-enkephalin and deltorphin-I, respectively. 1DMe dose dependently inhibited 75 to 80% of the cAMP production stimulated by forskolin. Preincubation with 1DMe halved the maximal inhibition of N-type Ca^{2+} channels by opioid agonists. In the presence of carbachol, acting on muscarinic receptors to release Ca^{2+} from the intracellular stores, deltorphin-I and 1DMe enhanced this release. Preincubation with 1DMe reduced the maximal effect of deltorphin-I by 40%, demonstrating an antiopioid effect in this experimental model for the first time. By using peptides corresponding to the carboxyl terminus of the $\alpha_{i1,2}$, α_{i3}, α_o, and α_s subunits of G proteins, which specifically uncouple receptors from G proteins, we demonstrated that μ-opioid and $NPFF_2$ receptors couple to the four subunits assayed. The Ca^{2+} release from the intracellular stores by 1DMe resulted from the coupling of $NPFF_2$ receptors with $G\alpha_o$ and $G\alpha_{i1,2}$, whereas the coupling with $G\alpha_s$ reduced the antiopioid effect of 1DMe in the modulation of N-type channels. This SH_2-D9 cell line now provides the opportunity to study the interaction between both receptors.

Neuropeptide FF (NPFF), FLFQPQRFamide, is representative of a family of mammalian amidated neuropeptides whose precursors pro-$NPFF_A$ and pro-$NPFF_B$ and G protein-coupled receptors $NPFF_1$ and $NPFF_2$ have been recently cloned (Zajac, 2001). Although NPFF does not interact with opioid receptors (Gouardères et al., 1998), a close relationship between neuropeptide FF and opioid systems has been clearly demonstrated in the central nervous system, especially in pain perception (Harrison et al., 1998; Roumy and Zajac, 1998; Panula et al., 1999). For instance, supraspinal injection of NPFF analogs, which has little or no effect in pain tests, decreases morphine-induced analgesia (antiopioid activity, Roumy and Zajac, 1998; Panula et al., 1999), whereas spinal administration induces a naloxone-sensitive analgesia and potentiates morphine-induced analgesia (pro-opioid activity, Roumy and Zajac, 1998; Panula et al., 1999).

In neurons, opioids, including nociceptin, 1) inhibit adenylyl cyclase activity and 2) stimulate inwardly rectifying K^+ channels and inhibit voltage-dependent Ca^{2+} currents by activating four types, μ, δ, κ, and Opioid Receptor-Like 1, of opioid receptors (Law et al., 2000). This leads to postsynaptic neuronal inhibition and to presynaptic inhibition of transmitter release, respectively. In contrast to opioids, no data report a direct modulation of K^+ and Ca^{2+} conductances by NPFF or analogs but rather describe a blockade of the opioid activity on ionic conductances when cells are pretreated with NPFF. NPFF or analogs, which are inactive by themselves, reverse the opioid-induced inhibition of Ca^{2+} conductance in $NPFF_2$ receptor-expressing neurons dissociated from rat dor-

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ABBREVIATIONS: NPFF, neuropeptide FF; $hNPFF_2$ receptor, human $NPFF_2$ receptor; 1DMe, [D-Tyr^1, (NMe)Phe^3]NPFF; SQA-NPFF, SQAFLFQPQRFa; EYF, EYFSLAAPQRFa; deltorphin-I, Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2; DAMGO, [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]-enkephalin; PTX, pertussis toxin; CTX, cholera toxin; NPY, neuropeptide Y; BSA, bovine serum albumin; KRH, Krebs-Ringer-HEPES; $[Ca^{2+}]_i$, intracellular calcium concentration; ANOVA, analysis of variance; H89, *N*-[2-(*p*-bromocinnamyl-amino)ethyl]-5-isoquinolinesulfonamide dihydrochloride; Ro 20-1724, 4-(3-butoxy-4methoxyphenyl)methyl-2-imidazolidone; Ro 31-8220, 2-(1-[3-(amidinothio)propyl]-1*H*-indol-3-yl)-3-(1-methylindol-3-yl) maleimide methane sulfonate salt.

sal root ganglion (Rebeyrolles et al., 1996), dorsal raphe (Roumy and Zajac, 1999), and $NPFF_1$-expressing neurons from the hypothalamic periventricular nucleus (Roumy et al., 2003). This functional antagonism also has been observed by others in different models such as acetylcholine release in the myenteric plexus of the guinea pig (Takeuchi et al., 2001), electrical response in hippocampus slices (Miller and Lupica, 1997), or Met-enkephalin release in the spinal cord (Ballet et al., 1999). In this latter case, blockade of presynaptic δ-opioid autoreceptors after activation of NPFF receptors leads to enhanced release of Met-enkephalin that activates μ-opioid receptors (Mauborgne et al., 2001). In addition, it concomitantly reduces the level of pronociceptive dynorphin (Ballet et al., 2002). Therefore, such an antiopioid activity, paradoxically, could account for the spinal opioid-dependent analgesia induced by NPFF.

Importantly, in experiments on isolated neurons, the effect of NPFF was restricted to opioid receptors and not to other G protein-coupled receptors, although involved in the same transduction pathway as, for example, $5\text{-}HT_{1A}$ receptors in the rat dorsal raphe nucleus (Roumy and Zajac, 1999). This suggests that the NPFF-induced inhibition is specific to opioid receptors and is not mediated by the spread out of an intracellular messenger or by a modification of the effector such as the phosphorylation of the voltage-gated Ca^{2+} channel. The mechanism of this cellular antiopioid effect is unknown and merits to be studied for bringing new information on G protein-coupled receptor signaling regulation.

To elucidate this mechanism, a model reproducing the cellular antiopioid effect of NPFF has been established by transfecting the neuroblastoma cell line SH-SY5Y with the human $NPFF_2$ ($hNPFF_2$) receptor, which is the most extensively characterized NPFF receptor. SH-SY5Y cells were chosen because they naturally express opioid (μ, δ, and Opioid Receptor-Like 1) receptors that inhibit voltage-dependent N-type Ca^{2+} channels (for review, see Vaughan et al., 1995). We present here the characterization of this cell line (SH_2-D9), with the demonstration that $NPFF_2$ receptors exert a functional antiopioid activity similar to that previously observed on isolated neurons. Furthermore, we demonstrate, for the first time, that $NPFF_2$ receptors antagonize the opioid-induced potentiation of Ca^{2+} release from intracellular stores triggered by activation of Gq-coupled muscarinic receptors. Therefore, this cell line constitutes a model of choice to study the interactions between opioid and NPFF receptors.

Materials and Methods

Materials. NPFF-related peptides [1DMe, [D-Tyr^1, (NMe)Phe^3]NPFF; SQA-NPFF, SQAFLFQPQRFa; EYF, EYFSLAAPQRFa], deltorphin-I (Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2), Cys-dermorphin (Tyr-D-Ala-Phe-Gly-Tyr-Pro-Lys-Cys-NH_2), and the peptides analogs to the C-terminal regions of the α subunits of G protein $\alpha_{i1,2}$ (345–354, [C]KNNLKDCGLF), α_{i3} (345–354: [C]KNNLKECGLY), α_o (345–354, [C]ANNLRGCGLY), and α_s (385–394, [C]RMHLRQYELL) were synthesized with an automated peptide synthesizer (model 433A; Applied Biosystems, Foster City, CA). The integrity of peptides was confirmed by mass spectrometry analysis. [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]-enkephalin (DAMGO) was purchased from Bachem (Bubendorf, Switzerland), levorphanol was from F. Hoffman-La Roche (Paris, France), clonidine was from Sigma/RBIR (Natick, MA) and ω-conotoxin GVIA was from Alomone Labs (Jerusalem, Israel). Pertussis toxin (PTX), cholera toxin (CTX), H89, 1,2-dimethoxy-*N*-methyl(1,3)benzodioxolo(5,6-*c*)phenanthridinium chloride (chelerythrine), and Ro 31-8220 were purchased from Sigma (Lyon, France). Porcine NPY was a generous gift from Dr. R. Quirion (McGill University, Montréal, QC, Canada). [^{3}H]DAMGO (67 Ci/mmol) and [^{3}H]adenine (26 Ci/mmol) were purchased from Amersham Biosciences Inc. (St. Quentin Fallavier, France). ^{125}I-EYF and ^{125}I-deltorphin-I were iodinated by electrophilic substitution, as described previously (Gouardères et al., 2001).

Cell Culture. Human neuroblastoma SH-SY5Y cells were kindly provided by F. Noble (Université René Descartes, Paris, France). Cells were grown in Dulbecco's modified Eagle's medium (4.5 g/l of glucose; GlutaMAX I) containing 10% fetal calf serum and 50 μg/ml gentamicin (Invitrogen, Cergy Pontoise, France), in a humidified atmosphere containing 5% CO_2. SH-SY5Y cells were transfected with the human $NPFF_2$ receptor subcloned into the bicistronic vector pEFIN3 (Kotani et al., 2001), by using FuGENE 6 according to the manufacturer's instructions (Roche Diagnostics, Indianapolis, IN). Recombinant cells were selected by addition of 400 μg/ml G418 (Geneticin; Invitrogen) to the culture medium. The SH_2-D9 clone, obtained by limit dilution, was chosen for all experiments. Wild-type and transfected cells were used undifferentiated between passages 8 to 20.

Binding on Cell Membrane. Membrane preparation was obtained as described previously (Mollereau et al., 2002). Binding of ^{125}I-EYF, a high-affinity $NPFF_2$ receptor agonist (Gouardères et al., 2001), was performed in polypropylene tubes in a final volume of 0.5 ml containing 5 to 15 μg of protein, 50 mM Tris-HCl, pH 7.4, 60 mM NaCl, 25 μM bestatin (Sigma), 0.1% bovine serum albumin (BSA), and the radioligand at the desired concentration. The nonspecific binding was determined in the presence of 1 μM EYF. For the binding of ^{125}I-deltorphin-I, a specific δ-opioid agonist, 10 μM levorphanol was used to determine the nonspecific binding, and the incubation buffer was 50 mM Tris-HCl, pH 7.4, 0.1% BSA, and the radioligand at the desired concentration. Binding of [^{3}H]DAMGO, a specific μ-opioid receptor agonist, was performed in polypropylene tubes in a final volume of 1 ml containing 100 μg of protein, 50 mM Tris-HCl, pH 7.4, and the radioligand at the desired concentration. The nonspecific binding was determined in the presence of 10 μM levorphanol.

After a 60-min incubation at 25°C, samples were rapidly filtered on Whatman GF/B filters preincubated in 50 mM Tris-HCl, pH 7.4, 0.1% BSA for the binding of ^{125}I-EYF and ^{125}I-deltorphin-I and 0.3% polyethylenimine for the binding of [^{3}H]DAMGO. The filters were rinsed three times with 4 ml of ice-cold 10 mM Tris-HCl, pH 7.4, containing 0.1% BSA for ^{125}I-EYF and ^{125}I-deltorphin-I. The bound radioactivity was measured with a gamma-counter (PerkinElmer Life and Analytical Sciences, Boston, MA) for ^{125}I-EYF and ^{125}I-deltorphin-I and with a liquid scintillation analyzer (PerkinElmer Life and Analytical Sciences) for [^{3}H]DAMGO.

Binding on Living Cells. Confluent cells, grown in 100-mm dishes, were rinsed and collected in ice-cold HEPES buffer (10 mM HEPES, pH 7.3, 150 mM NaCl, 2.5 mM KCl, 10 mM glucose, and 0.1% BSA). After centrifugation at 1000 rpm for 1 min at 4°C, cells were recovered in 2 ml of buffer. The cell suspension was divided and incubated for 20 min at room temperature with, or without, 1 μM 1DMe. The cell suspension (0.2 ml) was then added to polypropylene tubes in a final volume of 0.5 ml of HEPES buffer, containing 1 nM ^{125}I-deltorphin-I and 10 μM levorphanol for determination of nonspecific binding. After a 90-min incubation at 25°C, samples were rapidly filtered on Whatman GF/B filters preincubated in HEPES buffer. The filters were rinsed three times with 4 ml of ice-cold HEPES buffer, and the bound radioactivity was counted.

Measurement of cAMP. Cells ($4–5 \times 10^5$) were seeded into 24-well plates and incubated for 24 h. The culture medium was then replaced by 0.3 ml of fresh medium containing 0.1 μM adenine and 1 μCi of [^{3}H]adenine. After a 60-min incubation at 37°C under a 5% CO_2 in air atmosphere, the cells were rinsed twice with 0.5 ml of Krebs-Ringer-HEPES (KRH) (124 mM NaCl, 5 mM KCl, 1.25 mM

$MgSO_4$, 1.5 mM $CaCl_2$, 1.25 mM KH_2PO_4, 25 mM HEPES, 8 mM glucose, and 0.5 mg/ml BSA, pH 7.4). Prewarmed KRH (0.2 ml) was added to each well, and the reaction was initiated by addition of 0.1 ml of KRH containing 15 μM forskolin (Sigma), 0.3 mM 3-isobutyl-1-methylxanthine (Sigma), 0.3 mM Ro 20-1724 (Fisher, Illkirch, France) and the agonist to be tested. After 10 min at 37°C, the reaction was stopped by addition of 0.03 ml of 2.2 N HCl. The [^{3}H]cAMP content of each well was isolated by chromatography on acid alumina (Sigma) columns (Mollereau et al., 2002).

Measurement of $[Ca^{2+}]_i$. SH_2-D9 or SH-SY5Y cells were seeded at 3 to 5 $\times$ 10^5 cells in 35-mm Petri dishes and cultured for 24 or 48 h. On the day of the experiment, the culture medium was replaced with 1 ml of HEPES-buffered medium (without BSA), and the cells were incubated for 10 min. The medium was changed to 0.7 ml of HEPES buffer (without BSA) containing 3.6 μM Fluo-4 AM (Molecular Probes, Leiden, The Netherlands) and 0.1% Pluronic acid F127 (Sigma), and the cells were incubated for 30 min in the dark at 37°C. The medium was then replaced with 1 ml of HEPES medium containing 0.1% BSA (Euromedex, Souffelweyersheim, France), and the cells were further incubated for 30 min at room temperature in the dark to allow for complete Fluo-4 AM deesterification. All subsequent perfusing media contained 0.1% BSA.

Cells were viewed with a 40/0.65 objective, illuminated at 488 nm (10-nm bandwidth interference filter), and imaged with a cooled charge-coupled device camera (MicroMax 782 Y; Princeton Instruments, Evry, France) driven by MetaView software (Universal Imaging Corporation, Downingtown, PA). The average pixel intensity within user-defined regions of interest was measured and saved on a computer hard drive. To achieve a rate of measurement of 1 per second (with a 500-ms exposure time set by a Uniblitz shutter), 6 $\times$ 6 to 9 $\times$ 9 binning of pixels were used. The fluorescence intensity was expressed as F/F_0, where F and F_0 are the fluorescence intensity at any time and the mean resting fluorescence intensity preceding the first stimulation, respectively. In many cells, there was a slow linear decrease of F_0 as a function of time, and not as a function of previous illumination, that probably represents Fluo-4 leakage from the cells. This was corrected for by using the parameters of the linear regression of F_0 against time.

The cells were gravity perfused at a rate of 1.8 ml/min with a medium containing 150 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES. The pH was adjusted to 7.30 and 0.1% BSA was added.

Modulation of N-Type Ca^{2+} Channels. To study the modulation of N-type Ca^{2+} conductance, the L-type Ca^{2+} channels were blocked by adding 3 μM nifedipine in all perfusing media. Two Petri dishes seeded at the same cell density and cultured for the same duration were used. In the control culture, cells were returned to HEPES-buffered medium after incubation with Fluo-4, whereas for the test culture, this medium contained 1 to 1000 nM 1DMe, which also was added to all perfusing media. The cells were depolarized during 5 or 10 s with a 140 mM KCl medium (NaCl reduced to 12.5 mM). An electrically operated valve effected a rapid change of solution that was complete within 8 s. The response to a 5- or 10-s depolarization was recorded. The response to the same depolarization was recorded 5 min later, at the end of a 30-s perfusion of DAMGO, deltorphin-I, NPY, or clonidine. Five minutes later, a third response to the same depolarization was measured to test for recovery of the calcium transient. Only the cells that recovered from the opioid (or the other modulators) were included in the present results. For each Petri dish, the responses were recorded in two distinct microscope fields. Each experiment was performed on at least two different cultures. The reduction (%) of the depolarization-induced $[Ca^{2+}]_i$ transient by DAMGO was calculated as $100 \times [\Delta(F/F_0)_{DAMGO} - \Delta(F/F_0)_C]/\Delta(F/F_0)_C$, in which $\Delta(F/F_0)_C = ((F/F_0)_{max} - 1)_C$ and $\Delta(F/F_0)_{DAMGO} = ((F/F_0)_{max} - 1)_{DAMGO}$ are the magnitude of the fluorescence increase during depolarization in the control test and after 30-s perfusion with DAMGO, respectively.

In experiments designed to test the effects of protein kinase A or C inhibitors on the antiopioid activity of 1DMe, the control culture was returned to HEPES medium plus BSA plus the protein kinase inhibitor for 45 min, after Fluo-4 AM incubation, before perfusion was started. For the test culture, 100 nM 1DMe were added after 15 min of incubation with protein kinase inhibitors, and the cells were further incubated for 30 min. For both the control and test cultures all perfusing media contained the kinase inhibitor. Two inhibitors of protein kinase C were used: 1 μM chelerythrine and 0.1 μM Ro 31-8220. H89 (1 μM), a protein kinase A inhibitor, was also tested.

Interaction between Muscarinic Receptors and Opioid and/or NPFF$_2$ Receptors. For each experiment, two 35-mm culture dishes were used: one served as control and the other as the test culture. Regions of interest were laid over the all cell bodies present in the field. Unless otherwise indicated, the cells were perfused with 1 μM deltorphin-I for 40 s and/or 1 μM 1DMe for 40 s in the continuing presence of 5 μM carbachol. Because all cells in a field were stimulated by carbachol, the responses were represented by the mean $\pm$ S.E.M. of F/F_0 for all the cells, measured every second. However, the increase in fluorescence induced by deltorphin-I or 1DMe was measured in each cell.

To study a possible antiopioid effect of 1DMe on the response to deltorphin-I in the presence of carbachol, the cells were returned, after incubation with Fluo-4 AM, to either HEPES buffer or HEPES buffer plus 100 nM 1DMe, and all the perfusing media contained 100 nM 1DMe. The response to deltorphin-I (1–1000 nM, 40 s) in the presence of 5 μM carbachol was measured in control and 1DMe-treated cells. An identical experiment was performed in which the roles of deltorphin-I and 1DMe were reversed.

Identification of G Proteins Coupled to Opioid and NPFF$_2$ Receptors in SH_2-D9 Cells. Peptides corresponding to the last 10 C-terminal residues of G protein α subunits were tested for their ability to inhibit the specific binding of radiolabeled agonists and to block 1DMe and opioid responses in intracellular Ca^{2+} and cAMP assays. For functional assays, peptides (6 or 60 μg in 5 μl of water) were introduced in SH_2-D9 cells (1.5–3 $\times$ 10^6) by the Nucleofactor technology (Amaxa Biosystems, Cologne, Germany) according to the manufacturer's instructions. After electroporation, cells were rapidly plated in 35-mm dishes for Ca^{2+} imaging or in 24-well plates (5 $\times$ 10^5 cells/well) for cAMP and left to recover for 4 h in a humidified 37°C, 5% CO_2 incubator. To assess peptide delivery efficiency, cells were electroporated in the presence of 8 μg of dermorphin labeled with the fluorophore Alexa 488. Cells were left to recover for 4 h, washed four times, and observed with a fluorescence microscope.

Because electropermeabilization technique could not be applied for Ca^{2+} conductance measurement, 1.5 μg of Gα_s peptide was delivered into the cells with the Chariot transfection reagent (Active Motif, Rixensart, Belgium). Complexes were allowed to form for 30 min at room temperature and were then added to 50 to 70% confluent cells in 35-mm dishes. After a 2-h incubation according to manufacturer's instruction, the effect of 1DMe on the opioid-induced Ca^{2+} transient inhibition was investigated.

Data Analysis. Binding experiments and dose-response relationships were analyzed with Prism software (GraphPad Software Inc., San Diego, CA) using the appropriate functions. Statistical comparisons of more than two samples were done with one-way ANOVA followed by post hoc tests. Comparisons of two samples were done with the appropriate *t* test. In all cases, the level of significance was chosen as 0.05.

Results

Characterization of hNPFF$_2$-Transfected SH-SY5Y Cells (SH_2-D9 Clone). In SH-SY5Y membrane preparation, no specific binding of ^{125}I-EYF could be detected. In membranes from the SH_2-D9 clone, ^{125}I-EYF labeled one class of high-affinity sites (Fig. 1A; Table 1). Significantly, NPFF$_2$ receptors were expressed in the same range order as μ- and

δ-opioid receptors (Table 1). Preincubation of SH_2-D9 membranes, for 20 min with 1 μM 1DMe, did not change significantly the binding parameters of [^{3}H]DAMGO and ^{125}I-deltorphin-I (Table 1). In addition, the specific binding of 1 nM ^{125}I-deltorphin-I in living SH_2-D9 cells was not affected by preincubation with 1 μM 1DMe (20 min at room temperature), being 111 ± 19% (n = 3) of the control.

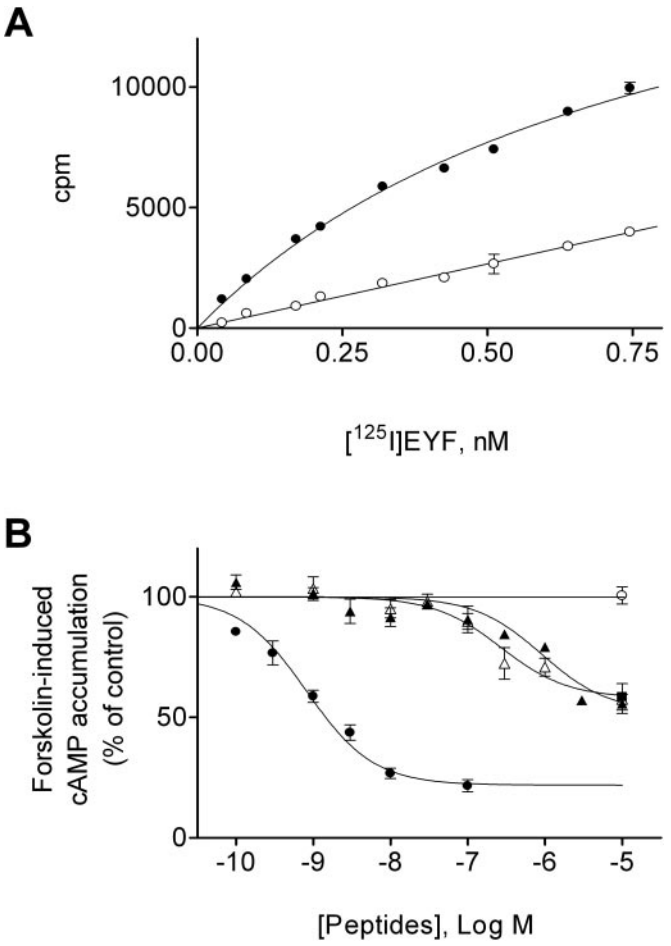

Fig. 1. Characterization of the $NPFF_2$ receptor expression in SH_2-D9 cells. A, representative saturation experiment showing total (●) and nonspecific (○) binding of ^{125}I-EYF to SH_2-D9 membranes. The points represent the mean (± S.E.M.) of measurements performed in triplicate. (For most points, the S.E.M. is smaller than the symbol size.) B, inhibition of intracellular cAMP accumulation in SH-SY5Y (open symbols) and SH_2-D9 (closed symbols) cells by 1DMe (●, ○ ± S.E.M.), DAMGO (▲, Δ± S.E.M.) and DAMGO in cells pretreated with 100 nM 1DMe (■ ± S.E.M.). Intracellular cAMP production was stimulated by 5 μM forskolin for 10 min at 37°C in the absence (control), or in the presence, of the agonist. Curves are the mean of at least three experiments performed in duplicate.

In SH-SY5Y and in SH_2-D9 cells, DAMGO inhibited 50% of the forskolin-induced cAMP production, with an EC_{50} of 529 ± 274 (n = 5) and 1114 ± 217 nM (n = 4), respectively (Fig. 1B). Likewise, in SH_2-D9 cells, 1DMe dose dependently inhibited with higher efficacy (75–80%) and potency (EC_{50} = 0.8 ± 0.1 nM, n = 6) the intracellular cAMP production (Fig. 1). It was totally inactive in SH-SY5Y cells (Fig. 1). When SH_2-D9 cells were preincubated with 1DMe (100 nM, 15 min, 37°C) and washed three times, no modification of the response to 10 μM DAMGO was observed (Fig. 1). The inhibition of adenylate cyclase by 1DMe and DAMGO was totally prevented by overnight PTX pretreatment (100 ng/ml), indicating that both μ-opioid and NPFF receptors mediated cAMP inhibition through a $G_{i/o}$ protein coupling.

Effect of $NPFF_2$ Receptor Activation on the Modulation of N-Type Ca^{2+} Channels by Opioids in SH_2-D9 Cells. In the presence of nifedipine to block the L-type Ca^{2+} channels, some SH-SY5Y and SH_2-D9 cells responded to depolarization (140 mM K^+, 5 or 10 s) by an increase in F/F_0 (Fig. 2A). This response was totally and irreversibly suppressed (n = 14) after incubation with ω-conotoxin GVIA (1 μM, 90 s), a highly specific antagonist of N-type calcium channels. When the depolarization was repeated at the end of a 30-s perfusion with 1 μM DAMGO, the magnitude of the $[Ca^{2+}]_i$ transient was reduced in all cells, compared with the control depolarization (Fig. 2A). The reduction of the F/F_0 transients by DAMGO was reversible and could be repeated at 10-min intervals without any sign of desensitization (Fig. 2A). The magnitude of the reduction was the same with 5- or 10-s depolarizations (−44.5 ± 1.2, n = 104 and −45.5 ± 2.0%, n = 67, respectively; $p > 0.05$, unpaired t test) and was identical in SH_2-D9 and SH-SY5Y cells (−44.9 ± 1.1%, n = 171 versus −48.0 ± 1.8%, n = 62, respectively; $p > 0.05$, unpaired t test). The reduction of the F/F_0 transients by DAMGO was dose-dependent with an EC_{50} value of 1.7 nM (Fig. 2B).

In SH_2-D9 cells preincubated (30 min) and perfused with 100 nM 1DMe, there was no change in either the resting $[Ca^{2+}]_i$ or the magnitude of the response to depolarization: $\Delta(F/F_0)$ = 2.233 ± 0.132 (n = 123) versus 2.227 ± 0.167 (n = 110) in control cells ($p > 0.05$, unpaired t test). However, the

TABLE 1

Binding parameters of [^{3}H]DAMGO, ^{125}I-deltorphin-I (^{125}I-Delt-I) and ^{125}I-EYF on membrane preparation from wild-type SH-SY5Y and $hNPFF_2$ receptor-transfected SH_2-D9 cells (control)

In SH_2-D9 cells, binding experiments also were performed on membranes preincubated (1DMe) for 20 min at room temperature with 1 μM 1DMe. Data are means ± S.E.M. of three to four experiments performed in duplicate.

	SH-SY5Y		SH_2-D9	
	K_D	B_{max}	K_D	B_{max}
	nM	*fmol/mg*	*nM*	*fmol/mg*
[^{3}H]DAMGO				
Control	0.54 ± 0.11	99 ± 2	0.58 ± 0.12	141 ± 14
1DMe			0.66 ± 0.11	141 ± 13
^{125}I-Delt-I				
Control	0.50 ± 0.09	303 ± 18	1.02 ± 0.11	254 ± 15
1DMe			1.27 ± 0.02	245 ± 12
^{125}I-EYF				
Control	N.D.	N.D.	0.42 ± 0.04	349 ± 48

N.D., not detectable.

reduction of $\Delta(F/F_0)$ by DAMGO was less after 1DMe than in control cells (Fig. 3A). The dose-response curve of DAMGO in the presence of 100 nM 1DMe (Fig. 2B) demonstrated that the EC_{50} value of DAMGO was unchanged (3.1 versus 1.7 nM in control cells) but that its maximal effect was approximately halved (−53.6% of the control) (Fig. 2B). By varying the 1DMe concentration and maintaining the DAMGO concentration constant at 1 μM, we found that 1DMe reduced the inhibitory effect of DAMGO on $\Delta(F/F_0)$ with an EC_{50} value of 4.2 nM and a maximal reduction of 40% (Fig. 3B). In SH-SY5Y cells, 100 nM 1DMe did not modify the magnitude of the inhibition of $\Delta(F/F_0)$ by DAMGO (Fig. 3B).

In SH_2-D9 cells, 100 nM SQA-NPFF, the 11-amino acid peptide contained in the human pro-$NPFF_A$ precursor, reduced the DAMGO-induced decrease in $\Delta(F/F_0)$ by the same amount as 100 nM 1DMe (−65.96% ± 6.04, n = 46 versus −59.95% ± 3.52, n = 67, respectively; $p > 0.05$, unpaired t test). In SH-SY5Y cells, 100 nM SQA-NPFF had no effect on the inhibition of $\Delta(F/F_0)$ by DAMGO.

To test for the possible involvement of protein kinases A and C in the antiopioid effect of 1DMe, two inhibitors of protein kinase C,1 μM chelerythrine and 1 μM Ro 31-8220, and one inhibitor of protein kinase A, 0.1 μM H89, were used. Neither chelerythrine nor H89 affected the responses to 1 μM DAMGO in the absence and presence of 100 nM 1DMe (not illustrated). Ro 31-8220 strongly reduced the response to 1 μM DAMGO in the absence of 1DMe so that it was difficult to assess whether 1DMe retained its antiopioid effect in presence of the inhibitor (data not shown).

SH-SY5Y cells also express δ-opioid, NPY Y_2, and α_2-ad-

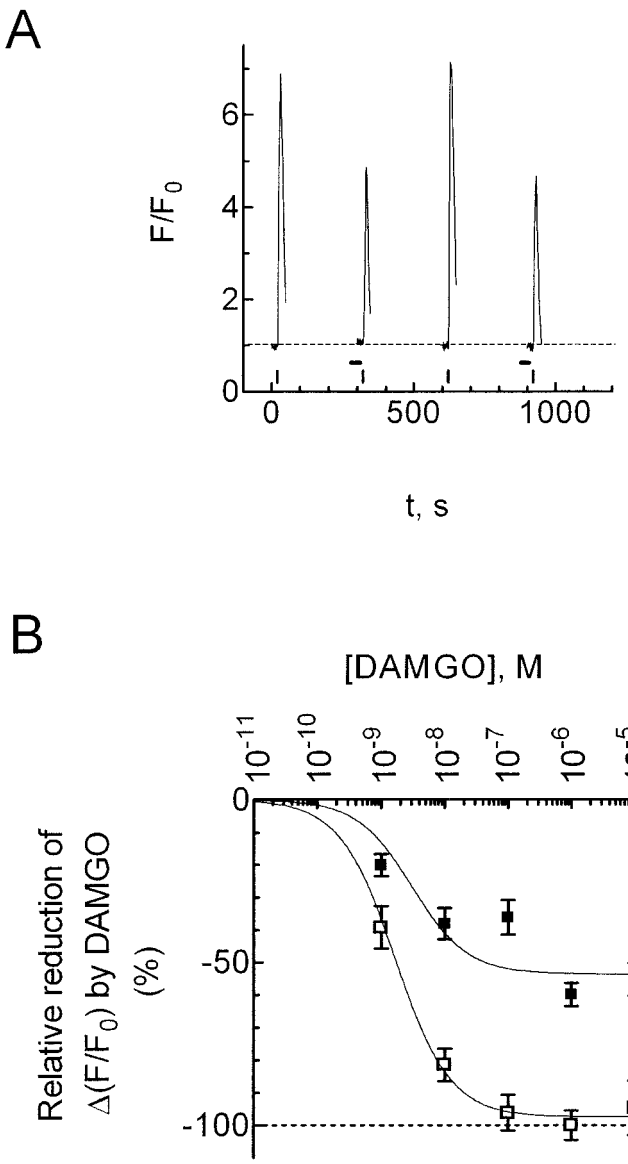

Fig. 2. The specific μ-opioid agonist DAMGO reduced the calcium transients induced by depolarization in SH_2-D9 cells. A, responses to 5-s depolarizations (vertical lines) in the absence and presence of DAMGO (thick horizontal lines). B, dose-response curves of the reduction of the depolarization-induced calcium transients by DAMGO in control (□ ± S.E.M.) and 1DMe-treated (■ ± S.E.M.) SH_2-D9 cells. The mean reduction of $\Delta(F/F_0)$ by 1 μM DAMGO was taken as 100%. From 10^{-9} to 10^{-5} M, the number of cells is 16, 37, 27, 58, and 43 in control and 24, 37, 29, 67, and 27 in 1DMe-pretreated SH_2-D9 cells, respectively.

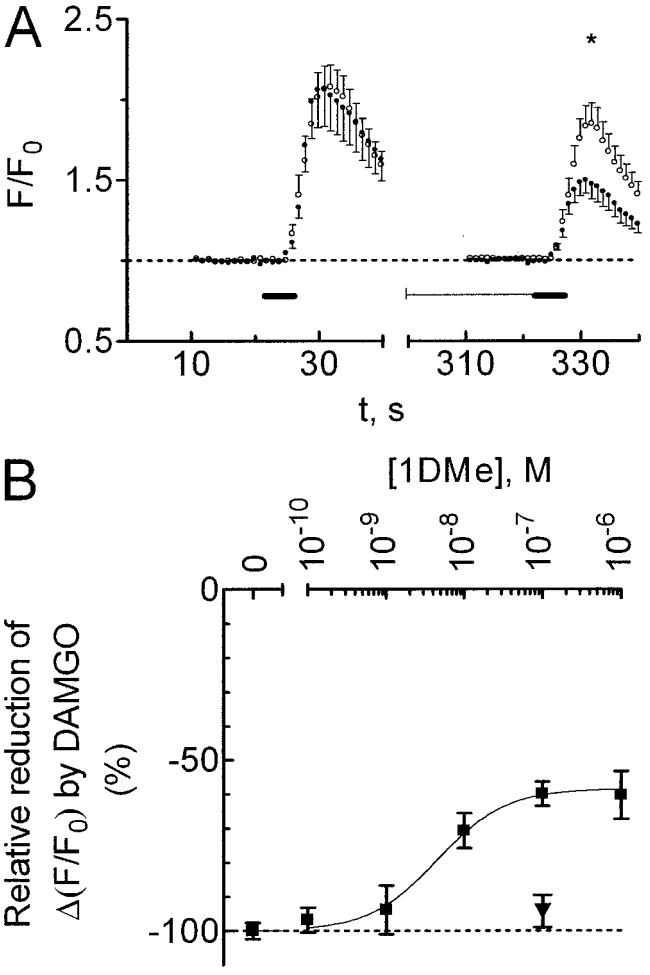

Fig. 3. Pretreatment with the NPFF agonist 1DMe reduced the inhibitory effect of DAMGO on N-type calcium channels. A, mean responses to 5-s depolarizations (thick horizontal lines) in 15 control (● − S.E.M.) and 19 1DMe-pretreated (○ + S.E.M.) SH_2-D9 cells, in control conditions (left) and at the end of a 30-s perfusion (thin horizontal line) with 1 μM DAMGO (right). The mean F/F_0 was calculated every second. Star (⋆) indicates that the response to depolarization at the end of the DAMGO perfusion is larger in 1DMe-treated than in control SH_2-D9 cells ($p < 0.05$, unpaired t test). B, dose-response curve of the effect of 1DMe (■ ± S.E.M.) on the inhibition of the response to depolarization by 1 μM DAMGO. The response to 1 μM DAMGO in the absence of 1DMe was taken as 100%. Number of cells is 171, 38, 17, 16, 58, and 18 from 0 to 10^{-6} M 1DMe, respectively. The NPFF agonist was without effect in SH-SY5Y cells (▼ ± S.E.M., n = 37).

renergic receptors (for review, see Vaughan et al., 1995). In SH_2-D9 cells, 1 μM deltorphin-I (30 s), 300 nM porcine NPY (30 s), and 10 μM clonidine (30 s) reduced the calcium transients triggered by depolarization (Fig. 4), although to a lesser extent than DAMGO ($p < 0.05$, one-way ANOVA followed by Bonferroni's multiple comparison test). In each case, 100 nM 1DMe significantly ($p < 0.05$) reduced the inhibitory effect of these agonists on N-type calcium conductance (Fig. 4).

Interactions of δ-Opioid and $NPFF_2$ Receptors with Muscarinic Receptors. In SH-SY5Y cells, opioid agonists enhance the release of Ca^{2+} from the intracellular stores triggered by carbachol acting on muscarinic receptors (Connor and Henderson, 1996). In all SH_2-D9 cells, perfusion with 5 μM carbachol produced an immediate increase in F/F_0 that decayed slowly toward a new steady state (Fig. 5, A and B) and that was not abolished by the removal of extracellular Ca^{2+} (not illustrated). Perfusions with deltorphin-I or DAMGO alone increased F/F_0 in less than 5% of the cells, whereas 1DMe alone never produced any change.

In the presence of 5 μM carbachol, 1 μM deltorphin-I and 1 μM DAMGO (40 s) increased F/F_0 in all the cells. The response to deltorphin-I was larger than that to DAMGO: $\Delta(F/F_0) = 3.59 \pm 0.15$ ($n = 84$) versus 0.85 ± 0.03 ($n = 90$), respectively ($p < 0.05$, unpaired t test). In view of this difference in magnitude, deltorphin-I was used in subsequent experiments. The NPFF agonist 1 μM 1DMe (40 s) also caused an increase in F/F_0 in all the cells, although of lesser magnitude than deltorphin-I: $\Delta(F/F_0) = 2.15 \pm 0.16$ versus 3.59 ± 0.15, respectively ($n = 84$, $p < 0.05$, paired t test). When applied for longer periods, in the presence of 5 μM carbachol, the time courses of the effects of deltorphin-I and 1DMe were different. During perfusion with 1 μM deltorphin-I, F/F_0 decreased to its predeltorphin-I level within 100 s (Fig. 5A), and upon switching back to carbachol alone, no change in F/F_0 was recorded (Fig. 5A). During perfusion with 1 μM 1DMe, the F/F_0 decrease was slower than with deltorphin-I (Fig. 5B), and F/F_0 remained above its pre-1DMe level during the 5 min of perfusion (Fig. 5B). As a consequence, upon switching back to carbachol alone a clear and fast decrease in

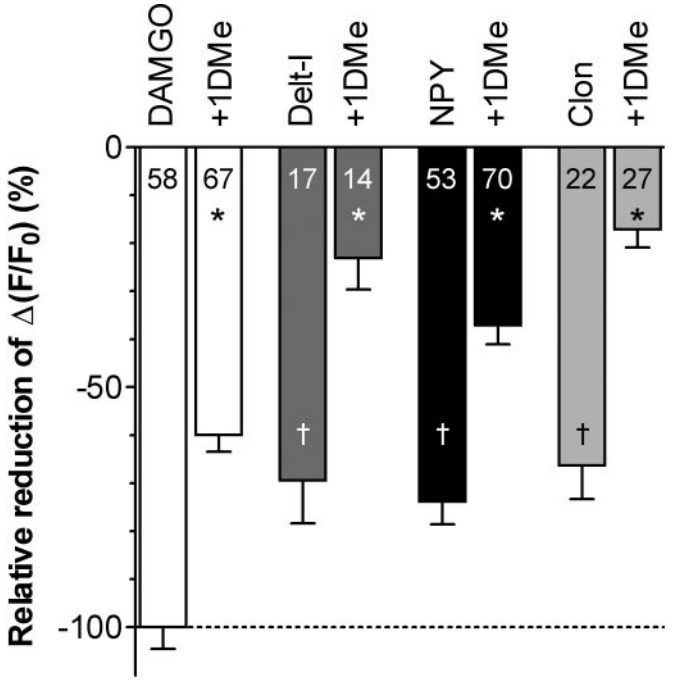

Fig. 4. Inhibitory effect of DAMGO, deltorphin-I (Delt-I) NPY, and clonidine (Clon) on the increase in F/F_0 induced by depolarization, in the absence and presence of 100 nM 1DMe. The response to DAMGO in the absence of 1DMe was taken as 100%. Significantly different ($\star$, $p < 0.05$) from its respective control. Significantly different (†, $p < 0.05$) from the response to DAMGO in the absence of 1DMe. One-way ANOVA followed by Bonferroni's multiple comparison test. The number of cells in each situation is indicated in the columns.

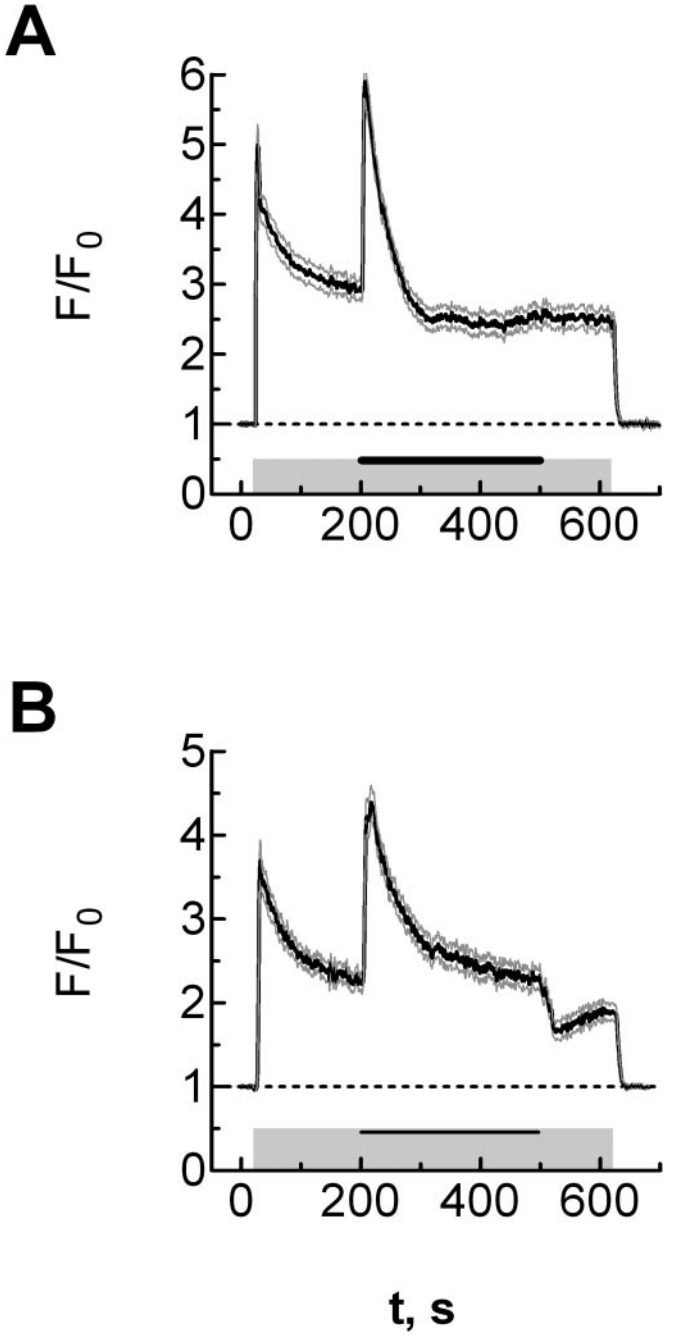

Fig. 5. Responses to deltorphin-I (A) and 1DMe (B) in the presence of carbachol. A, mean (black line) F/F_0, (±S.E.M., gray lines), calculated every second for the 35 cells present in the microscope field during application of 1 μM deltorphin-I (thick horizontal line) in the continuing presence of 5 μM carbachol (gray area). B, mean (black line) F/F_0, (±S.E.M., gray lines), calculated every second for 25 cells during application of 1 μM 1DMe (thick horizontal line) in the continuing presence of 5 μM carbachol (gray area).

F/F$_0$ was recorded (Fig. 5B). Removing extracellular Ca^{2+} did not alter the responses to deltorphin-I or 1DMe in the presence of carbachol (not illustrated). In SH-SY5Y cells, perfusion with 1 μM 1DMe (40 s) plus 5 μM carbachol caused no change in F/F$_0$, whereas 1 μM deltorphin-I (40 s) produced the same increase as in SH$_2$-D9 cells (not illustrated).

To test whether 1DMe could have an antiopioid effect on the response to deltorphin-I, we measured the dose-response relationship of 1 to 1000 nM deltorphin-I (40 s) in the continuing presence of 5 μM carbachol in control SH$_2$-D9 cells and in cells pretreated for 30 min and perfused with 100 nM 1DMe. In the presence of 100 nM 1DMe, the increase in F/F$_0$ in response to deltorphin-I was reduced (Fig. 6A). The EC$_{50}$ value for deltorphin-I was not significantly altered (5.3 versus 7.1 nM in control cells), but the maximal effect was reduced by 40% (Fig. 6B), indicating that the inhibition was not competitive. When SH$_2$-D9 cells were preincubated with 100 nM deltorphin-I before perfusion with media containing 100 nM deltorphin-I, the response to 1 μM 1DMe (40 s) in the presence of carbachol was identical to that of control cells [Δ(F/F$_0$) = 2.74 $\pm$ 0.20, n = 37 versus 2.23 $\pm$ 0.23, n = 19, in control cells; $p > 0.05$, unpaired t test]. Thus, the interaction between opioid and NPFF$_2$ receptors is not reciprocal.

Identification of G Proteins Associated with Opioid and NPFF$_2$ Receptors in SH$_2$-D9 Cells. Peptides corresponding to the carboxyl terminus of the Gα subunits of G proteins, which represent an important site of interaction with the receptor, have been reported to specifically uncouple receptors from G proteins in several systems, such as adenosinergic, adrenergic, and serotonergic, leading either to a low- or high-affinity state of the receptors (Gilchrist et al., 1998; Chang et al., 2000; Mazzoni et al., 2000). The G$\alpha_{i1,2}$ Gα_{i3}, Gα_o, and Gα_s inhibitory peptides were therefore tested for their ability to modulate the specific binding of ^{125}I-EYF and [^{3}H]DAMGO (Fig. 7). The G$\alpha_{i1,2}$, Gα_{i3}, and Gα_o synthetic peptides inhibited (maximum 25–30%) the binding of [^{3}H]DAMGO. In contrast, G$\alpha_{i1,2}$ peptide displayed a strong dose-dependent inhibition (up to 75% at 300 μM) of the specific binding of ^{125}I-EYF. A smaller inhibition (50% at 300 μM) was observed for the Gα_o peptide, whereas a slight (25%) increase in binding was recorded for Gα_{i3}. Surprisingly, the Gα_s peptide was the most potent inhibitor of both NPFF and opioid receptor binding. It was as efficacious (70% inhibition at 300 μM) as the G$\alpha_{i1,2}$ peptide to inhibit the ^{125}I-EYF-specific binding. Although the effect was smaller than with ^{125}I-EYF, it produced the greatest reduction of the [^{3}H]DAMGO-specific binding. Thus, NPFF and opioid receptors interact with all Gα subunits assayed.

To characterize more precisely the functional association of G proteins with opioid and NPFF receptors, inhibitory peptides were then tested for their ability to block cellular responses. In SH$_2$-D9 cells that were electroporated in the presence of Alexa 488 dermorphin (8 μg), 77% of the cells were fluorescent, whereas no fluorescence was detected in cells incubated with the labeled peptide but not electroporated (not illustrated). Electroporation was thus an efficient method to deliver the Gα peptides into SH$_2$-D9 cells, although differences in loading efficiency between the different peptides could not be excluded.

SH$_2$-D9 cells were electroporated in the absence and presence of 50 or 500 μM G$\alpha_{i1,2}$, Gα_{i3}, or Gα_o peptides, because 100 ng/ml PTX (18 h) suppressed the responses to 1 μM deltorphin-I (40 s) and 1 μM 1DMe (40 s) in the presence of 5 μM carbachol (not illustrated). As shown in Fig. 8, the response to deltorphin-I was reduced by G$\alpha_{i1,2}$ and Gα_o pep-

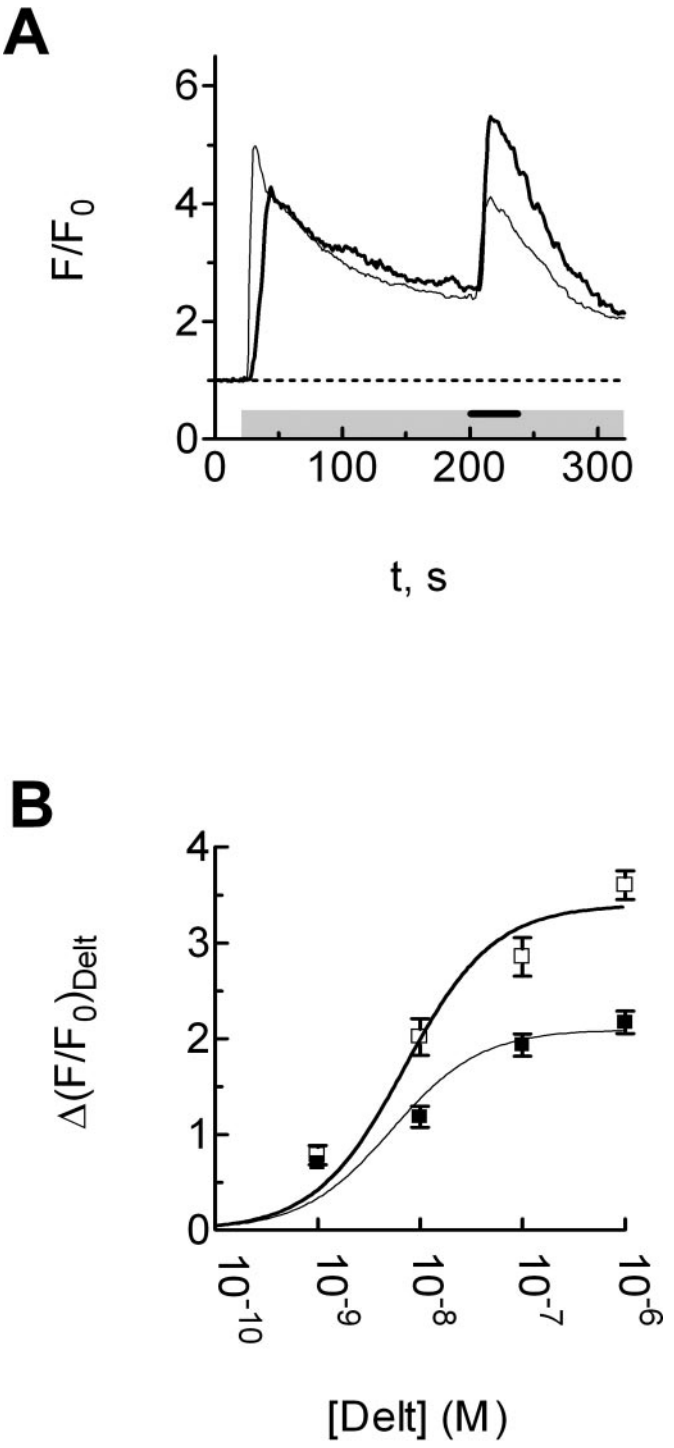

Fig. 6. Response to deltorphin-I in the continuing presence of carbachol is reduced in cells preincubated and perfused with 1DMe. A, mean F/F$_0$, calculated every second, for 33 control cells (thick line) and 44 cells preincubated with 100 nM 1DMe (thin line) during application of 1 μM deltorphin-I (thick horizontal line) in the continuing presence of 5 μM carbachol (gray area). B, increase in F/F$_0$ in response to deltorphin-I in the continuing presence of carbachol in SH$_2$-D9 cells in the absence (□ $\pm$ S.E.M.) and presence (■ $\pm$ S.E.M.) of 100 nM 1DMe. From 10^{-9} to 10^{-6} M, the number of cells is 26, 33, 70, and 84 and 42, 44, 85, and 92 in control and 1DMe-treated cells, respectively.

tides at 50 and 500 μM, whereas $G\alpha_{i3}$ only was efficient at 500 μM. The activation of the δ-opioid receptor in the presence of carbachol released Ca^{2+} from intracellular stores through a preferential coupling with $G\alpha_{i1,2}$, $G\alpha_o$, and to a lesser extent to $G\alpha_{i3}$. The response to 1DMe was reduced by the $G\alpha_o$ peptide at 50 and 500 μM and by the $G\alpha_{i1,2}$ peptide at 500 μM. The $G\alpha_{i3}$ peptide, at 500 μM concentration, caused a nonsignificant reduction of the response to 1DMe (Fig. 8), indicating that the activation of the $NPFF_2$ receptor, in the presence of carbachol, released Ca^{2+} from intracellular stores through a preferential coupling with $G\alpha_o$ and to a lesser extent with $G\alpha_{i1,2}$.

The inhibitory effect of DAMGO on N-type Ca^{2+}-channel was reduced to 24% of its control value ($p < 0.05$, unpaired t test) after pretreatment with 100 ng/ml PTX (20–22 h), precluding the measurement of the antiopioid effect of 1DMe. Only we could test the effect of the $G\alpha_s$ peptide, after delivery within the cells by the Chariot peptide carrier: the $G\alpha_s$ peptide did not change the inhibitory effect of DAMGO, but increased the antiopioid activity of 1DMe (Fig. 9). To confirm the involvement of $G\alpha_s$, SH_2-D9 cells were incubated overnight with 500 ng/ml CTX. This resulted in a very low density of cells that were unresponsive to depolarization, so that it was not possible to measure the antiopioid activity of 1DMe.

To exclude a nonspecific effect of the $G\alpha_s$ peptide, the inhibition of the forskolin-stimulated production of cAMP by 1 nM 1DMe was compared in SH_2-D9 cells electroporated in the presence of $G\alpha_{i1,2}$ or $G\alpha_s$ peptides (60 μg). The effect of 1 nM 1DMe in cells electroporated with $G\alpha_{i1,2}$, was reduced to 74.3 ± 5.8% ($n = 4$) of that measured in the absence of peptide ($p < 0.05$, unpaired t test), whereas it was not modified in cells electroporated with $G\alpha_s$ (108.7 ± 6.4% of control cells, $n = 3$, $p > 0.05$, unpaired t test).

Discussion

We describe in the present study a cellular model (human $NPFF_2$ receptor transfected SH-SY5Y cells: SH_2-D9) that reproduces the functional antagonism exerted by NPFF on opioid activity in neurons, thus allowing the possibility to explore the molecular mechanisms responsible for this process.

SH_2-D9 cells express high-affinity $NPFF_2$ receptors with a B_{max} of the same order of magnitude (100–300 fmol/mg) as for μ- and δ-opioid receptors (Kazmi and Mishra, 1987; Toll et al., 1997; Noble et al., 2000; this study). Activation of both μ-opioid and $NPFF_2$ receptors inhibit adenylate cyclase, in a PTX-sensitive way, as already demonstrated in SH-SY5Y cells for opioid receptors (Kazmi and Mishra, 1987) and in $NPFF_2$-transfected Chinese hamster ovary cells (Kotani et al., 2001). We have, therefore, obtained a neuroblastoma cell line expressing high-affinity and functional opioid and

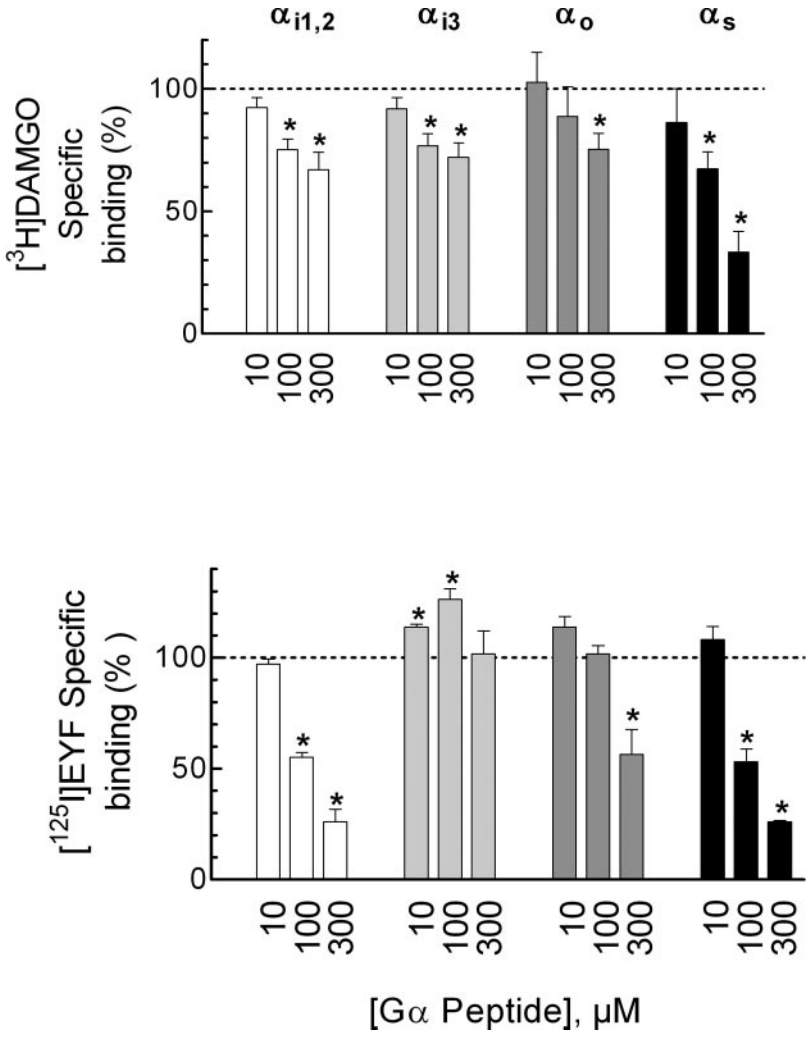

Fig. 7. Inhibition of specific [^{3}H]DAMGO and ^{125}I-EYF binding by $G\alpha_{i1,2}$, $G\alpha_{i3}$, $G\alpha_o$, and $G\alpha_s$ peptides. Membranes from SH_2-D9 cells were incubated for 60 min at 25°C with 0.1 nM ^{125}I-EYF or 1 nM [^{3}H]DAMGO in the absence (control) or in the presence of increasing concentrations of Gα peptides. Columns represent mean ± S.E.M., expressed as percentage of control specific binding, of three to five experiments performed in duplicate. Significantly (⋆, $p < 0.05$) different from the control. One-way ANOVA followed by Bonferroni's multiple comparison test.

NPFF$_2$ receptors. We demonstrate that activation of NPFF$_2$ receptors, in this cell line, exerts an antiopioid effect in two experimental paradigms: modulation of N-type voltage-activated Ca^{2+} channels as observed in neurons (Roumy and Zajac, 1999) and coincident signaling between Gq-coupled receptors (muscarinic) and opioid receptors.

In all SH$_2$-D9 cells responding to depolarization in the presence of nifedipine (a blocker of the L-type Ca^{2+} channels), DAMGO, a highly specific μ-opioid agonist reduces the magnitude of the depolarization-induced increase in $[Ca^{2+}]_i$. In cells preincubated with an agonist of NPFF$_2$ receptors (1DMe), the effect of DAMGO on the Ca^{2+} transient is reduced noncompetitively, with no change in the EC_{50} value and a reduction of 40 to 50% of its maximal effect. This antiopioid effect is caused by the activation of the NPFF$_2$ receptor, because it is absent in nontransfected SH-SY5Y cells. It is identical to that demonstrated in dorsal root ganglion (Rebeyrolles et al., 1996) and dorsal raphe neurons (Roumy and Zajac, 1999). In contrast to the specificity toward opioid receptors established in neurons (Roumy and Zajac, 1999) and in in vitro models (Takeuchi et al., 2001), the activation of NPFF$_2$ receptors in SH$_2$-D9 cells also reduces the inhibition of N-type Ca^{2+} channels induced by NPY Y$_2$ and α_2-adrenergic receptors. It should be recognized, however, that the interaction of NPFF$_2$ receptors with other G protein-coupled receptors has not been exhaustively studied in isolated neurons and thus it cannot be entirely excluded that the activation of NPFF$_2$ receptors could antagonize the activity of receptors other than the opioids. On the other hand, this lack of specificity may relate to the nature of the SH-SY5Y cells, which are undifferentiated neuroblast-like cells that can spontaneously transdifferentiate into an epithelial-like S cell (Ross et al., 1983) and be induced to differentiate to a mature neuronal phenotype. It is therefore possible that the compartmentation of receptors and/or G proteins within the membrane of these cells might be very different from that of mature neurons. This could be approached experimentally by differentiating SH$_2$-D9 cells.

In SH-SY5Y cells, μ- and δ-opioid receptor agonists are able to release Ca^{2+} from the intracellular stores only when applied in the presence of carbachol, acting at the muscarinic receptor, that causes Ca^{2+} release through activation of G_q (Connor and Henderson, 1996). In the presence of 1 μM carbachol, DAMGO and [D-Pen^2,D-Pen^5]-enkephalin, μ- and δ-specific agonists, increase $[Ca^{2+}]_i$ with an EC_{50} value of 270 and 10 nM, respectively (Connor and Henderson, 1996). Opioid agonists act through stimulation of phospholipase C by G$\beta\gamma$ subunits from $G_{i/o}$ proteins, although an increase in inositol trisphosphate is still debated (Smart et al., 1995; Yeo et al., 2001). In SH$_2$-D9 but not in SH-SY5Y cells, 1DMe increases $[Ca^{2+}]_i$ in the presence of 5 μM carbachol, as do the

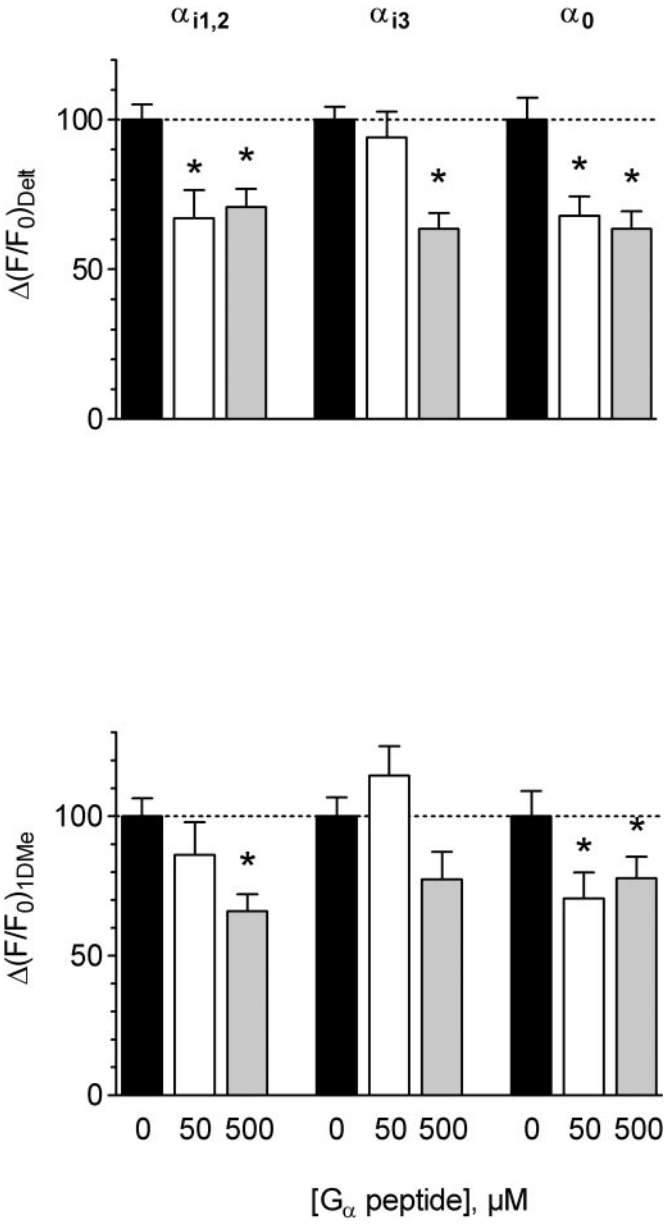

Fig. 8. Increase in F/F_0 induced by perfusion with deltorphin-I (1 μM, top) or 1DMe (1 μM, bottom) in the presence of 5 μM carbachol in SH$_2$-D9 cells electroporated in the absence and presence of the indicated Gα peptides at 50 or 500 μM. Significantly different ($\star$, $p < 0.05$) from its respective control. One-way ANOVA followed by Bonferroni's multiple comparison test. The number of cells in each situation is indicated in the columns.

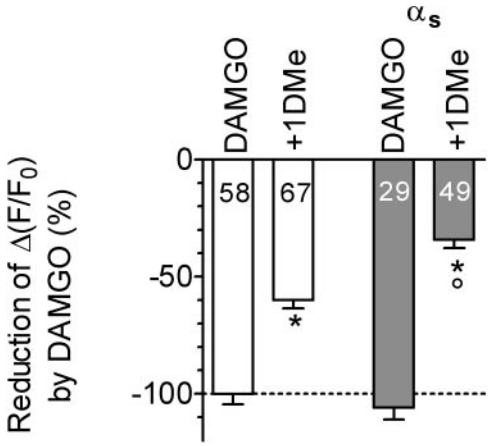

Fig. 9. Effect of the Gα_s peptide on the antiopioid activity of 1DMe in the inhibition of N-type calcium channels by DAMGO. The reduction, by DAMGO, of the increase in F/F_0 induced by depolarization was measured in the absence (DAMGO) and presence (+1DMe) of 100 nM 1DMe in control or α_s-treated SH$_2$-D9 cells. Significantly different ($\star$, $p < 0.05$) from their respective control. Significantly different ($^\circ$, $p < 0.05$) from the reduction of $\Delta(F/F_0)$ in the presence of 1DMe in control cells. One-way ANOVA followed by Bonferroni's multiple comparison test. The number of cells in each situation is indicated in the columns.

opioid agonists in both cell lines. However, there may exist subtle differences between the mechanism of response to opioid and NPFF agonists because the time courses of the responses to 5-min applications of deltorphin-I and 1DMe are not identical. It should be pointed out that this is the first report of coincident signaling between NPFF receptors and G_q-coupled receptors.

Furthermore, pretreatment of SH_2-D9 cells with 100 nM 1DMe reduces the response to deltorphin-I, whereas preincubation with deltorphin-I does not affect the response to 1DMe, demonstrating that the interaction between opioid and NPFF receptors is not reciprocal. Therefore, as for the modulation of N-type Ca^{2+} channels, $NPFF_2$ receptors exert a noncompetitive antiopioid activity on the release of intracellular Ca^{2+}.

Thus, we demonstrate, for the first time, that $NPFF_2$ receptors have a cellular antiopioid activity in two experimental situations where the final targets, an N-type voltage-sensitive calcium channel and the inositol triphosphate-gated Ca^{2+} channel, are different. This suggests that a modification of these channels (e.g., a phosphorylation) reducing their sensitivity to the activation of opioid receptors is not responsible for the antiopioid effect of NPFF agonists but that an upstream modification of the signaling cascade should be considered. A decrease in opioid agonist affinity or receptor number does not explain the antiopioid effect of NPFF because the binding parameters of opioid agonists in membranes or living cells remained unchanged after pretreatment with 1DMe. A competition for a common pool of G protein, resulting in decreased signaling of opioid receptors, might be responsible for the antiopioid effect of NPFF, as in the case of human cannabinoid receptor that reduces noradrenaline- and somatostatin-induced inhibition of Ca^{2+} channels (for review, see Cordeaux and Hill, 2002). However, we would expect the interaction between opioid and $NPFF_2$ receptors to be competitive, which is not the case, and the opioid agonists to reduce the Ca^{2+} release induced by 1DMe in the presence of carbachol, which is not the case either.

We have taken advantage of the inhibitory properties of peptides corresponding to the carboxyl terminus of the $G\alpha$ subunits (Gilchrist et al., 1998) to carry a more precise characterization of the G proteins associated with $NPFF_2$ and opioid receptors in SH-SY5Y cells. As expected from pertussis toxin experiments, the specific binding to NPFF receptors is strongly decreased by $G\alpha_{i1,2}$ and to a lesser extent by $G\alpha_o$ peptide. Accordingly, these two peptides decrease the effect of 1DMe in the presence of carbachol, suggesting that NPFF receptors are preferentially coupled to $G\alpha_{i1,2}$ and $G\alpha_o$ proteins. However, even if it does not block the functional effect of NPFF in the carbachol assay, an interaction with the $G\alpha_{i3}$ subunit is not excluded because a 25% increase in binding is observed with the $G\alpha_{i3}$ peptide. Thus, our results indicate that $NPFF_2$ receptors are probably coupled to $G\alpha_{i1,2}$, $G\alpha_o$, and $G\alpha_{i3}$ proteins. They are in accordance with previous experiments performed in human embryonic kidney 293 and COS7 cells cotransfected with chimeric G proteins, showing an interaction between NPFF receptors with $G\alpha_{q/i2}$ and $G\alpha_{q/o}$ (Elshourbagy et al., 2000) as well as $G\alpha_{q/i3}$ and $G\alpha_{q/z}$ (Bonini et al., 2000).

The $G\alpha_s$ peptide strongly inhibits the binding to NPFF receptors and potentiates the antiopioid effect of 1DMe in Ca^{2+} conductance measurements. That the action of the $G\alpha_s$ peptide is specific is suggested by the fact that although the $G\alpha_{i1,2}$ peptide reduces the inhibitory effect of 1DMe on the forskolin-induced cAMP production, the $G\alpha_s$ peptide is inactive. Some data also suggest a coupling between NPFF receptors and $G\alpha_s$. Analogs of NPFF, at concentrations in the micromolar range, stimulate adenylate cyclase in membranes from the mouse olfactory bulb (Gherardi and Zajac, 1997), and a cellular response to NPFF through the activation of the chimeric $G\alpha_{q/s}$ has been demonstrated (Bonini et al., 2000). Furthermore, the antiopioid activity of NPFF on the nociceptin-induced inhibition of Ca^{2+} conductances in isolated rat dorsal raphe neurons is prevented by CTX (Roumy and Zajac, 2001). Together, these observations firmly suggest that $NPFF_2$ receptors couple to G_s, although their activation does not clearly increase cAMP production. Furthermore, this coupling does not mediate NPFF antiopioid activity, but it is involved in its modulation.

As described previously in SH-SY5Y (Carter and Medzihradsky, 1993; Laugwitz et al., 1993), opioid receptors are coupled to $G\alpha_{i1,2}$, $G\alpha_{i3}$, and $G\alpha_o$ in SH_2-D9 cells. In addition, we find that the binding of [^{3}H]DAMGO is strongly inhibited by the $G\alpha_s$ peptide, but this does not result in a reduced effect of the opioid agonist on the N-type Ca^{2+} channels measured at a saturating concentration. The existence of G_s-coupled excitatory opioid receptors has been suggested (Crain and Shen, 2000), but such a coupling through G_s is not involved in either the inhibition of the N-type Ca^{2+} channels or the cross-talk with G_q-coupled receptors because both effects are suppressed by PTX.

Together, these results indicate that $NPFF_2$ receptors exert a functional antagonism on opioid receptors that is not reciprocal. There are many peptides, including the opioid peptides themselves, able to modulate the activity of opioid receptors, but to our knowledge only NPFF and cholecystokinin (Heinricher et al., 2001) exert a cellular antiopioid activity. The SH_2-D9 model provides the opportunity to characterize the molecular mechanisms involved in the interaction between NPFF and opioid receptors.

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