δ-Opioid Receptors Stimulate the Metabolic Sensor AMP-Activated Protein Kinase through Coincident Signaling with G_{q/11}-Coupled Receptors

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ABSTRACT
AMP-activated protein kinase (AMPK) and δ-opioid receptors (DORs) are both involved in controlling cell survival, energy metabolism, and food intake, but little is known on the interaction between these two signaling molecules. Here we show that activation of human DORs stably expressed in Chinese hamster ovary (CHO) cells increased AMPK activity and AMPK phosphorylation in vitro and in vivo. In CHO cells, AMPK activation was prevented by PKA, transforming growth factor-β, and protein kinase C inhibitors. Conversely, the DOR effect was reduced by Ca^{2+}/calmodulin-dependent protein kinase (CaMKK) inhibition, apyrase treatment, G_{q/11} antagonism, and blockade of P2 purinergic receptors. Apyrase treatment also depressed DOR stimulation of intracellular Ca^{2+} concentration, whereas P2 receptor antagonism blocked DOR stimulation of inositol phosphate accumulation. In SH-SY5Y neuroblastoma cells and primary olfactory bulb neurons, DOR activation failed to affect AMPK phosphorylation per se but potentiated the stimulation by either muscarinic agonists or 2-methyl-thio-ADP. Sequestration of G protein βγ subunits (G_{βγ}) blocked the DOR potentiation of AMPK phosphorylation induced by oxotremorine-M. In CHO cells, the DOR activator 5-aminomimidazole-4-carboxamide-1-β-d-ribonucleoside stimulated AMPK phosphorylation and glucose uptake, whereas pharmacological inhibition of AMPK, expression of a dominant-negative mutant of AMPKα1, and P2Y receptor blockade reduced DOR-stimulated glucose uptake. The data indicate that in different cell systems, DOR activation up-regulates AMPK through a G_{βγ} dependent synergistic interaction with G_{q/11}-coupled receptors, potentiating Ca^{2+} release and CaMKKβ-dependent AMPK phosphorylation. In CHO cells, this coincident signaling mechanism is involved in DOR-induced glucose uptake.

Introduction
AMP-activated protein kinase (AMPK) is a multisubstrate serine/threonine protein kinase that is activated under conditions of metabolic stress, such as glucose deprivation and hypoxia/ischemia, causing depletion of intracellular ATP and an increase of AMP/ATP ratio. AMPK is a heterotrimeric complex composed of a catalytic α subunit and regulatory β and γ subunits. In mammals, two α (α1 and α2), two β (β1 and β2), and three γ (γ1, γ2, and γ3) subunits encoded by distinct genes have been identified (Towler and Hardie, 2007).

ABBREVIATIONS: ACC, acetyl-coenzyme A carboxylase; ADV, adenosivir; AICAR, 5-aminomimidazole-4-carboxamide-1-β-d-ribonucleoside; AMPK, AMP-activated protein kinase; ANOVA, analysis of variance; BSA, bovine serum albumin; CaMKK, Ca^{2+}/calmodulin-dependent protein kinase; CCh, carbachol; CHO, Chinese hamster ovary; CHO/DOR, CHO cells stably expressing human DOR; compound C, 6-[4-[2-(1-piperidinyl)ethoxy]phenyl]-3-(4-pyridinyl)-pyrazolo[1,5-a]pyrimidine; dBcAMP, dibutyryl-cAMP; DN, dominant-negative; DOR, δ-opioid receptor; DPDPE, [D-Pen(2,5)]-enkephalin; DTT, dithiothreitol; FCS, fetal calf serum; GLUT, glucose transporter; Go 6983, 3-[1-[3-(dimethylamino)propyl]-5-methoxy-1H-indol-3-yl]-4-(1H-indol-3-yl)-1H-pyrole-2,5-dione; GRK2-CT, C-terminal region of G protein-coupled receptor kinase 2; HPLC, high-performance liquid chromatography; IGF-1, insulin-like growth factor-1; IP, inositol phosphate; LDH, lactate dehydrogenase; MAP, mitogen-activated protein; MeSADP, 2-methylthio ADP; MRS 2179, N^6-methyl 2′-deoxyadenosine 3′,5′-bisphosphate; naltrindole, 17-(cyclopropylmethyl)-6,7-dehydro-4,5-epoxy-3,14-dihydroxy-6,7-2′,3′-indolomorphinan; NTI, naltrindole; Oxo-M, oxotremorine-M; PBS, phosphate-buffered saline; PD 98059, 2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one; P3K, phosphatidylinositol 3-kinase; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; PPADS, pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid; PTX, Bordetella pertussis toxin; 2′′-cAMPS, 2′′-O-(2′-deoxyribose) cyclic AMP; SAM, S-adenosyl-methionine; SAMS peptide, HMRSAMSGGHLKRR; SNG 80, (−)-4-[(1R)-N-(22S,SR)-4-allyl-2,5-dimethyl-1-piperazinyl]-3-methoxybenzyl]-N,N-diethyl-benzamide; SP600125, anthra[1–9-c]pyrazolo[6(2H)]-one; SpotCAMS, adenosine-3′,5′-cyclic monophosphorothioate, Sp-isomer; STO-609, 7-oxo-7H-benzimidazo[2,1-a]benz[e]isoquinoline-3-carboxylic acid; TAK1, transforming growth factor-β-activated kinase 1.
AMPK is regulated through distinct mechanisms. AMPK activity is at least 50- to 100-fold enhanced by phosphorylation at Thr172 within the α subunit T-loop activation domain. In mammals, a major upstream protein kinase is the constitutively active tumor suppressor liver kinase B1 in complex with the regulatory proteins STE20-related adaptor and MO25 (Towler and Hardie, 2007). AMPK can also be regulated in a Ca^{2+}-dependent manner through phosphorylation at Thr172 by the Ca^{2+}/calmodulin-dependent protein kinase kinase (CaMKK) β (Towler and Hardie, 2007). In addition, evidence for the involvement of the transforming growth factor-β-activated kinase 1 (TAK1), a member of the mitogen-activated protein (MAP) kinase kinase family, has also been provided (Momiclovic et al., 2006). On the other hand, AMPK binds to γ subunit cystathionine β-synthase domains and activates AMPK through an allosteric mechanism, as well as promoting the phosphorylation by upstream kinase and inhibiting Thr172 dephosphorylation by phosphatases (Oakhill et al., 2011).

Once activated, AMPK tends to maintain energy homeostasis by switching on catabolic pathways that produce ATP and inhibiting ATP-consuming events. Thus, AMPK activates fatty acid oxidation through phosphorylation and inhibition of acetyl-coenzyme A carboxylase (ACC). The inhibition of ACC causes a decrease of malonyl-coenzyme with the consequent activation of mitochondrial carnitine palmitoyltransferase-1. AMPK also inhibits protein synthesis by phosphorylating and activating the elongation factor-2 kinase, which stops the elongation step in translation and by inhibiting the mammalian target of rapamycin pathway, which stimulates protein synthesis and cell growth (Kahn et al., 2005). AMPK promotes glycogenolysis and glucose uptake by enhancing the membrane expression of the glucose transporter GLUT4 in muscle and the membrane translocation and/or activity of GLUT1 in different cell types (Towler and Hardie, 2007). In hypothalamic neurons, AMPK has been identified as a key signaling molecule mediating the central control of appetite by orexigenic and anorectic hormones (Kahn et al., 2005; Towler and Hardie, 2007). By regulating energy metabolism, AMPK may control cell survival. Accumulating evidence indicates that activation of AMPK plays a neuroprotective role under conditions of reduced energy supply or hypoxia, whereas dysregulation of AMPK is associated with neurodegeneration (Ronnett et al., 2009).

Similar to AMPK, DORs have been reported to stimulate glucose uptake in skeletal muscle and transfected cells (Evans et al., 2001; Olianas et al., 2011) and to exert neuroprotective effects under hypoxic and ischemic insults (Bofetiado et al., 1996; Zhang et al., 2002). Moreover, it has been shown that DOR agonists increase food intake by acting on distinct brain areas, including the hypothalamus (Glass et al., 1999). However, despite these functional similarities, no information has so far been provided linking AMPK activity with DOR signaling. In the present study, we have investigated the regulation of AMPK by DOR stably expressed in Chinese hamster ovary (CHO) cells and endogenously present in SH-SY5Y neuroblastoma cells and primary olfactory bulb neurons.

Materials and Methods

Materials. (+)-4-(αR)-a-((2S,5R)-4-Allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-N,N-diethylbenzamide (SNC 80), 17-(cyclopropylmethyl)-6,7-dehydro-4,5a-epoxy-3,14-dihydroxy-6,7-2',3'-indolomorphinan (naltrindole) hydrochloride (NTI), 2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one (PD 98059), 4-[5-(4-fluorophenyl)-2-(4-methylsulphonyl)phenyl]-1H-imidazo[4,5-f]pyridine (SB203580), arthra[1-9-cyprazol-6(2H)]-one (SP600125), 5-aminomidoazide-4-carboxamidel-β-0-ribonucleoside (AICAR), N^6-methyl-2'-deoxyadenosine 3',5'-bisphosphate (MRS 2179), and pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS) tetrasodium salt were all from Tocris Bioscience (Bristol, UK). The peptide HMRASGGLHVKRR (SAMPS peptide) and [α-25,5]-enephalin (DPDPE) was purchased from Bachem AG (Bubendorf, Switzerland). 2-[(3-Dimethylaminopropyl)indol-3-yl]-3-(indol-3-yl)maleimide (Go 6850; also termed bisindolylmaleimide I and GF 10920X), 3-[1-[(3-dimethylamino)propyl]-5-methoxy-1H-indol-3-yl]-4-(1H-indol-3-yl)-1H-pyrrrole-2,5-dione (Go 6893), 7-oxo-7H-benzo[1,2-b]benzimidazole-[2,1-β]j-nitrobenz-3-carboxylic acid (STO-609), ionomycin, and 2-deoxy-o-glucose were from Calbiochem (La Jolla, CA). Adenosine-3',5'-cyclic monophosphorothioate, Rp-isomer (Rp-cAMPS) and adenosine-3',5'-cyclic monophosphorothioate, Sp-isomer (Sp-cAMPS) were from Biomol GmbH (Hamburg, Germany). The transforming growth factor-β-activated kinase 1 (TAK1) inhibitor (5Z)-7-oxozaenosol (antibiotic LL1640-2) was from Santa Cruz Biotechnology (Santa Cruz, CA). The cyclic depsipeptide YM 254890, isolated from culture broth of Chromobacterium sp. QS3666, was generously provided by Dr. Jun Takasaki (Yamanouchi Pharmaceutical Co. Ltd., Tsukuba, Ibaraki, Japan). Bordetella pertussis toxin (PTX), 6-[4-[2-(1-piperidin-1-yl)ethoxy]-phenyl]-3-(4-pteridinyl)-pyrazolo[1,5-a]pyrimidine (compound C) dihydrochloride, 2-methylthio ADP (MeSADP), dibutyryl-cAMP (dBcAMP), apyrase, phosphatase inhibitor cocktail 1, protease inhibitor cocktail, and the other reagents were all from Sigma-Aldrich (St. Louis, MO).

Cell Culture and Transfections. CHO-K1 cells (American Type Culture Collection, Manassas, VA) were grown at 37°C in a humidified atmosphere (5% CO_2) in Ham's F12 medium containing 1-glutamine and sodium bicarbonate and supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin/100 μg/ml streptomycin (Invitrogen, Carlsbad, CA). CHO-K1 cells stably expressing the human DORs (CHO/DOR cells) were developed by transfecting the cells with pcDNA3.1-Hygr(+)vector encoding the human DORs (Top Gene Technologies, Montreal, QC, Canada) using PolyFect (QIAGEN Technologies, Montreal, QC, Canada) according to the manufacturer's instructions. Cells were selected by their resistance to 1 mg/ml hygromycin (Invitrogen) for 4 weeks, and cell clones were isolated by using cloning cylinders. The cell clone used in the present study had a receptor density of ~1500 fmol/mg protein determined by saturation binding with the radioligand [3H]HNTI (PerkinElmer Life and Analytical Sciences, Wallingford, MA). Cells were maintained in Ham’s F12 medium containing 1-glutamine and sodium bicarbonate and supplemented with 10% FCS, 100 U/ml penicillin/100 μg/ml streptomycin, and 350 μg/ml hygromycin.

SH-SY5Y cells were obtained from the European Cell Culture Collection (Salisbury, UK) and grown in Ham’s F12/15 medium (1:1) supplemented with 2 mM l-glutamine, 1% nonessential amino acids, 10% FCS, and 100 U/ml penicillin/100 μg/ml streptomycin (Invitrogen) at 37°C in a humidified atmosphere of 5% CO_2 in air. Cells were used to the 16th to 18th passages.

For transient transfection with the minigene encoding the C-terminal region (amino acids 495-689) of G protein-coupled receptor kinase 2 (GRK2-CT) (pRK5 BARK1 minigene plasmid; Addgene, Cambridge MA), SH-SY5Y cells were seeded in antibiotic-free medium on six-well plates at the density of 7 x 10^5 cells/well. Twenty-four hours later, the medium was removed and substituted with Opti-MEM medium (Invitrogen). Cells were transfected with either GRK2-CT minigene (4 μg/well) or empty vector for 16 h using Lipofectamine 2000 (Invitrogen) as transfectant. Thereafter, cells were transfected to normal growth medium, serum-starved overnight, and analyzed 48 h after transfection.
Adenoviral Infections. CHO/DOR cells were seeded on 24-well plates at a density of $10^5$ cells/well 24 h before infection. Thereafter, the medium was removed and changed to a fresh medium containing 2% FCS (200 μM/l). Cells were infected with 100 plaque-forming units/cell of either null control recombinant adenovirus (ADV) or dominant-negative (DN) AMPKα1 recombinant ADV (Eton Bioscience, San Diego, CA) for 12 h. The DN mutant contains a D159A mutation in the ATP binding domain and lacks the ATP binding capacity. The mutant competes with wild-type AMPKα1 for the binding with β and γ subunits (Woods et al., 2000). Cells were then transferred to normal growth medium, serum-starved overnight, and analyzed 48 h after infection.

Preparation of Primary Cultures of Mouse Olfactory Bulb Neurons. Primary cultures of neural cells of mouse olfactory bulbs were prepared from 1-day-old CD-1 mice (Harlan Laboratories, Udine, Italy). The animals were anesthetized by hypothermia and sacrificed by decapitation. The brains were immediately immersed in an ice-cold complete Neurobasal A medium containing B-27 serum-free supplement (Invitrogen), 0.5 mM l-glutamine, 50 μM β-mercaptoethanol, and 100 U/ml penicillin-100 μg/ml streptomycin (Invitrogen), placed on the dorsal surface and the olfactory bulbs were removed by cutting the olfactory tracts. After removal of the meninges, the bulbs were transferred to fresh medium and minced into small fragments under a dissecting microscope. The tissue fragments were collected by sedimentation and incubated in the presence of 0.2% trypsin (type IX from porcine pancreas; Sigma-Aldrich) dissolved in Neurobasal A medium for 30 min at 30°C. Thereafter, soybean trypsin inhibitor (final concentration, 1%) and DNase type I (0.1 mg/ml) were added, and the incubation was continued for additional 5 to 10 min. After gentle trituration with fire-polished Pasteur pipettes, the cell suspension was layered at the top of a 4% bovine serum albumin (BSA) solution in complete Neurobasal A medium and centrifuged at 1200 g for 10 min at room temperature. The cell pellet was resuspended in complete Neurobasal A medium. Cells were plated on six-well plates precoated with 0.01% L-poly-lysine at a density of 10^5/well. The cells were placed in a humidified incubator and aliquots were taken for protein determination twice the cell suspension through a 26-gauge needle. The lysate was centrifuged at 20,800g for 5 min at 4°C, and the supernatant was collected and assayed for protein content. Aliquots containing equal amount of protein (500–600 μg) were incubated with 1.5 μg of anti-AMPKα1/2 subunit antibody (Santa Cruz Biotechnology) for 2 h at 4°C with constant rotation. Thereafter, 20 μl of protein A/G agarose slurry, previously equilibrated in lysis buffer, were added, and the incubation was continued for 1 h at 4°C. The beads were then washed three times with a washing buffer containing 20 mM Tris-HCl, pH 7.4, 140 mM NaCl, 5 mM sodium pyrophosphate, 50 mM NaF, 2 mM dithiothreitol (DTT), 0.25 M sucrose, 1% Nonidet P-40, 1% protease inhibitor cocktail, 0.5% phosphatase inhibitor cocktail, 2 mM sodium orthovanadate, and 1 mM phenylmethylsulfonyl fluoride. Cell lysis was obtained by aspirating the cell suspension through a 25-gauge needle. The lysate was centrifuged at 20,800g for 5 min at 4°C, and the supernatant was collected and assayed for AMPK activity. Aliquots containing equal amount of protein (500–600 μg) were incubated with 1.5 μg of anti-AMPKα1/2 subunit antibody (Santa Cruz Biotechnology) for 2 h at 4°C with constant rotation. Thereafter, 20 μl of protein A/G agarose slurry, previously equilibrated in lysis buffer, were added, and the incubation was continued for 1 h at 4°C. The beads were then washed three times with a washing buffer containing 20 mM Tris-HCl, pH 7.4, 140 mM NaCl, 1% Nonidet P-40, and 1 mM DTT, and one with assay buffer containing 40 mM HEPES/NaOH, pH 7.0, 80 mM NaCl, 8% glycerol, 0.8 mM EDTA, 0.8 mM DTT, 5 mM MgCl2, and 200 μM AMP. Beads were finally resuspended in 20 μl of assay mixture containing assay buffer supplemented with 50 μM ATP, 200 μM SAMPS peptide, and 3 μCi of [γ-32P]ATP (3000 Ci/mmol; PerkinElmer) and incubated for 15 min at 30°C. Blanks were prepared by omitting the SAMPS peptide. Preliminary experiments indicated that the enzyme activity was linear with time within this incubation period. Reaction was stopped by adding EDTA (final concentration, 90 mM) and centrifuging at 8000g for 2 min at 4°C. An aliquot of the supernatant was spotted onto a 1 × 2-mm phosphocellulose paper strip (Whatman, Clifton, NJ) that was air-dried and washed four times with 1% phosphoric acid. The 32P radioactivity absorbed onto the strips was measured by liquid scintillation counting. Assays were run in triplicates.

Measurement of Intracellular Free Ca^{2+}. Intracellular concentration of free Ca^{2+} was measured in confluent monolayers of CHO/DOR and SH-SY5Y cells using the Ca^{2+}-sensitive dye fura-2. Cells were grown on polyl-lysine-coated 10-mm diameter round glass coverslips individually placed into the well of 24-well plates. The cells were incubated in the presence of 5 μM fura-2/acetoxymethyl ester (Calbiochem, San Diego, CA), 0.05% BSA, and 0.1% (v/v)
Pluronic acid F-127 for 1 h at 37°C in Krebs-HEPES medium supplemented with 1.2 mM CaCl₂ and 1 mM probenecid. Thereafter, the cells were washed three times with the same medium without CaCl₂ and kept at 37°C. The coverslip was placed in a coverslip holder (PerkinElmer Life and Analytical Sciences, Waltham, MA) and transferred with the medium to a quartz cuvette. Five minutes before the beginning of the assay a 100× solution of CaCl₂ was added to give a final Ca²⁺ concentration of 1.2 mM. The cuvette was placed in a luminescence spectrometer (LS-50B; PerkinElmer Life and Analytical Sciences) equipped with a thermostatic chamber set at 37°C and operated through a computer program by using the Intracellular Biochemistry software (PerkinElmer Life and Analytical Sciences). The program also yielded the free Ca²⁺ concentration values. Test agents were injected into the bathing medium in a volume of 10 μl with a Hamilton syringe at the indicated time points. The samples were alternatively excited at 340 and 380 nm and the emission was recorded at 510 nm. Maximum (R_max) and minimum (R_min) emission values were determined by adding the Ca²⁺ ionophore ionomycin (20 μM) following by 15 mM EGTA (pH adjusted to 10.0 with Tris base), respectively. Autofluorescence was measured in parallel samples unloaded with fura-2/acetoxymethyl ester.

Accumulation of [³H]inositol Phosphates. CHO/DOR confluent cultures grown in six-well plates were incubated in serum-free Ham’s F10 medium (Invitrogen) supplemented with 2.5 μCi/ml [myo-³H]inositol (88 Ci/mmol; PT6-271 stabilizer; GE Healthcare) for 24 h. Cells were washed with PBS and incubated in freshly oxygenated Krebs-HEPES buffer supplemented with 1.2 mM CaCl₂ and 10 mM LiCl for 20 min at 37°C. Thereafter, either vehicle or SNC 80 was added, and the incubation was continued for 40 min. When used, MRS 2179 was added 10 min before SNC 80. The incubation was stopped by aspirating the medium and washing the cells three times with ice-cold Krebs-HEPES buffer containing 1.2 mM CaCl₂, 10 mM d-glucose, and 0.2 mM phloretin. Cells were solubilized by adding 0.1% SDS and cell-trapped radioactivity was measured by liquid scintillation counting. Nonspecific uptake was determined by adding 20 μM cytochalasin B to parallel samples, and this value was subtracted from that of each experimental sample. Assays were run in duplicate.

Statistical Analysis. Results were reported as mean ± S.E.M. of n independent experiments. Concentration-response curves were analyzed by the program GraphPad Prism (San Diego, CA). Statistical analysis was performed by either Student’s unpaired t test or one-way analysis of variance (ANOVA) followed by Newman-Keuls post hoc test as appropriate.

Results

Activation of DORs Induces AMPK Phosphorylation. Exposure of CHO/DOR cells to the DOR agonist SNC 80 caused a rapid increase of AMPK phosphorylation at Thr172, which was significant at 2 min, reached a maximum at 5 min, and then rapidly declined reaching basal values at 20 min (Fig. 1A). The stimulatory effect of SNC 80 was concentration-dependent with an EC₅₀ value of 5.1 ± 0.6 nM and a maximal effect corresponding to approximately 3-fold increase of basal value (p < 0.001) (Fig. 1B). Like SNC 80, DPDPE, a peptidic DOR agonist, maximally stimulated AMPK phosphorylation by 2.9 ± 0.3-fold (p < 0.001) with an EC₅₀ value of 2.1 ± 0.05 nM (Fig. 1C).

The stimulation of AMPK phosphorylation by SNC 80 (100 nM) was completely blocked by the selective DOR antagonist NTI (100 nM), which per se had no effect (Fig. 1D). Moreover, cell treatment with PTX, which impairs receptor-G protein interactions by ADP-ribosylating Gₛᵢ proteins, totally prevented the stimulatory effects of either SNC 80 (100 nM) or DPDPE (100 nM) (Fig. 1E).

Activation of DORs Enhances AMPK Activity. To assess whether the increased AMPK phosphorylation was associated with a change in enzyme activity, cells were treated with the receptor agonists, and AMPK activity was measured after immunoprecipitation. As shown in Fig. 2A, SNC 80 (100 nM) and DPDPE (100 nM) caused a significant stimulation of AMPK activity (95 ± 5 and 90 ± 6% increase of control activity, respectively; p < 0.001), and this effect was completely blocked by NTI (100 nM).

To explore whether the increased AMPK activity translated into an enhanced phosphorylation of endogenous substrates, we examined the phosphorylation state of ACC, a main downstream target of AMPK (Kahn et al., 2005). As shown in Fig. 2B, cell treatment with SNC 80 (100 nM) caused a significant increase of ACC phosphorylation at Ser79, which was completely prevented by NTI (100 nM).

Activation of DORs Fails to Affect Intracellular ADP/ATP Ratio. To explore the mechanisms mediating the acti-
vation of AMPK, the effects of DOR activation on intracellular adenine nucleotide levels were examined. CHO/DOR cells were treated with either vehicle or 100 nM NTI for 5 min and then exposed to either vehicle, 100 nM SNC 80, or 100 nM DPDPE for additional 5 min. AMPKα1/2 was immunoprecipitated from cell extracts and AMPK activity was assayed as described under Materials and Methods. Values are reported as percentage of control (vehicle + vehicle) and are the mean ± S.E.M. of three experiments. B, DOR activation increases ACC phosphorylation at Ser79. Cells were preincubated with either vehicle or 100 nM NTI and then exposed to either vehicle or 100 nM SNC 80 for 5 min. The levels of phospho-ACC (pACC) were determined in cell extracts by Western blot and normalized to the levels of actin. Denisometeric ratios are reported as percentage of control and are the mean ± S.E.M. of four experiments. ***, p < 0.001 versus control by ANOVA followed by Newman-Keuls post hoc test. C, representative HPLC chromatograms of adenine nucleotide levels in cell extracts obtained after treatment with either vehicle or SNC 80 (100 nM) for 5 min. Data are representative of three separate experiments. mAU, milliabsorbance units.
Rp-cAMPS at a concentration (50 μM) effective in inhibiting PKA activity (Rothermel and Botelho, 1988) failed to affect AMPK phosphorylation per se and did not affect the stimulation induced by SNC 80 (100 nM). Conversely, exposure to the cAMP analogs dBcAMP (2 mM) and Sp-cAMPS (500 μM) inhibited SNC 80-induced AMPK phosphorylation (Fig. 3, B and C), indicating that an increased activity of the cAMP/PKA pathway down-regulated the opioid response.

Besides inhibiting cAMP formation, DORs have been shown to activate a network of signaling pathways comprising MAP kinases and PKC isoforms (Quock et al., 1999; Tegeder and Geisslinger, 2004), which may affect AMPK phosphorylation. As shown in Fig. 4, A to D, the SNC 80 (100 nM) stimulatory effect was not altered by cell treatment with either the TAK1 inhibitor (5Z)-7-oxozeaenol (100 nM) for 30 min (A), the MAP kinase kinase inhibitor PD98059 (50 μM) for 60 min (B), the p38 MAP kinase inhibitor SB203580 (10 μM) for 30 min (C), or the JNK inhibitor SP600125 (10 μM) for 30 min (D). Moreover, the DOR response was insensitive to both Go 6850 (1 μM), which inhibits conventional PKC isoforms, and Go6983 (1 μM), a broad-spectrum inhibitor of PKC isoforms (Fig. 4E). The concentrations of the inhibitors employed were previously found to be effective in blocking their target kinases (Hofmann, 1997; Ninomiya-Tsuji et al., 2003; Bain et al., 2007).

The CaMKK Inhibitor STO-609 Attenuates DOR-Induced AMPK and ACC Phosphorylation. DORs have been shown to regulate intracellular Ca2+ levels (Connor and Henderson 1996; Yoon et al., 1999; Yeo et al., 2001), and...
CaMKKβ is one of the main upstream kinase regulating AMPK (Towler and Hardie, 2007). Exposure of CHO/DOR cells to 100 nM ionomycin in the presence, but not in the absence, of extracellular Ca\(^{2+}\) induced a marked increase of AMPK phosphorylation (Fig. 5A), indicating the occurrence of a Ca\(^{2+}\)-dependent mechanism of AMPK regulation. Western blot analysis with an antibody that recognizes CaMKKβ showed the presence in CHO/DOR cell extracts of an immunoreactive band of \(\sim 65\) kDa, which corresponds to the molecular mass of CaMKKβ (Fig. 5B). Pretreatment of CHO/DOR cells with 10 \(\mu\)M STO-609, a relatively specific and cell-permeable inhibitor of CaMKKβ (Tokumitsu et al., 2002; Bain et al., 2007), reduced the SNC 80 (100 nM)-induced AMPK phosphorylation by 70\% (\(p < 0.01\)) (Fig. 5C). Moreover, this treatment almost completely suppressed the DOR stimulation of ACC phosphorylation (Fig. 5D). These data indicate that in CHO/DOR cells the Ca\(^{2+}\)/CaMKKβ pathway is involved in AMPK activation induced by DOR stimulation.

**DORs Stimulate AMPK through a Cooperative Interaction with Endogenously Activated P2Y Receptors.** Previous studies have reported that, when expressed in CHO cells, \(\alpha_2\)-adrenergic receptors and DORs mobilized intracellular Ca\(^{2+}\) by enhancing the stimulation of phospholipase C (PLC) \(\beta\) by endogenously expressed G\(_{q11}\)-coupled P2Y purinergic receptors (Akerman et al., 1998; Yeo et al., 2001). It was therefore important to examine whether this type of receptor interaction was involved in DOR regulation of AMPK phosphorylation. HPLC analysis of adenine nucleotide content in CHO/DOR extracellular medium after 1-h incubation indicated the presence of ATP, ADP, and AMP at the concentration of 48 ± 5, 250 ± 60, and 610 ± 100 nM, respectively (\(n = 7\)). The extracellular concentrations of adenine nucleotides were not significantly altered by 5-min incubation with SNC 80 (100 nM). Measurement of LDH activity in the extracellular medium after 5, 15, 20, and 60 min of incubation yielded values not significantly different from blank values (nonconditioned medium) (results not shown).

SNC 80 (100 nM) caused a rapid and transient increase in intracellular free Ca\(^{2+}\) concentrations from a resting level of 60 ± 8 nM to a peak value of 180 ± 20 nM (\(p < 0.001\), \(n = 15\)). The stimulatory effect reached a peak after approximately 20 s, gradually returned to resting levels within 2 min and was completely blocked by cell pretreatment with 100 nM NTI (Fig. 6, A and B). Addition to the medium of 2 U/ml apyrase, which hydrolyzes ATP and ADP to AMP, almost completely suppressed the opioid-induced increase of intracellular Ca\(^{2+}\) (Fig. 6C), indicating that the effect was dependent on the presence of extracellular ATP and/or ADP. The increase of intracellular Ca\(^{2+}\) was associated with stimulation of PLC, as demonstrated by the increased accumulation of \[^3H\]IPs in response to SNC 80 (100 nM) (Fig. 6D). The stimulatory effect was completely prevented by cell pretreatment with MRS 2179 (3 \(\mu\)M), a selective P2Y1 purinergic receptor antagonist.

Next, the dependence of DOR regulation of AMPK on endogenous P2 purinergic receptor activity was examined. We found that the stimulatory effect of SNC 80 on AMPK phosphorylation was greatly suppressed by cell treatment with apyrase (2 U/ml) (Fig. 7A). In the same cell samples, the apyrase treatment failed to affect the stimulation of extracellular signal-regulated protein kinases 1 and 2 phosphorylation (Fig. 7B).
lation by SNC 80 (100 nM), indicating that the treatment did not exert a global inhibition of DOR signaling (results not shown). Moreover, cell exposure to either PPADS (100 μM), a nonselective P2 receptor antagonist, or MRS 2179 (1 μM) inhibited DOR stimulation of AMPK phosphorylation by 45% (p < 0.05, n = 4) and 70% (p < 0.001, n = 4), respectively (Fig. 7, B and C). Finally, the stimulatory effect was suppressed by 80% (p < 0.001, n = 5) after cell treatment with the Gq/11 antagonist YM 254890 (10 μM), a cyclic depsipeptide that blocks Gq/11 activation by inhibiting the exchange of GDP with GTP in the α subunit (Takasaki et al., 2004). Collectively, these data indicate that in CHO/DOR cells, DOR activation enhances AMPK through a mechanism involving the potentiation of endogenous Gq/11-coupled P2Y receptors, which leads to enhanced stimulation of PLCβ, increased intracellular Ca2⁺ and activation of CaMKKβ.

In SH-SY5Y Cells and Primary Olfactory Bulb Neurons, DOR Activation Enhances AMPK Phosphorylation in Synergy with Gq/11-Coupled Receptors. We next investigated whether DOR expressed under native conditions could activate AMPK in synergy with Gq/11-coupled receptors. To this end, the human neuroblastoma cell line SH-SY5Y and primary cultures of mouse olfactory bulb neurons were employed. Previous studies have shown that in SH-SY5Y cells DOR activation enhanced the intracellular Ca2⁺ mobilization induced by stimulation of Gq/11-coupled muscarinic M3 receptors (Connor and Henderson 1996; Yeo et al., 2001). Moreover, it has recently been reported that in these cells activation of M3 muscarinic receptors increased AMPK activity through CaMKKβ (Thornton et al., 2008). In agreement with these observations, we found that exposure of SH-SY5Y cells to the cholinergic agonist carbachol (CCh; 100 nM) caused a significant increase of AMPK phosphorylation, which was almost completely blocked in the presence of the CaMKK inhibitor STO-609 (Fig. 8A). In these cells, the addition of SNC 80 (100 nM) failed to affect AMPK phosphorylation per se, but increased the stimulatory effects elicited by submaximal concentrations of CCh (100 nM) and oxotremorine-M (Oxo-M; 300 nM), a selective muscarinic receptor agonist, by 90% (p < 0.01, n = 5) and 60% (p < 0.05, n =...
and three (D) experiments. E, expression of the G protein \( G_{\alpha 11} \) failed to affect intracellular Ca\(^{2+} \) mobilization in empty vector-transfected cells, but the addition of SNC 80 (100 nM) increased AMPK phosphorylation as effectively as in empty vector-transfected cells. In these cells, the combined addition of 100 nM SNC 80 with 1 μM Oxo-M increased the Ca\(^{2+} \) peak up to 210 ± 12 nM (\( n = 5 \)), a value not significantly different from that obtained with CCh alone.

We then examined the ability of DORs naturally expressed in olfactory bulb neurons to regulate the state of AMPK phosphorylation. As observed in SH-SY5Y cells, incubation of primary neuronal cultures with SNC 80 (100 nM) failed to affect AMPK phosphorylation (Fig. 8F). Oxo-M (1 μM) also had no effect on basal AMPK phosphorylation, but when combined with SNC 80 (100 nM), a significant enhancement was observed. Addition of MeSADP (10 μM), a P2Y1 purinergic receptor agonist, increased phospho-AMPK expression by 60% (\( p < 0.05, n = 4 \)), and this effect was potentiated by 90% (\( p < 0.05, n = 4 \)) when DOR were concomitantly stimulated (Fig. 8F).

**Involvement of AMPK in DOR Stimulation of Glucose Uptake.** In CHO/DOR cells, exposure to the AMPK activator AICAR (2 mM) enhanced AMPK phosphorylation by approximately 20-fold (\( p < 0.001, n = 3 \)), without affecting the expression of the total enzyme protein (Fig. 9A). Under
similar experimental conditions, AICAR (2 mM) stimulated 2-deoxy-D-glucose uptake by 105 ± 6% \( (p < 0.001, n = 5) \) (Fig. 9B). As reported previously (Olianas et al., 2011), DOR activation by SNC 80 (100 nM) increased glucose uptake by 140 ± 8% \( (p < 0.001, n = 6) \) (Fig. 9C). Pretreatment with the AMPK inhibitor compound C (10 and 25 \( \mu \)M) reduced the stimulatory effect of SNC 80 (100 nM) by 37 ± 6 and 43 ± 6%, respectively \( (p < 0.05, n = 4) \) (Fig. 9C). Because compound C is not fully specific for AMPK (Bain et al., 2007), we have further examined the involvement of AMPK by adenovirus expression of an AMPKa1 DN mutant. As shown in Fig. 9D, in cells infected with the AMPK mutant DOR-stimulated glucose uptake was decreased by 40 ± 6% \( (p < 0.05, n = 4) \). In addition, cell treatment with MRS 2179 (1 \( \mu \)M) reduced the DOR-stimulated glucose uptake by 30 ± 3% \( (p < 0.05, n = 4) \) (Fig. 9E).

Discussion

The present study constitutes the first demonstration that in different cell types, DOR activation increases AMPK activity through coincidence signaling with \( \text{G}_{\text{q/11}} \)-coupled receptors. This conditional activation of AMPK by a \( \text{G}_{\text{i/o}} \)-coupled receptor has not been described previously. Indeed, the stimulation of a number of \( \text{G}_{\text{i/o}} \)-coupled receptors, including \( \alpha_2 \)-adrenergic, muscarinic M2 and M4, prostanoid EP3, and f-Met-Leu-Phe receptors, has been found to be without effect on AMPK activity (Kishi et al., 2000; Hutchinson et al., 2005; Merlin et al., 2010). The observation that in SH-SY5Y cells and in primary olfactory bulb neurons, DOR stimulation per se failed to affect AMPK phosphorylation is consistent with these studies, indicating a lack of a direct coupling of DOR to AMPK. However, the synergistic interaction with \( \text{G}_{\text{q/11}} \)-coupled receptors observed in these cells and the requirement of concurrent activation of endogenous P2Y purinergic receptors in CHO/DOR cells revealed a novel mechanism by which DOR, and possibly other \( \text{G}_{\text{i/o}} \)-coupled receptors, can be functionally linked to AMPK regulation.

Depletion of intracellular ATP with the consequent increase of AMP/ATP ratio is a major mechanism for AMPK activation by cellular stressors (Hardie and Hawley, 2001). Because DORs have been reported to affect energy homeostasis (Evans et al., 2001; Olianas et al., 2011), initially it was important to determine the effects of DOR stimulation on intracellular AMP/ATP ratio. AMP levels in CHO cells were too low to be accurately measured with HPLC analysis. Therefore, we examined the ADP/ATP ratio as a surrogate

**Materials and Methods.** Values are the mean ± S.E.M. of five experiments. C, the AMPK inhibitor compound C reduces DOR stimulation of glucose uptake. Cells were pretreated with either vehicle or the indicated concentrations of compound C (Comp C) for 1 h and then exposed to either vehicle or 100 nM SNC 80. Values are the mean ± S.E.M. of four to six experiments. D, expression of AMPKa1 DN mutant reduces DOR-stimulated glucose uptake. Cells infected with either null control adenovirus (null ADV) or ADV encoding the AMPKa1 DN mutant (AMPKa1 DN) were treated with either vehicle or 100 nM SNC 80. Values are reported as percentage of control (null ADV infected cells treated with vehicle) and are the mean ± S.E.M. of four experiments. E, antagonism of P2Y1 purinergic receptors attenuates DOR stimulation of glucose uptake. Cells were pretreated with either vehicle or the P2Y1 receptor antagonist MRS 2179 (1 \( \mu \)M) for 5 min before the addition of either vehicle or 100 nM SNC 80. Values are the mean ± S.E.M. of four experiments. ***, \( p < 0.001 \) versus control by Student’s \( t \) test (B) and ANOVA followed by Newman-Keuls post hoc test (B–E).
index, because cellular AMP/ATP ratio varies as the square of the ADP/ATP ratio (Hardie and Hawley, 2001). Moreover, recent studies have shown that, like AMP, ADP regulates AMPK by protecting the enzyme from dephosphorylation, demonstrating that both ADP/ATP and AMP/ATP ratios contribute to AMPK regulation (Oakhill et al., 2011; Xiao et al., 2011). DOR stimulation failed to significantly affect the relative concentrations of intracellular ADP and ATP, indicating that AMPK activation occurred independently of changes in the adenine nucleotide levels. Because DOR effects depend on Gαq/11 signaling, this finding is consistent with the observation that different Gαq/11-coupled receptors have been found to activate AMPK without affecting the AMP/ATP ratio (Hutchinson et al., 2008).

Although DORs are known to trigger a variety of intracellular signaling cascades that may directly or indirectly affect AMPK, only agents acting on the synergistic interaction with Gαq/11-coupled receptors were found to affect DOR regulation of AMPK. For instance, cell treatment with either inhibitors of TAK1 and MAP kinases or antagonists of different PKC isoforms had no effect on DOR stimulation. The inhibition of the cAMP/PKA pathway, which is commonly triggered by DORs (Quock et al., 1999), has been considered as a possible mechanism by which Gαo-coupled receptors may control AMPK activity (Hutchinson et al., 2008). In fact, PKA can inactivate AMPK by either phosphorylating the α subunit at Ser485/491 or inhibiting CaM KKβ activity (Hurley et al., 2006). However, DOR stimulation of AMPK did not seem to be consequent to inhibition of the cAMP/PKA pathway, because CHO/DOR cell treatment with the selective PKA antagonist Rp-cAMPS failed to mimic or to change the DOR stimulation of AMPK. The lack of effect by Rp-cAMPS was probably due to a low level of tonic inhibition of AMPK by PKA, rather than to the absence of AMPK regulation by PKA. In fact, cell treatment with the PKA activators dB-cAMP and Sp-cAMPS inhibited DOR stimulation of AMPK, indicating that this response could be down-regulated by activation of PKA signaling. It is also important to mention that PKA has been shown to curtail the activation of PLCβ by Gβγ (Liu and Simon, 1996). Therefore, the PKA inhibition of DOR-induced AMPK activation might also have derived from an impaired activation of PLCβ.

In CHO/DOR cells, DOR stimulation of AMPK phosphorylation was found to be completely dependent on concurrent activation of endogenous purinergic receptors. In fact, this response as well as DOR stimulation of [3H]IP accumulation and intracellular free Ca2+ mobilization were suppressed in cells treated with either apyrase, P2 purinergic receptor antagonists or the Gαq/11 antagonist YM 254890. These data strongly indicate the occurrence of a synergistic interaction with Gαq/11-coupled P2Y receptors, particularly with the P2Y1 receptor, which is endogenously expressed in CHO cells (Marcet et al., 2004). Analysis of extracellular adenine nucleotide concentrations indicated that CHO/DOR cells accumulated substantial amounts of ATP, ADP, and AMP in the incubation medium within the 1-h time interval preceding the cell treatments. It is unlikely that the accumulation of adenine nucleotides was due to lytic cell death, because no significant LDH activity could be measured in the extracellular medium. It has been shown that CHO cells can release ATP (Abraham et al., 1993), although the precise mechanisms have not been identified. In addition to exocytosis, several nonexocytotic pathways can mediate ATP release in response to different stimuli, including mechanical stress, hypoxia, ischemia, and ischemic preconditioning (Sabirov and Okada, 2005). Released ATP and ADP formed from ATP by the action of ectonucleotidases may reach concentrations sufficiently high to stimulate P2 purinergic receptors. Under these conditions, concomitant activation of DOR may potentiate P2Y receptor signaling enhancing AMPK activity. The finding that SNC 80 increases AMPK phosphorylation by MeSADP in olfactory bulb cultures indicates that this receptor cross-talk is not limited to CHO/DOR cells but can occur in other cell types that coexpress the receptors and the associated signaling molecules. In the central nervous system activation of P2 purinergic receptors has been reported to impair cell survival and to cause neurodegeneration (Burnstock, 2006). However, selective activation of P2Y1 receptors has been found to exert neuroprotective effects against oxidative stress and brain ischemia (Shinozaki et al., 2005; Zheng et al., 2010). Neuroprotective effects of DOR agonists in cellular and animal models of neuronal hypoxia/ischemia have also been observed (Bo fungi et al., 1996; Zhang et al., 2002). In the light of the present results, it will be of interest to investigate whether the activation of the two receptors exhibits a synergistic effect on cell survival and whether this response involves a cooperative regulation of AMPK.

In either SH-SY5Y cells or primary olfactory bulb neurons, DOR activation failed per se to induce AMPK phosphorylation. This result suggests that in these cells, unlike CHO/DOR cells, the permissive effect due to the basal stimulation of Gαq/11-coupled receptors by extracellular adenine nucleotides is absent. In these cell systems, however, the addition of SNC 80 enhanced the AMPK phosphorylation elicited by either muscarinic or P2Y1 purinergic receptor activation. In SH-SY5Y cells, SNC 80 failed to potentiate AMPK phosphorylation by nicotinic, which, by opening the ionotropic nicotinic receptors, increases intracellular Ca2+ independently of Gαq/11 and PLCβ activation (Ridley et al., 2002). Moreover, in SH-SY5Y cells, sequestration of Gβγ by transfection with GRK2-CT failed to affect the Oxo-M stimulation of AMPK phosphorylation but prevented the SNC 80-induced enhancement, indicating the involvement of Gβγ in the opio id action. In agreement with previous studies (Yeo et al., 2001), cell transfection with GRK2-CT reduced DOR potentiation of CCh-induced elevation of cytosolic free Ca2+, indicating that Gβγ is also required for the coincident signaling between DOR and M2 receptors in the regulation of Ca2+ mobilization. The present study indicates that in these cells, a downstream effect of the synergistic interaction between the two receptor systems is the up-regulation of AMPK phosphorylation through CaMKKβ.

A number of studies have provided evidence that AMPK is involved in the regulation of glucose uptake via GLUT4 and GLUT1 glucose transporters, although the precise mechanisms are not completely known (Towler and Hardie, 2007). We have recently reported that in CHO/DOR cells, activation of DOR stimulate GLUT1-dependent glucose uptake through a mechanism involving transactivation of insulin-like growth factor 1 (IGF1) receptor tyrosine kinase and phosphatidinositol 3-kinase (PI3K) pathway (Olias et al., 2011). There is evidence for the occurrence in different cell types of a crosstalk between AMPK and insulin receptor signaling (Towler and Hardie, 2007), and it has been reported that AMPK may
regulate PI3K activity (Jakobsen et al., 2001; Tao et al., 2010). We therefore examined whether DOR enhancement of AMPK activation contributed to the stimulation of glucose uptake. We found that in CHO/DOR cells, the AMPK activator AICAR markedly increased AMPK phosphorylation and significantly stimulated glucose uptake, suggesting the possible regulation of glucose transport by enhancing IGF-1 receptor/PI3K signaling or that this enzyme mediates only a portion of the DOR-stimulated glucose uptake. Whether AMPK regulates glucose transport by enhancing IGF-1 receptor/PI3K signaling or through additional pathways remains to be assessed.

In conclusion, the present study demonstrates that DOR activates AMPK, a key enzyme in the cellular adaptation to metabolic insults, through a coincident signaling with Gq/11-coupled receptors contributed to DOR stimulation of glucose uptake. However, the partial reduction obtained with AMPK inhibitors suggests that this enzyme mediates only a portion of the DOR-stimulated glucose uptake. Whether AMPK regulates glucose transport by enhancing IGF-1 receptor/PI3K signaling or through additional pathways remains to be assessed.

In conclusion, the present study demonstrates that DOR stimulation of glucose uptake was significantly inhibited by either compound C, expression of a DN mutant of AMPKα1 or antagonism of P2Y1 purinergic receptors. Collectively, these data indicate that AMPK activation through the synergistic interaction with Gq/11-coupled receptors contributed to DOR stimulation of glucose uptake. However, the partial reduction obtained with AMPK inhibitors suggests that this enzyme mediates only a portion of the DOR-stimulated glucose uptake. Whether AMPK regulates glucose transport by enhancing IGF-1 receptor/PI3K signaling or through additional pathways remains to be assessed.

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