

# $\delta$ -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  $\delta$ -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 on Thr172. DOR-induced AMPK phosphorylation was prevented by pertussis toxin, reduced by protein kinase A (PKA) activators, and unaffected by PKA, transforming growth factor- $\beta$ -activated kinase 1, mitogen-activated protein kinase, and protein kinase C inhibitors. Conversely, the DOR effect was reduced by  $Ca^{2+}$ /calmodulin-dependent protein kinase 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 accu-

mulation. 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  $\beta\gamma$  subunits ( $G\beta\gamma$ ) blocked the DOR potentiation of AMPK phosphorylation induced by oxotremorine-M. In CHO cells, the AMPK activator 5-aminoimidazole-4-carboxamide-1- $\beta$ -D-ribose nucleoside stimulated AMPK phosphorylation and glucose uptake, whereas pharmacological inhibition of AMPK, expression of a dominant-negative mutant of AMPK $\alpha$ 1, and P2Y receptor blockade reduced DOR-stimulated glucose uptake. The data indicate that in different cell systems, DOR activation up-regulates AMPK through a  $G\beta\gamma$ -dependent synergistic interaction with  $G_{q/11}$ -coupled receptors, potentiating  $Ca^{2+}$  release and CaMKK $\beta$ -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 condi-

tions 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  $\alpha$  subunit and regulatory  $\beta$  and  $\gamma$  subunits. In mammals, two  $\alpha$  ( $\alpha$ 1 and  $\alpha$ 2), two  $\beta$  ( $\beta$ 1 and  $\beta$ 2), and three  $\gamma$  ( $\gamma$ 1,  $\gamma$ 2, and  $\gamma$ 3) subunits encoded by distinct genes have been identified (Towler and Hardie, 2007).

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**ABBREVIATIONS:** ACC, acetyl-coenzyme A carboxylase; ADV, adenovirus; AICAR, 5-aminoimidazole-4-carboxamide-1- $\beta$ -D-ribose nucleoside; AMPK, AMP-activated protein kinase; ANOVA, analysis of variance; BSA, bovine serum albumin; CaMKK,  $Ca^{2+}$ /calmodulin-dependent protein kinase 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,  $\delta$ -opioid receptors; 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-pyrrole-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 $\alpha$ -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; PI3K, phosphatidylinositol 3-kinase; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; PPADS, pyridoxal phosphate-6-azophenyl-2',4'-disulfonic acid; PTX, *Bordetella pertussis* toxin; Rp-cAMPS, adenosine-3',5'-cyclic monophosphorothioate, Rp-isomer; SB203580, 4-[5-(4-fluorophenyl)-2-[4-(methylsulphonyl)phenyl]-1H-imidazol-4-yl]pyridine; SAMS peptide, HMRSAMSGHLHLVKRR; SNC 80, (+)-4-[( $\alpha$ R)- $\alpha$ -((2S,5R)-4-Allyl-2,5-dimethyl-1-piperaziny)-3-methoxybenzyl]-N,N-diethylbenzamide; SP600125, anthra[1-9-cd]pyrazol-6(2H)-one; Sp-cAMPS, adenosine-3',5'-cyclic monophosphorothioate, Sp-isomer; STO-609, 7-oxo-7H-benzimidazo[2,1-a]benz[de]isoquinoline-3-carboxylic acid; TAK1, transforming growth factor- $\beta$ -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  $\alpha$  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  $\text{Ca}^{2+}$ -dependent manner through phosphorylation at Thr172 by the  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase kinase (CaMKK)  $\beta$  (Towler and Hardie, 2007). In addition, evidence for the involvement of the transforming growth factor- $\beta$ -activated kinase 1 (TAK1), a member of the mitogen-activated protein (MAP) kinase kinase kinase family, has also been provided (Momcilovic et al., 2006). On the other hand, AMP binds to  $\gamma$  subunit cystathionine  $\beta$ -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 palmytoyltransferase-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 (Ronnelt et al., 2009).

Similar to AMPK, DORs have been reported to stimulate glucose uptake in skeletal muscle and transfected cells (Evans et al., 2001; Olanas 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-[( $\alpha R$ )- $\alpha$ -(2*S*,5*R*)-4-Allyl-2,5-dimethyl-1-piperaziny]-3-methoxybenzyl]-*N,N*-diethylbenzamide (SNC 80),

17-(cyclopropylmethyl)-6,7-dehydro-4,5 $\alpha$ -epoxy-3,14-dihydroxy-6,7-2',3'-indolomorphinan (naltrindole) hydrochloride (NTI), 2-(2-amino-3-methoxyphenyl)-4*H*-1-benzopyran-4-one (PD 98059), 4-[5-(4-fluorophenyl)-2-[4-(methylsulphonyl)phenyl]-1*H*-imidazol-4-yl]pyridine (SB203580), anthra[1-9-*cd*]pyrazol-6(2*H*)-one (SP600125), 5-aminoimidazole-4-carboxamide-1- $\beta$ -D-ribose nucleoside (AICAR), *N*<sup>6</sup>-methyl 2'-deoxyadenosine 3',5'-bisphosphate (MRS 2179), and pyridoxal phosphate-6-azophenyl-2',4'-disulfonic acid (PPADS) tetrasodium salt were from Tocris Bioscience (Bristol, UK). The peptide HMRSAMSGLHLVKRR (SAMS peptide) and [D-Pen(2,5)]-enkephalin (DPDPE) was purchased from Bachem AG (Bubendorf, Switzerland). 2-[1-(3-Dimethylaminopropyl)indol-3-yl]-3-(indol-3-yl)maleimide (Go 6850; also termed bisindolylmaleimide I and GF 109203X), 3-[1-[3-(dimethylamino)propyl]-5-methoxy-1*H*-indol-3-yl]-4-(1*H*-indol-3-yl)-1*H*-pyrrole-2,5-dione (Go 6983), 7-oxo-7*H*-benzimidazo[2,1-*a*]benz[*de*]isoquinoline-3-carboxylic acid (STO-609), ionomycin, and 2-deoxy-D-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- $\beta$ -activated kinase 1 (TAK1) inhibitor (5*Z*)-7-oxozeaenol (antibiotic LLZ1640-2) was from Santa Cruz Biotechnology (Santa Cruz, CA). The cyclic decapeptide YM 254890, isolated from culture broth of *Cromobacterium* sp. QS3666, was generously provided by Dr. Jun Takasaki (Yamanouchi Pharmaceutical Co. Ltd., Tsukuba, Ibaraki, Japan). *Bordetella pertussis* toxin (PTX), 6-[4-[2-(1-piperidinylethoxy)phenyl]-3-(4-pyridinyl)-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 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%  $\text{CO}_2$ ) in Ham's F12 medium containing L-glutamine and sodium bicarbonate and supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin/100  $\mu\text{g/ml}$  streptomycin (Invitrogen, Carlsbad, CA). CHO-K1 cells stably expressing the human DORs (CHO/DOR cells) were developed by transfecting the cells with pcDNA3.1-Hygro(+)-vector encoding the human DORs (Top Gene Technologies, Montreal, QC, Canada) using PolyFect (QIAGEN GmbH, Hilden, Germany) as transfection reagent following 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  $\sim 1500$  fmol/mg protein determined by saturation binding with the radioligand [<sup>3</sup>H]NTI (PerkinElmer Life and Analytical Sciences, Waltham, MA). Cells were maintained in Ham's F12 medium containing L-glutamine and sodium bicarbonate and supplemented with 10% FCS, 100 U/ml penicillin/100  $\mu\text{g/ml}$  streptomycin, and 350  $\mu\text{g/ml}$  hygromycin.

SH-SY5Y cells were obtained from the European Cell Culture Collection (Salisbury, UK) and grown in Ham's F12/minimal essential medium (1:1) supplemented with 2 mM L-glutamine, 1% nonessential amino acids, 10% FCS, and 100 U/ml penicillin/100  $\mu\text{g/ml}$  streptomycin (Invitrogen) at 37°C in a humidified atmosphere of 5%  $\text{CO}_2$  in air. Cells were used up 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  $7 \times 10^5$  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  $\mu\text{g/well}$ ) or empty vector for 16 h using Lipofectamine 2000 (Invitrogen) as transfectant. Thereafter, cells were transferred 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  $\mu$ l/well). Cells were infected with 100 plaque-forming units/cell of either null control recombinant adenovirus (ADV) or dominant-negative (DN) AMPK $\alpha$ 1 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 AMPK $\alpha$ 1 for the binding with  $\beta$  and  $\gamma$  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  $\mu$ M  $\beta$ -mercaptoethanol, and 100 U/ml penicillin-100  $\mu$ g/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 the density of  $10^6$ /well. The cells were placed in a humidified incubator and maintained at 37°C in 5% CO<sub>2</sub>. Six hours after plating the medium was removed. Thereafter, the medium was changed every 4 days. Cells were used 8 to 10 days after plating. Experiments were performed according to the principles of laboratory animal care (Law on animal experiments in Italy, D.L. 116/92).

**Cell Treatment, Preparation of Cell Extracts, and Western Blotting.** CHO/DOR and SH-SY5Y cells were incubated in serum-free medium for 18 to 24 h. The medium was renewed 1 h before treatments, and the cells were exposed to the various agents as specified in the text. When the effect of ionomycin was examined, serum-starved CHO/DOR cells were washed twice with phosphate-buffered saline (PBS), pH 7.40, and incubated in Krebs-HEPES buffer containing 25 mM HEPES/NaOH, pH 7.4, 125 mM NaCl, 1.2 mM Mg<sub>2</sub>SO<sub>4</sub>, 10 mM glucose, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 3.8 mM KCl supplemented with either 0.1 mM EGTA (no Ca<sup>2+</sup>) or 1.2 mM CaCl<sub>2</sub> (+Ca<sup>2+</sup>) for 20 min at 37°C. Olfactory bulb neurons were incubated in Neurobasal A medium without B-27 supplement for 5 to 6 h, washed with PBS and incubated in Krebs-HEPES buffer containing 1.2 mM CaCl<sub>2</sub> for 20 min at 37°C. Agents were dissolved in either saline or dimethyl sulfoxide. The final dimethyl sulfoxide concentration was equal to or less than 0.5%. Control samples were incubated in the presence of an equal concentration of vehicle. After treatments, cells were washed briefly with ice-cold PBS, and cell extracts were prepared by scraping the cells in an ice-cold lysis buffer containing PBS, 0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 2 mM EDTA, 2 mM EGTA, 4 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 10 mM sodium fluoride, 20 nM okadaic acid, 0.1% phosphatase inhibitor cocktail 1, 1% protease inhibitor cocktail, and 1 mM phenylmethylsulfonyl fluoride. The samples were sonicated for 5 s in ice-bath and aliquots were taken for protein determination. Aliquots containing equal amount of protein were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and proteins were electrophoretically transferred to polyvinylidene difluoride membranes

(Hybond-P; GE Healthcare, Piscataway, NJ). The efficiency of the transfer was controlled by gel staining and by following the transfer of prestained protein standards (Santa Cruz Biotechnology, Santa Cruz, CA). Nonspecific binding sites were blocked by incubation in 20 mM Tris-HCl, 137 mM NaCl, and 0.05% Tween 20, pH 7.6 (Tris-buffered saline-Tween 20) containing 5% BSA for 1 h. After washing with Tris-buffered saline-Tween 20, the membranes were incubated overnight at 4°C with the following primary antibodies: rabbit polyclonal anti-phospho-AMPK $\alpha$  (Thr172) 1:2000; Cell Signaling Technology, Danvers, MA); rabbit polyclonal anti-phospho-ACC (Ser79) (1:1000; Millipore, Temecula, CA); rabbit polyclonal anti-AMPK $\alpha$ 1/2 (1:1000; Santa Cruz Biotechnology), mouse monoclonal anti-GRK 2 (1:500; Santa Cruz Biotechnology), and goat polyclonal anti-CaMKK $\beta$  (1:500; Santa Cruz Biotechnology); rabbit polyclonal anti-actin (1:2000; Sigma-Aldrich, St. Louis, MO). The membranes were then incubated with a horseradish peroxidase-conjugated secondary antibody (1:10,000) of the appropriate species (Santa Cruz), and immunoreactive bands were detected by using ECL Plus and ECL Hyperfilm (GE Healthcare). The size of the immunoreactive bands was determined by using molecular weight standards detected with a specific antibody suitable for the ECL system (Santa Cruz). Band densities were determined by densitometric analysis using Image Scanner III (GE Healthcare, Milan, Italy) and National Institutes of Health ImageJ software (<http://rsbweb.nih.gov/ij/>). The optical density of phosphoprotein bands was normalized to the density of the corresponding total protein band or actin band.

**Assay of AMPK Activity.** AMPK activity was assayed after immunoprecipitation by using a site-specific peptide corresponding to the Ser79 phosphorylation site of ACC (SAMS peptide) as substrate. Cells were serum-starved for 24 h, processed as specified above, and incubated in the presence of the experimental agents. Cells were then washed with ice-cold PBS and scraped in a ice-cold lysis buffer containing 20 mM Tris-HCl, pH 7.0, 50 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 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  $\mu$ g) were incubated with 1.5  $\mu$ g of anti-AMPK $\alpha$ 1/2 subunit antibody (Santa Cruz Biotechnology) for 2 h at 4°C with constant rotation. Thereafter, 20  $\mu$ l 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 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 once 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 MgCl<sub>2</sub>, and 200  $\mu$ M AMP. Beads were finally resuspended in 20  $\mu$ l of assay mixture containing assay buffer supplemented with 50  $\mu$ M ATP, 200  $\mu$ M SAMS peptide, and 3  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP (3000 Ci/mmol; PerkinElmer) and incubated for 15 min at 30°C. Blanks were prepared by omitting the SAMS 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  $\times$  2-cm phosphocellulose paper strip (Whatman, Clifton, NJ) that was air-dried and washed four times with 1% phosphoric acid. The <sup>32</sup>P radioactivity absorbed onto the strips was measured by liquid scintillation counting. Assays were run in triplicates.

**Measurement of Intracellular Free Ca<sup>2+</sup>.** Intracellular concentration of free Ca<sup>2+</sup> was measured in confluent monolayers of CHO/DOR and SH-SY5Y cells using the Ca<sup>2+</sup>-sensitive dye fura-2. Cells were grown onto poly(L-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  $\mu$ M fura-2/acetoxymethyl ester (Calbiochem, San Diego, CA), 0.05% BSA, and 0.15% (v/v)



Pluronic acid F-127 for 1 h at 37°C in Krebs-HEPES medium supplemented with 1.2 mM CaCl<sub>2</sub> and 1 mM probenecid. Thereafter, the cells were washed three times with the same medium without CaCl<sub>2</sub> 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 CaCl<sub>2</sub> was added to give a final Ca<sup>2+</sup> 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 Ca<sup>2+</sup> concentration values. Test agents were injected into the bathing medium in a volume of 10  $\mu$ l 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 Ca<sup>2+</sup> ionophore ionomycin (20  $\mu$ M) followed 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 [<sup>3</sup>H]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  $\mu$ Ci/ml [*myo*-<sup>3</sup>H]inositol (88 Ci/mmol with 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 CaCl<sub>2</sub> 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 adding 0.6 ml of 0.25 N HCl. The cells were scraped and transferred to tubes containing 1.2 ml of chloroform-methanol [1:2 (v/v)]. The samples were shaken, and 0.5 ml of chloroform were added. The samples were centrifuged at 1000g for 10 min, and the upper aqueous phase was adjusted to pH 7.0 with the addition of 1.5 M NH<sub>4</sub>OH and applied to Dowex 1  $\times$  8 columns in the formate form (Bio-Rad Laboratories, Hercules, CA). The columns were washed with 20 bed volumes of H<sub>2</sub>O, 20 bed volumes of 5 mM *myo*-inositol, and 16 bed volumes of 5 mM sodium tetraborate in 50 mM sodium formate. [<sup>3</sup>H]IPs were eluted by adding 6 bed volumes of 1 M ammonium formate in 0.1 M formic acid. The radioactivity present in the eluate and the organic phase was determined by liquid scintillation counting. For each sample, the accumulation of [<sup>3</sup>H]IPs was corrected for the amount of radioactivity incorporated in the organic phase, and the percentage of total radioactivity converted to [<sup>3</sup>H]IPs was calculated. Assays were run in triplicates.

**Measurement of Adenine Nucleotide Levels.** Serum-starved CHO/DOR cells were incubated for 1 h in serum-free medium and exposed to the test agents as specified. The medium was aspirated, centrifuged at 150 g for 15 min, and stored at -80°C until assayed. The cells were washed with PBS and lysed by the addition of 7% perchloric acid. After 30-min incubation on ice, the cell extracts were centrifuged at 20,800g for 10 min at 4°C, and the supernatants were adjusted to pH 7.0 by the addition of K<sub>2</sub>CO<sub>3</sub> and centrifuged as above. The samples were analyzed for nucleotide contents by high-performance liquid chromatography (HPLC). The HPLC apparatus was a solvent delivery module (125S; Beckman Coulter, Fullerton, CA) equipped with a diode array 168 detector and Gold Nouveau software and a Waters (Milford, MA) symmetry C<sub>18</sub> column. The flow rate was 0.2 ml/min and the concentration of nucleotides was determined from measurement of peak area at 266 nm.

**Assay of Lactate Dehydrogenase Activity.** LDH activity of medium samples was assayed by using the LDH-cytotoxicity assay kit II obtained from BioVision Research Products (Mountain View, CA) according to the manufacturer's instructions.

**Assay of 2-Deoxy-D-Glucose Uptake.** The measurement of 2-deoxy-D-glucose uptake by CHO/DOR cells was performed as described previously (Olianas et al., 2011). In brief, confluent cell monolayers

were incubated in serum-free Ham's F12 medium for 12 h and treated with the agents or the corresponding vehicles as specified in the figure legends. The concentration of the agents was kept constant throughout the subsequent incubation step. The cells were then washed twice and incubated with Krebs-HEPES containing 1.2 mM CaCl<sub>2</sub> and lacking glucose for 20 min at 37°C. Receptor agonists were then added, and the incubation was continued for 15 min. Control samples received an equal volume of vehicle. The reaction was started by the addition of 1  $\mu$ Ci/ml [<sup>3</sup>H]2-deoxy-D-glucose (8.0 Ci/mmol; PerkinElmer Life and Analytical Sciences) together with unlabeled 2-deoxy-D-glucose to reach a final concentration of 1 mM and the uptake was measured for a period of 8 min. The incubation was stopped by aspirating the medium and washing the cells three times with ice-cold Krebs-HEPES buffer containing 1.2 mM CaCl<sub>2</sub>, 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  $\mu$ M cytochalasin B to parallel samples, and this value was subtracted from that of each experimental sample. Assays were run in duplicate.

**Statistical Analysis.** Results are reported as mean  $\pm$  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.

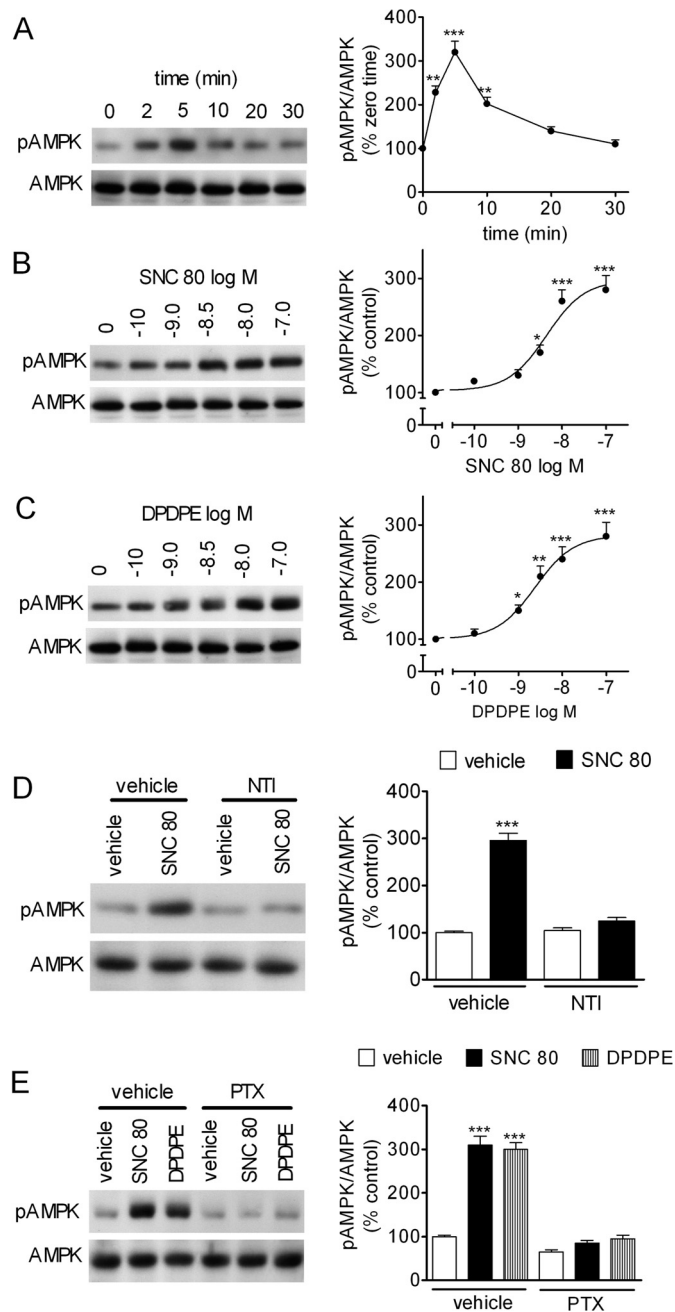
Exposure of CHO/DOR cells to the DOR agonist SNC 80 caused a rapid increase of AMPK phosphorylation at Thr172, which was significant at 2 min, reached a maximum at 5 min, and then rapidly declined reaching basal values at 20 min (Fig. 1A). The stimulatory effect of SNC 80 was concentration-dependent with an EC<sub>50</sub> value of 5.1  $\pm$  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  $\pm$  0.3-fold ( $p < 0.001$ ) with an EC<sub>50</sub> value of 2.1  $\pm$  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 G<sub>i/o</sub> proteins, totally prevented the stimulatory effects of either SNC 80 (100 nM) or DPDPE (100 nM) (Fig. 1E).

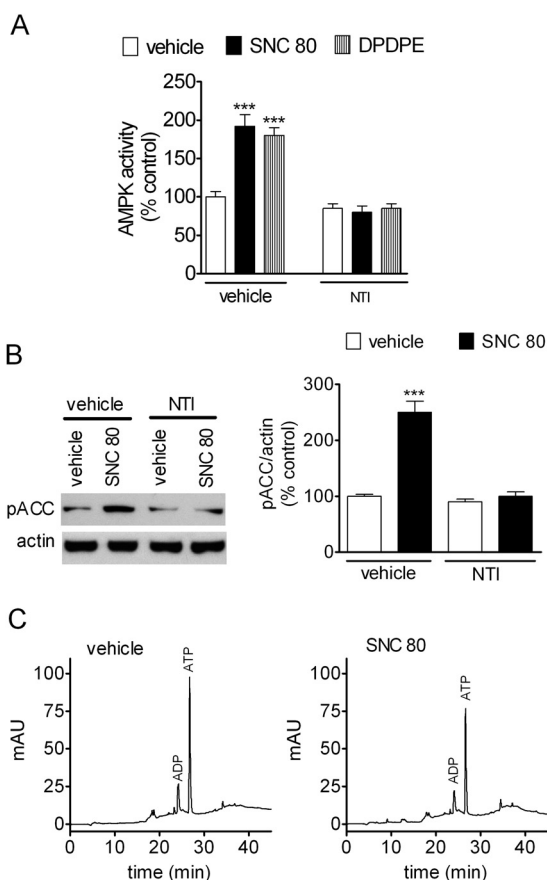
**Activation of DORs Enhances AMPK Activity.** To assess whether the increased AMPK phosphorylation was associated with a change in enzyme activity, cells were treated with the receptor agonists, and AMPK activity was measured after immunoprecipitation. As shown in Fig. 2A, SNC 80 (100 nM) and DPDPE (100 nM) caused a significant stimulation of AMPK activity (95  $\pm$  5 and 90  $\pm$  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-



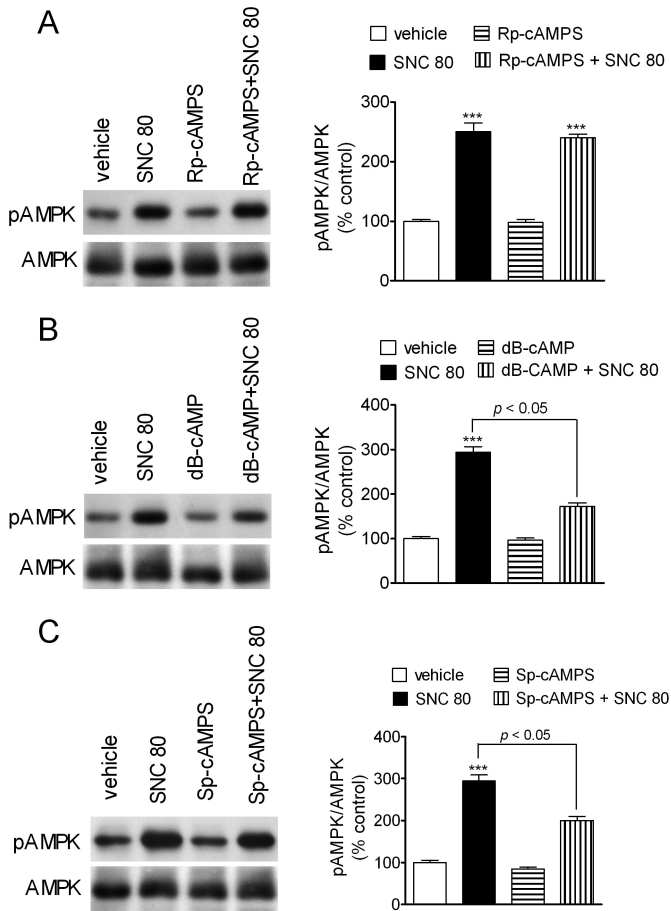
**Fig. 1.** DOR activation induces AMPK phosphorylation at Thr172 in CHO/DOR cells. **A**, time course of SNC 80-induced phosphorylation of AMPK. Cells were incubated with SNC 80 (100 nM) for the indicated time periods and phospho-AMPK (pAMPK) and total AMPK immunoreactivities were measured in cell extracts by Western blot. Densitometric values are reported as percentage of the corresponding vehicle-treated sample (zero time) and are the mean  $\pm$  S.E.M. of four experiments. \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$  versus zero time. **B** and **C**, concentration-dependent stimulation of AMPK phosphorylation by SNC 80 and DPDPE, respectively. Cells were incubated in the presence of the indicated concentrations of the DOR agonists for 5 min. Values are reported as percentage of control (vehicle) and are the mean  $\pm$  S.E.M. of three experiments. **D**, antagonism of SNC 80-induced AMPK phosphorylation by NTI. Cells were preincubated for 5 min with either vehicle or 100 nM NTI and then exposed to either vehicle or 100 nM SNC 80 for additional 5 min. Values are reported as percentage of control (vehicle + vehicle) and are the mean  $\pm$  S.E.M. of three experiments. **E**, PTX treatment prevents DOR stimulation of AMPK phosphorylation. Cells were treated for 24 h with either vehicle or 100 ng/ml PTX and then exposed to either vehicle, 100 nM SNC 80, or 100 nM DPDPE for 5 min. Values are the mean  $\pm$  S.E.M. of three experiments. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$  versus control by ANOVA followed by Newman-Keuls post hoc test.



**Fig. 2.** DOR stimulation increases AMPK activity in CHO/DOR cells without changing the ADP/ATP ratio. **A**, cells were pretreated 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. AMPK $\alpha$ /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  $\pm$  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. Densitometric ratios are reported as percentage of control and are the mean  $\pm$  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.

vation of AMPK, the effects of DOR activation on intracellular adenine nucleotide levels were examined. CHO/DOR cells were treated with either vehicle or SNC 80 (100 nM), and intracellular adenine nucleotides were measured by HPLC. AMP concentration was too low to be accurately determined; therefore, the levels of ADP and ATP were considered (Fig. 2C). Cell treatment with SNC 80 (100 nM) failed to affect the ADP/ATP ratio. Values were as follows: vehicle,  $0.143 \pm 0.005$ ; SNC 80,  $0.133 \pm 0.006$  ( $p > 0.05$ ;  $n = 3$ ).

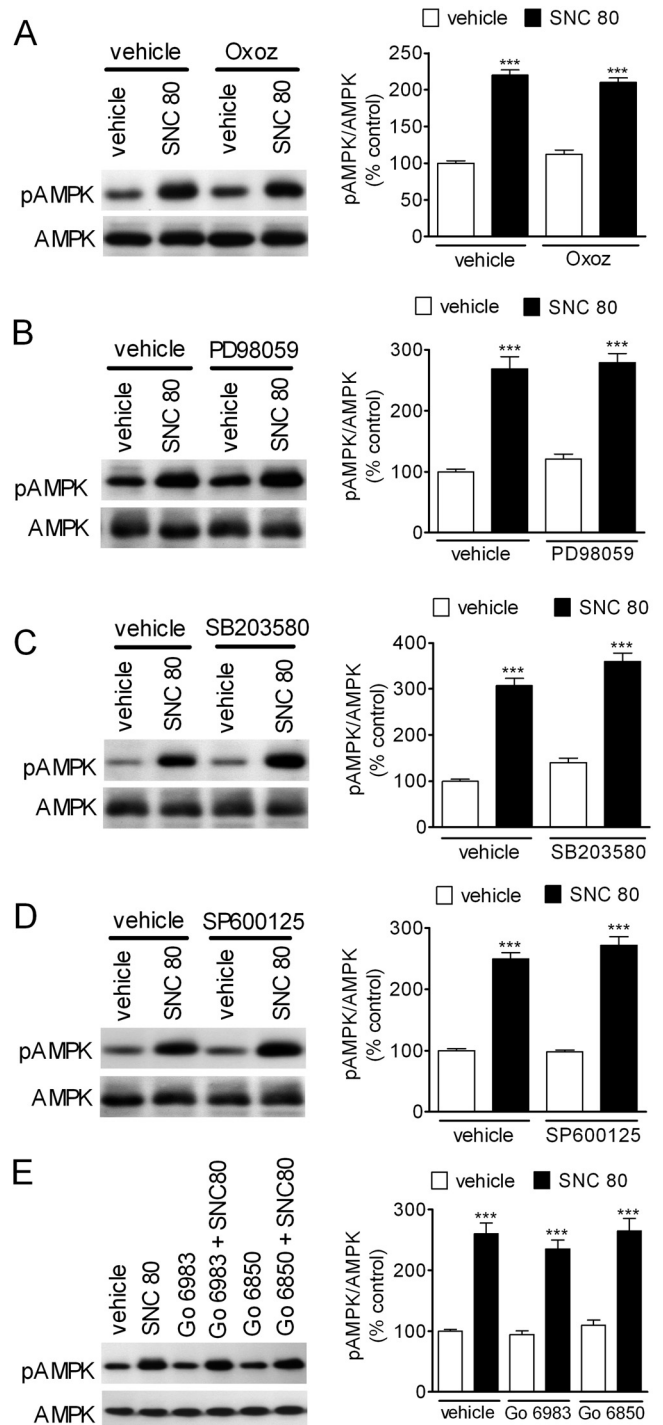
**Effects of Agents Acting on PKA, TAK1, MAP Kinases, and PKC.** DORs are known to be coupled to inhibition of cAMP formation (Quock et al., 1999) and the cAMP/PKA pathway has been shown to exert an inhibitory control on AMPK activity (Hurley et al., 2006). If DOR activation were stimulating AMPK by inhibiting cAMP, a similar effect would have been obtained by impairing PKA activation. As shown in Fig. 3A, cell treatment with the selective cAMP/PKA antagonist



**Fig. 3.** Effects of PKA modulators on DOR stimulation of AMPK phosphorylation. Cells were preincubated for 30 min with either vehicle or Rp-cAMPS (50  $\mu$ M) (A), dBcAMP (2 mM) (B), or Sp-cAMPS (500  $\mu$ M) (C), and then exposed to either vehicle or 100 nM SNC 80 for 5 min. Values are expressed as percentage of control (vehicle + vehicle) and are the mean  $\pm$  S.E.M. of four experiments. \*\*\*,  $p < 0.001$  versus control by ANOVA followed by Newman-Keuls post hoc test.

Rp-cAMPS at a concentration (50  $\mu$ M) 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  $\mu$ M) 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), the MAP kinase kinase inhibitor PD 98059 (50  $\mu$ M), the p38 MAP kinase inhibitor SB 203580 (10  $\mu$ M), or the c-Jun N-terminal kinase (JNK) inhibitor SP 600125 (10  $\mu$ M). Moreover, the DOR response was insensitive to both Go 6850 (1  $\mu$ M), which inhibits conventional PKC isoforms, and Go6983 (1  $\mu$ M), 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).



**Fig. 4.** DOR stimulation of AMPK phosphorylation is not affected by TAK1, MAP kinase and PKC inhibitors. Cells were preincubated with either vehicle, the TAK1 inhibitor (5Z)-7-oxozeaenol (Oxoz; 100 nM) for 30 min (A), the MAP kinase kinase inhibitor PD98059 (50  $\mu$ M) for 60 min (B), the p38 MAP kinase inhibitor SB203580 (10  $\mu$ M) for 30 min (C), the JNK inhibitor SP600125 (10  $\mu$ M) for 30 min (D), or the PKC inhibitors Go 6983 and Go 6850 (both at 1  $\mu$ M) for 30 min (E). Thereafter, cells were exposed to either vehicle or 100 nM SNC 80 for 5 min. Values are expressed as percentage of control and are the mean  $\pm$  S.E.M. of three experiments. \*\*\*,  $p < 0.001$  versus control by ANOVA followed by Newman-Keuls post hoc test.

**The CaMKK Inhibitor STO-609 Attenuates DOR-Induced AMPK and ACC Phosphorylation.** DORs have been shown to regulate intracellular  $Ca^{2+}$  levels (Connor and Henderson 1996; Yoon et al., 1999; Yeo et al., 2001), and



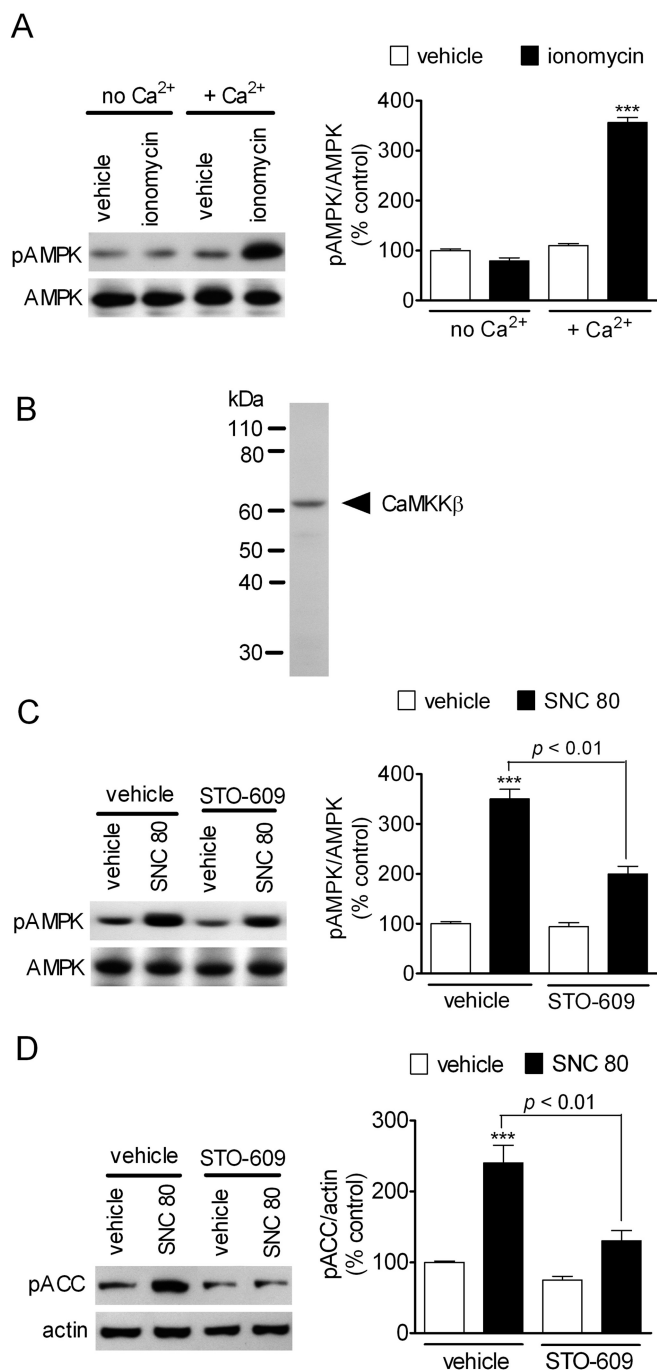
CaMKK $\beta$  is one of the main upstream kinase regulating AMPK (Towler and Hardie, 2007). Exposure of CHO/DOR cells to 100 nM ionomycin in the presence, but not in the absence, of extracellular Ca<sup>2+</sup> induced a marked increase of AMPK phosphorylation (Fig. 5A), indicating the occurrence of a Ca<sup>2+</sup>-dependent mechanism of AMPK regulation. Western blot analysis with an antibody that recognizes CaMKK $\beta$  showed the presence in CHO/DOR cell extracts of an immunoreactive band of ~65 kDa, which corresponds to the molecular mass of CaMKK $\beta$  (Fig. 5B). Pretreatment of CHO/DOR cells with 10  $\mu$ M STO-609, a relatively specific and cell-permeable inhibitor of CaMKK $\beta$  (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<sup>2+</sup>/CaMKK $\beta$  pathway is involved in AMPK activation induced by DOR stimulation.

#### DORs Stimulate AMPK through a Cooperative Interaction with Endogenously Activated P2Y Receptors.

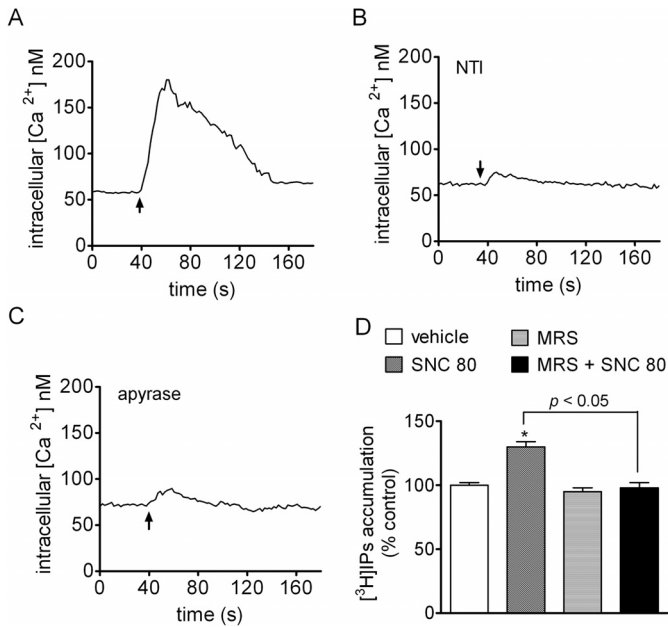
Previous studies have reported that, when expressed in CHO cells,  $\alpha_2$ -adrenergic receptors and DORs mobilized intracellular Ca<sup>2+</sup> by enhancing the stimulation of phospholipase C (PLC)  $\beta$  by endogenously expressed G<sub>q/11</sub>-coupled P2Y purinergic receptors (Akerman et 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  $48 \pm 5$ ,  $250 \pm 60$ , and  $610 \pm 100$  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<sup>2+</sup> concentrations from a resting level of  $60 \pm 8$  nM to a peak value of  $180 \pm 20$  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<sup>2+</sup> (Fig. 6C), indicating that the effect was dependent on the presence of extracellular ATP and/or ADP. The increase of intracellular Ca<sup>2+</sup> was associated with stimulation of PLC, as demonstrated by the increased accumulation of [<sup>3</sup>H]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



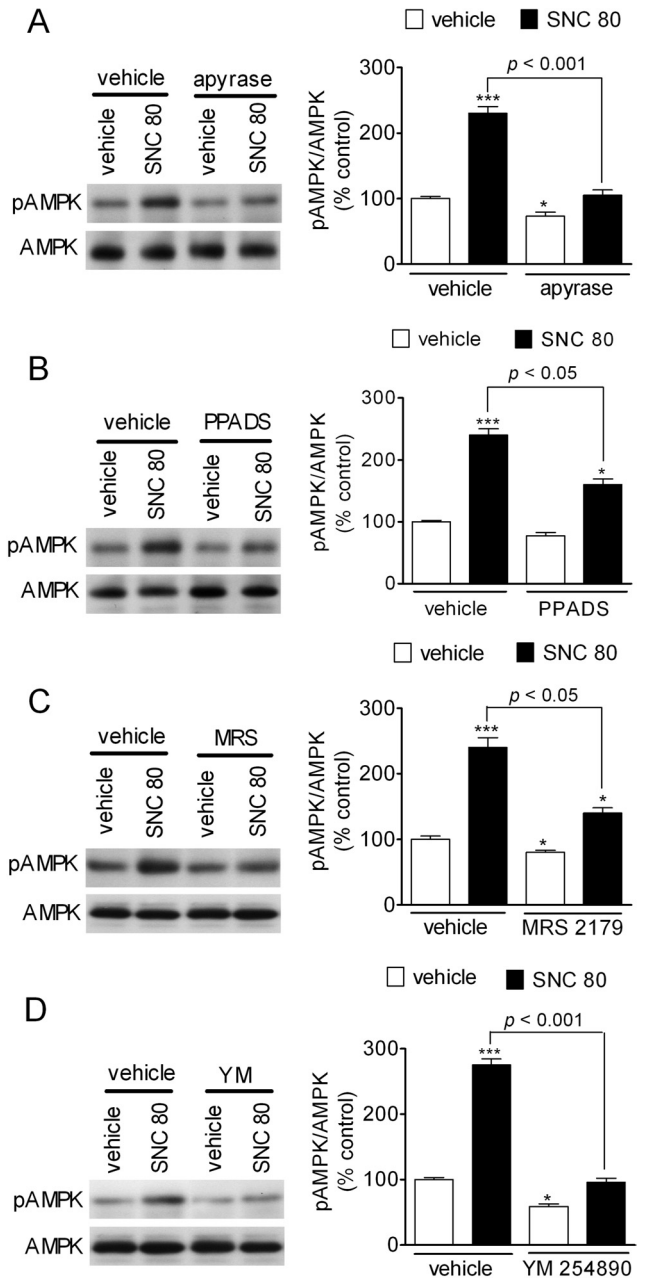
**Fig. 5.** Involvement of a Ca<sup>2+</sup>/CaMKK $\beta$  pathway in DOR activation of AMPK. A, Ca<sup>2+</sup>-dependent stimulation of AMPK phosphorylation by ionomycin. CHO/DOR cells were treated with either vehicle or 100 nM ionomycin in a Krebs-HEPES buffer lacking CaCl<sub>2</sub> and supplemented with either 0.1 mM EGTA (no Ca<sup>2+</sup>) or 1.2 mM CaCl<sub>2</sub> (+Ca<sup>2+</sup>) for 5 min. Phospho-AMPK levels were measured in cell extracts by Western blot and normalized to the levels of total AMPK. Densitometric ratios are reported as percentage of control and are the mean  $\pm$  S.E.M. of three experiments. B, expression of CaMKK $\beta$  in CHO/DOR cells. Cell extracts (10  $\mu$ g of protein) were loaded onto a 10% polyacrylamide gel and subjected to SDS-PAGE. CaMKK $\beta$  was detected by immunoblot analysis. Molecular size markers are indicated on the left. Data are representative of three experiments. C and D, the CaMKK $\beta$  inhibitor STO-609 blocks DOR activation of AMPK. CHO/DOR cells were preincubated with either vehicle or 10  $\mu$ M STO-609 for 30 min and then exposed to either vehicle or 100 nM SNC 80 for 5 min. Phospho-AMPK (C) and phospho-ACC (D) levels were measured in cell extracts by Western blot and normalized to the levels of total AMPK and actin, respectively. Densitometric ratios are reported as percentage of control and are the mean  $\pm$  S.E.M. of four experiments. \*\*\*,  $p < 0.001$  versus control by ANOVA followed by Newman-Keuls post hoc test.



**Fig. 6.** DOR stimulation of intracellular  $\text{Ca}^{2+}$  mobilization and  $[^3\text{H}]\text{IP}$  accumulation in CHO/DOR cells. The traces represent continuous records of intracellular  $\text{Ca}^{2+}$  concentrations determined as described under *Materials and Methods*. Cells were treated with either 100 nM SNC 80 (A), pretreated with either 100 nM NTI for 5 min (B) or 2 U/ml apyrase for 4 min (C), and then exposed to 100 nM SNC 80. The arrows indicate the time of SNC 80 addition. Data are representative of five experiments for each treatment. D, stimulation of  $[^3\text{H}]\text{IP}$  accumulation by SNC 80. Cells pre-labeled with  $[^3\text{H}]\text{inositol}$  were treated with either vehicle or 3  $\mu\text{M}$  MRS 2179 (MRS) for 10 min and then incubated with either vehicle or 100 nM SNC 80 for 40 min. Values are reported as percentage of control and are the mean  $\pm$  S.E.M. of four experiments. \*,  $p < 0.05$  versus control by ANOVA followed by Newman-Keuls post hoc test.

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  $\mu\text{M}$ ), a nonselective P2 receptor antagonist, or MRS 2179 (1  $\mu\text{M}$ ) 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% ( $p < 0.001$ ,  $n = 3$ ) after cell treatment with the  $G_{\alpha_{q11}}$  antagonist YM 254890 (10  $\mu\text{M}$ ), a cyclic depsipeptide that blocks  $G_{\alpha_{q11}}$  activation by inhibiting the exchange of GDP with GTP in the  $\alpha$  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  $G_{\alpha_{q11}}$ -coupled P2Y receptors, which leads to enhanced stimulation of PLC $\beta$ , increased intracellular  $\text{Ca}^{2+}$  and activation of CaMKK $\beta$ .

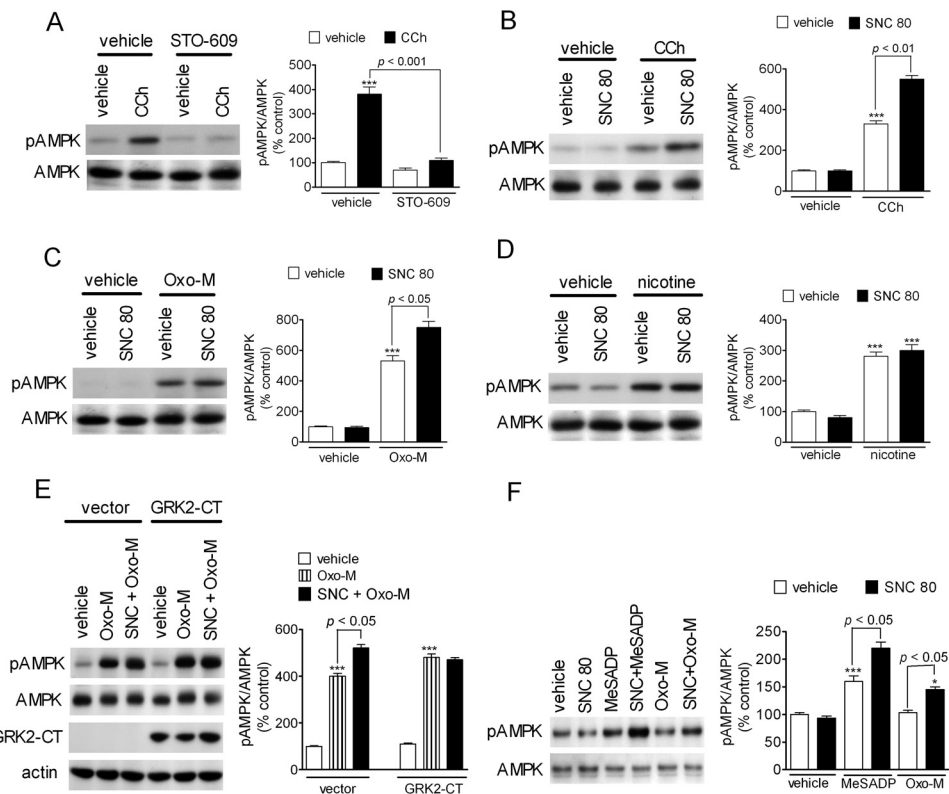
**In SH-SY5Y Cells and Primary Olfactory Bulb Neurons, DOR Activation Enhances AMPK Phosphorylation in Synergy with  $G_{\alpha_{q11}}$ -Coupled Receptors.** We next investigated whether DOR expressed under native conditions could activate AMPK in synergy with  $G_{\alpha_{q11}}$ -coupled receptors. 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  $\text{Ca}^{2+}$  mobilization induced by stimulation of  $G_{\alpha_{q11}}$ -coupled muscarinic  $M_3$  receptors (Connor and Henderson 1996; Yeo et al., 2001). Moreover, it has recently been reported that in these cells activation of  $M_3$  muscarinic receptors increased



**Fig. 7.** DOR stimulate AMPK through a cooperative interaction with endogenously activated P2Y receptors. CHO/DOR cells were pretreated with either vehicle or 2 U/ml apyrase for 4 min (A), 100  $\mu\text{M}$  PPADS for 10 min (B), 1  $\mu\text{M}$  MRS 2179 (MRS) for 10 min (C), or 10  $\mu\text{M}$  YM 254890 (YM) for 20 min (D) and then exposed to either vehicle or 100 nM SNC 80 for 5 min. Values are the mean  $\pm$  S.E.M. of four (A and D) and five (B and C) experiments. \*,  $p < 0.05$ ; \*\*\*,  $p < 0.001$  versus control by ANOVA followed by Newman-Keuls post hoc test.

AMPK activity through CaMKK $\beta$  (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% ( $p < 0.05$ ,  $n =$





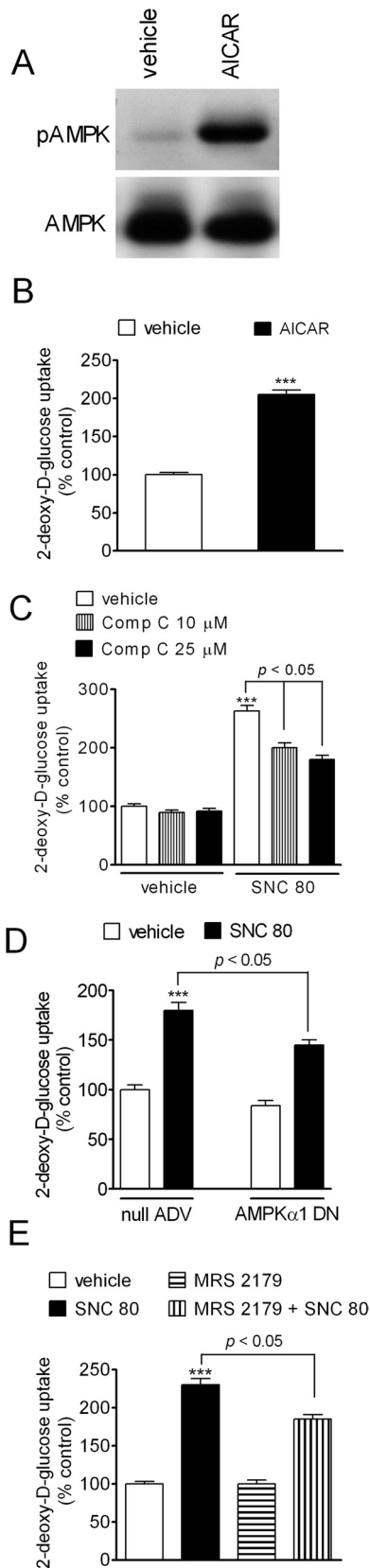
**Fig. 8.** DOR activation enhances AMPK phosphorylation in SH-SY5Y cells and primary olfactory bulb neurons in synergy with  $G_{\alpha/11}$ -coupled receptors. A, STO-609 blocks CCh-induced AMPK phosphorylation in SH-SY5Y cells. Cells were pretreated for 1 h with either vehicle or 10  $\mu$ M STO-609 and then exposed to either vehicle or 100 nM CCh for 5 min. Values are the mean  $\pm$  S.E.M. of three experiments. B to D, in SH-SY5Y cells, DORs potentiate muscarinic but not nicotinic stimulation of AMPK phosphorylation. Cells were preincubated for 5 min with either vehicle or 100 nM SNC 80 and then exposed to either vehicle or 100 nM CCh (B), 300 nM Oxo-M (C), or 10  $\mu$ M nicotine (D) for 5 min. Values are the mean  $\pm$  S.E.M. of five (B), four (C), and three (D) experiments. E, expression of the  $G\beta\gamma$  scavenger GRK2-CT impairs DOR potentiation of muscarinic-induced AMPK phosphorylation. SH-SY5Y cells transfected with either empty vector (vector) or GRK2-CT minigene were pretreated with either vehicle or 100 nM SNC 80 for 5 min and then exposed to either vehicle or 1  $\mu$ M Oxo-M for 5 min. Values are expressed as percentage of control (empty vector-transfected cells treated with vehicle) and are the mean  $\pm$  S.E.M. of four separate experiments. F, effects of DOR stimulation on AMPK phosphorylation in primary olfactory bulb neurons. Cells were pretreated with either vehicle or 100 nM SNC-80 for 5 min and then exposed to either vehicle, 10  $\mu$ M MeSADP or 1  $\mu$ M Oxo-M for an additional 5 min. Values are the mean  $\pm$  S.E.M. of four experiments. \*,  $p < 0.05$ ; \*\*\*,  $p < 0.001$  versus control by ANOVA followed by Newman-Keuls post hoc test.

4), respectively (Fig. 8, B and C). Cell treatment with nicotine (10  $\mu$ M) to activate endogenous cholinergic nicotinic receptors also caused a significant increase of AMPK phosphorylation, but this response was not potentiated by concomitant DOR stimulation by SNC 80 (Fig. 8D). DOR have been shown to mobilize intracellular  $Ca^{2+}$  through the action of G protein  $\beta\gamma$  subunits ( $G\beta\gamma$ ) (Yoon et al., 1999, 2001). To investigate the role of  $G\beta\gamma$  in DOR potentiation of muscarinic stimulation of AMPK, SH-SY5Y were transfected with a minigene encoding GRK2-CT, which binds and neutralizes  $G\beta\gamma$  through its pleckstrin homology domain (Koch et al., 1994). In cells expressing GRK2-CT, exposure to Oxo-M (1  $\mu$ M) increased AMPK phosphorylation as effectively as in empty vector-transfected cells, but the addition of SNC 80 (100 nM) failed to potentiate the muscarinic response (Fig. 8E). Analysis of  $Ca^{2+}$  mobilization in empty vector- and GRK2-CT-transfected SH-SY5Y cells indicated that blockade of  $G\beta\gamma$  prevented DOR potentiation of CCh-induced stimulation of intracellular free  $Ca^{2+}$ . Thus, in empty-vector-transfected cells, the addition of 1  $\mu$ M CCh increased free  $Ca^{2+}$  levels from  $49 \pm 5$  to  $165 \pm 7$  nM (mean  $\pm$  S.E.M. of five experiments,  $p < 0.001$  by Student's  $t$  test). The addition of SNC 80 (100 nM) failed to affect intracellular  $Ca^{2+}$ , but, when added together with 1  $\mu$ M CCh, increased the  $Ca^{2+}$  levels up to

$270 \pm 9$  nM ( $p < 0.05$  versus CCh alone,  $n = 5$ ). In GRK2-CT-transfected cells, 1  $\mu$ M CCh enhanced the intracellular  $Ca^{2+}$  from  $43 \pm 6$  to  $185 \pm 8$  nM ( $n = 5$ ,  $p > 0.05$  versus CCh effect in empty vector-transfected cells). In these cells, the combined addition of 100 nM SNC 80 with 1  $\mu$ M CCh increased the  $Ca^{2+}$  peak up to  $210 \pm 12$  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  $\mu$ M) 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  $\mu$ M), 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



**Fig. 9.** Involvement of AMPK in DOR stimulation of glucose uptake in CHO/DOR cells. A, cells were preincubated with either vehicle or the AMPK activator AICAR (2 mM) for 1 h and AMPK phosphorylation was analyzed by Western blot. The immunoblot is representative of three experiments. B, stimulation of glucose uptake by AICAR. Cells were treated as above and glucose uptake was determined as described under

similar experimental conditions, AICAR (2 mM) stimulated 2-deoxy-D-glucose uptake by  $105 \pm 6\%$  ( $p < 0.001$ ,  $n = 5$ ) (Fig. 9B). As reported previously (Olianas et al., 2011), DOR activation by SNC 80 (100 nM) increased glucose uptake by  $140 \pm 8\%$  ( $p < 0.001$ ,  $n = 6$ ) (Fig. 9C). Pretreatment with the AMPK inhibitor compound C (10 and 25  $\mu$ M) reduced the stimulatory effect of SNC 80 (100 nM) by  $37 \pm 6$  and  $43 \pm 6\%$ , 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 AMPK $\alpha$ 1 DN mutant. As shown in Fig. 9D, in cells infected with the AMPK mutant DOR-stimulated glucose uptake was decreased by  $40 \pm 6\%$  ( $p < 0.05$ ,  $n = 4$ ). In addition, cell treatment with MRS 2179 (1  $\mu$ M) reduced the DOR-stimulated glucose uptake by  $30 \pm 3\%$  ( $p < 0.05$ ,  $n = 4$ ) (Fig. 9E).

### Discussion

The present study constitutes the first demonstration that in different cell types, DOR activation increases AMPK activity through coincidence signaling with  $G_{q/11}$ -coupled receptors. This conditional activation of AMPK by a  $G_{i/o}$ -coupled receptor has not been described previously. Indeed, the stimulation of a number of  $G_{i/o}$ -coupled receptors, including  $\alpha_2$ -adrenergic, muscarinic  $M_2$  and  $M_4$ , prostanoid EP3 $\alpha$ , 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  $G_{q/11}$ -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  $G_{i/o}$ -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  $\pm$  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  $\pm$  S.E.M. of four to six experiments. D, expression of AMPK $\alpha$ 1 DN mutant reduces DOR-stimulated glucose uptake. Cells infected with either null control adenovirus (null ADV) or ADV encoding the AMPK $\alpha$ 1 DN mutant (AMPK $\alpha$ 1 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  $\pm$  S.E.M. of four experiments. E, antagonism of P2Y1 purinergic receptors attenuates DOR stimulation of glucose uptake. Cells were preincubated 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  $\pm$  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 (C–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 depended on  $G_{q/11}$  signaling, this finding is consistent with the observation that different  $G_{q/11}$ -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  $G_{q/11}$ -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  $G_{i/o}$ -coupled receptors may control AMPK activity (Hutchinson et al., 2008). In fact, PKA can inactivate AMPK by either phosphorylating the  $\alpha$  subunit at Ser485/491 or inhibiting CaMKK $\beta$  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 PLC $\beta$  by  $G\beta\gamma$  (Liu and Simon, 1996). Therefore, the PKA inhibition of DOR-induced AMPK activation might also have derived from an impaired activation of PLC $\beta$ .

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 [ $^3$ H]IP accumulation and intracellular free  $Ca^{2+}$  mobilization were suppressed in cells treated with either apyrase, P2 purinergic receptor antagonists or the  $G_{q/11}$  antagonist YM 254890. These data strongly indicate the occurrence of a synergistic interaction with  $G_{q/11}$ -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 (Bofetiado 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  $G_{q/11}$ -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 induced by nicotine, which, by opening the ionotropic nicotinic receptors, increases intracellular  $Ca^{2+}$  independently of  $G_{q/11}$  and PLC $\beta$  activation (Ridley et al., 2002). Moreover, in SH-SY5Y cells, sequestration of  $G\beta\gamma$  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  $G\beta\gamma$  in the opioid 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  $Ca^{2+}$ , indicating that  $G\beta\gamma$  is also required for the coincident signaling between DOR and  $M_3$  receptors in the regulation of  $Ca^{2+}$  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 CaMKK $\beta$ .

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 factor1 (IGF1) receptor tyrosine kinase and phosphatidylinositol 3-kinase (PI3K) pathway (Olianas et al., 2011). There is evidence for the occurrence in different cell types of a cross-talk 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 activity 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 AMPK in these cells. The DOR stimulation of glucose uptake was significantly inhibited by either compound C, expression of a DN mutant of AMPK $\alpha$ 1 or antagonism of P2Y1 purinergic receptors. Collectively, these data indicate that AMPK activation through the synergistic interaction with G $_{\alpha 11}$ -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 activate AMPK, a key enzyme in the cellular adaptation to metabolic insults, through a coincident signaling with G $_{\alpha 11}$ -coupled receptors. This regulatory mechanism may be relevant for the beneficial effect of DOR activation in conditions of limited energy supply.

#### Authorship Contributions

*Participated in research design:* M. C. Olianas and Onali.

*Conducted experiments:* Olianas, Dedoni, and Olianas.

*Performed data analysis:* Olianas, Dedoni, and Olianas.

*Wrote or contributed to the writing of the manuscript:* M. C. Olianas and Onali.

#### References

- Abraham EH, Prat AG, Gerweck L, Seneveratne T, Arceci RJ, Kramer R, Guidotti G, and Cantiello HF (1993) The multidrug resistance (*mdr1*) gene product functions as an ATP channel. *Proc Natl Acad Sci USA* **90**:312–316.
- Akerman KE, Näsman J, Lund PE, Shariatmadari R, and Kukkonen JP (1998) Endogenous extracellular purine nucleotides redirect  $\alpha_2$ -adrenoceptor signaling. *FEBS Lett* **430**:209–212.
- Bain J, Plater L, Elliott M, Shpuro N, Hastie CJ, McLauchlan H, Klevernic I, Arthur JS, Alessi DR, and Cohen P (2007) The selectivity of protein kinase inhibitors: a further update. *Biochem J* **408**:297–315.
- Bofetiado DM, Mayfield KP, and D'Alecy LG (1996) Alkaloid  $\delta$  agonist BW373U86 increases hypoxic tolerance. *Anesth Analg* **82**:1237–1241.
- Burnstock G (2006) Pathophysiology and therapeutic potential of purinergic signaling. *Pharmacol Rev* **58**:58–86.
- Connor M and Henderson G (1996)  $\delta$ - and  $\mu$ -Opioid receptor mobilization of intracellular calcium in SH-SY5Y human neuroblastoma cells. *Br J Pharmacol* **117**:333–340.
- Evans AA, Tunnicliffe G, Knights P, Bailey CJ, and Smith ME (2001) Delta opioid receptors mediate glucose uptake in skeletal muscle of lean and obese-diabetic (*ob/ob*) mice. *Metabolism* **50**:1402–1408.
- Glass MJ, Billington CJ, and Levine AS (1999) Opioids and food intake: distributed functional neural pathways? *Neuropeptides* **33**:360–368.
- Hardie DG and Hawley SA (2001) AMP-activated protein kinase: the energy charge hypothesis revisited. *BioEssays* **23**:1112–1119.
- Hofmann J (1997) The potential for isoenzyme-selective modulation of protein kinase C. *FASEB J* **11**:649–669.
- Hurley RL, Barré LK, Wood SD, Anderson KA, Kemp BE, Means AR, and Witters LA (2006) Regulation of AMP-activated protein kinase by multisite phosphorylation in response to agents that elevate cellular cAMP. *J Biol Chem* **281**:36662–36672.
- Hutchinson DS, Chernogubova E, Dallner OS, Cannon B, and Bengtsson T (2005)  $\beta$ -Adrenoceptors, but not  $\alpha$ -adrenoceptors, stimulate AMP-activated protein kinase in brown adipocytes independently of uncoupling protein-1. *Diabetologia* **48**:2386–2395.
- Hutchinson DS, Summers RJ, and Bengtsson T (2008) Regulation of AMP-activated protein kinase activity by G-protein coupled receptors: potential utility in treatment of diabetes and heart disease. *Pharmacol Ther* **119**:291–310.
- Jakobsen SN, Hardie DG, Morrice N, and Tornqvist HE (2001) 5'-AMP-activated protein kinase phosphorylates IRS-1 on Ser-789 in mouse C2C12 myotubes in response to 5-aminoimidazole-4-carboxamide riboside. *J Biol Chem* **276**:46912–46916.
- Kahn BB, Alquier T, Carling D, and Hardie DG (2005) AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism. *Cell Metab* **1**:15–25.
- Kishi K, Yuasa T, Minami A, Yamada M, Hagi A, Hayashi H, Kemp BE, Witters LA, and Ebina Y (2000) AMP-activated protein kinase is activated by the stimulations of G $_{\alpha i}$ -coupled receptors. *Biochem Biophys Res Commun* **276**:16–22.
- Koch WJ, Hawes BE, Ingles J, Luttrell LM, and Lefkowitz RJ (1994) Cellular expression of the carboxyl terminus of a G protein-coupled receptor kinase attenuates G $_{\beta\gamma}$ -mediated signaling. *J Biol Chem* **269**:6193–6197.
- Liu M and Simon MI (1996) Regulation by cAMP-dependent protein kinase of a G-protein-mediated phospholipase C. *Nature* **382**:83–87.
- Marcet B, Chappe V, Delmas P, and Verrier B (2004) Pharmacological and signaling properties of endogenous P2Y1 receptors in cystic fibrosis transmembrane conductance regulator-expressing Chinese hamster ovary cells. *J Pharmacol Exp Ther* **309**:533–539.
- Merlin J, Evans BA, Csikasz RI, Bengtsson T, Summers RJ, and Hutchinson DS (2010) The M3-muscarinic acetylcholine receptor stimulates glucose uptake in L6 skeletal muscle by a CaMKK-AMPK-dependent mechanism. *Cell Signal* **22**:1104–1113.
- Momcilovic M, Hong SP, and Carlson M (2006) Mammalian TAK1 activates Snf1 protein kinase in yeast and phosphorylates AMP-activated protein kinase in vitro. *J Biol Chem* **281**:25336–25343.
- Ninomiya-Tsuji J, Kajino T, Ono K, Ohtomo T, Matsumoto M, Shiina M, Mihara M, Tsuchiya M, and Matsumoto K (2003) A resorcylic acid lactone, 5Z-7-oxozeaenol, prevents inflammation by inhibiting the catalytic activity of TAK1 MAPK kinase. *J Biol Chem* **278**:18485–18490.
- Oakhill JS, Steel R, Chen ZP, Scott JW, Ling N, Tam S, and Kemp BE (2011) AMPK is a direct adenylate charge-regulated protein kinase. *Science* **332**:1433–1435.
- Olianas MC, Dedoni S, and Onali P (2011)  $\delta$ -Opioid receptors stimulate GLUT1-mediated glucose uptake through Src- and IGF-1 receptor-dependent activation of PI3-kinase signalling in CHO cells. *Br J Pharmacol* **163**:624–637.
- Quock RM, Burkey TH, Varga E, Hosohata Y, Hosohata K, Cowell SM, Slate CA, Ehler FJ, Roeske WR, and Yamamura HI (1999) The  $\delta$ -opioid receptor: molecular pharmacology, signal transduction, and the determination of drug efficacy. *Pharmacol Rev* **51**:503–532.
- Ridley DL, Pakkanen J, and Wonnacott S (2002) Effects of chronic drug treatments on increases in intracellular calcium mediated by nicotinic acetylcholine receptors in SH-SY5Y cells. *Br J Pharmacol* **135**:1051–1059.
- Ronnett GV, Ramamurthy S, Kleman AM, Landree LE, and Aja S (2009) AMPK in the brain: its roles in energy balance and neuroprotection. *J Neurochem* **109**:17–23.
- Rothermel JD and Parker Botelho LH (1988) A mechanistic and kinetic analysis of the interactions of the diastereoisomers of adenosine 3',5'-(cyclic)phosphorothioate with purified cyclic AMP-dependent protein kinase. *Biochem J* **251**:757–762.
- Sabirov RZ and Okada Y (2005) ATP release via anion channels. *Purinergic Signal* **1**:311–328.
- Shinozaki Y, Koizumi S, Ishida S, Sawada J, Ohno Y, and Inoue K (2005) Cytoprotection against oxidative stress-induced damage of astrocyte by extracellular ATP via P2Y1 receptors. *Glia* **49**:288–300.
- Takasaki J, Saito T, Taniguchi M, Kawasaki T, Moritani Y, Hayashi K, and Kobori M (2004) A novel G $_{\alpha 11}$ -selective inhibitor. *J Biol Chem* **279**:47438–47445.
- Tao R, Gong J, Luo X, Zang M, Guo W, Wen R, and Luo Z (2010) AMPK exerts dual regulatory effects on the PI3K pathway. *J Mol Signal* **5**:1.
- Tegeder I and Geisslinger G (2004) Opioids as modulators of cell death and survival—unraveling mechanisms and revealing new indications. *Pharmacol Rev* **56**:351–369.
- Thornton C, Sardini A, and Carling D (2008) Muscarinic receptor activation of AMP-activated protein kinase inhibits orexigenic neuropeptide mRNA expression. *J Biol Chem* **283**:17116–17122.
- Tokumitsu H, Inuzuka H, Ishikawa Y, Ikeda M, Saji I, and Kobayashi R (2002) STO-609, a specific inhibitor of the Ca $^{2+}$ /calmodulin-dependent protein kinase kinase. *J Biol Chem* **277**:15813–15818.
- Towler MC and Hardie DG (2007) AMP-activated protein kinase in metabolic control and insulin signaling. *Circ Res* **100**:328–341.
- Woods A, Azzout-Marniche D, Foretz M, Stein SC, Lemarchand P, Ferré P, Foufelle F, and Carling D (2000) Characterization of the role of AMP-activated protein kinase in the regulation of glucose-activated gene expression using constitutively active and dominant negative forms of the kinase. *Mol Cell Biol* **20**:6704–6711.
- Xiao B, Sanders MJ, Underwood E, Heath R, Mayer FV, Carmena D, Jing C, Walker PA, Eccleston JF, Haire LF, et al. (2011) Structure of mammalian AMPK and its regulation by ADP. *Nature* **472**:230–233.
- Yeo A, Samways DS, Fowler CE, Gunn-Moore F, and Henderson G (2001) Coincident signalling between the Gi/Go-coupled  $\delta$ -opioid receptor and the Gq-coupled m3 muscarinic receptor at the level of intracellular free calcium in SH-SY5Y cells. *J Neurochem* **76**:1688–1700.
- Yoon SH, Lo TM, Loh HH, and Thayer SA (1999)  $\delta$ -Opioid-induced liberation of G $\beta\gamma$  mobilizes Ca $^{2+}$  stores in NG108–15 cells. *Mol Pharmacol* **56**:902–908.
- Zhang J, Gibney GT, Zhao P, and Xia Y (2002) Neuroprotective role of  $\delta$ -opioid receptors in cortical neurons. *Am J Physiol Cell Physiol* **282**:C1225–C1234.
- Zheng W, Watts LT, Holstein DM, Prajapati SI, Keller C, Grass EH, Walter CA, and Lechleiter JD (2010) Purinergic receptor stimulation reduces cytotoxic edema and brain infarcts in mouse induced by photothrombosis by energizing glial mitochondria. *PLoS ONE* **5**:e14401.

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