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RESEARCH PAPER

δ-Opioid receptors stimulate GLUT1-mediated glucose uptake through Src- and IGF-1 receptor-dependent activation of PI3-kinase signalling in CHO cells

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BACKGROUND AND PURPOSE
Although opioids have been reported to affect glucose homeostasis, relatively little is known on the role of δ-opioid receptors. We have investigated the regulation of glucose transport by human δ-opioid receptors expressed in Chinese hamster ovary cells.

EXPERIMENTAL APPROACH
The uptake of [^{3}H]-2-deoxy-D-glucose and 3-*O*-[methyl-[^{3}H]]-D-glucose in response to δ-opioid receptor ligands and the expression of GLUT1, GLUT3 and GLUT4 glucose transporters were examined. Moreover, the effects of intracellular signal transduction inhibitors on δ-opioid receptor-regulated [^{3}H]-2-deoxy-D-glucose uptake and protein phosphorylation were investigated.

KEY RESULTS
Activation of δ-opioid receptors rapidly stimulated [^{3}H]-2-deoxy-D-glucose and 3-*O*-[methyl-[^{3}H]]-D-glucose uptakes, which were blocked by the GLUT inhibitors cytochalasin B and phloretin. The stimulation of [^{3}H]-2-deoxy-D-glucose uptake that occurred without a change in plasma membrane GLUT1 – required the coupling to G_i/G_o proteins – was independent of cAMP and extracellular signal-regulated protein kinases, and was suppressed by blockade of Src and insulin-like growth factor-1 receptor (IGF-1R) tyrosine kinases. Inhibition of phosphatidylinositol 3-kinase (PI3K) by wortmannin or LY294002 and by PI3Kα, but not γ, isoform-selective inhibitors greatly reduced the δ-opioid receptor stimulation of glucose uptake. Moreover, the response was attenuated by overexpressing a dominant-negative kinase-deficient Akt form and by chemical inhibition of Akt. Stimulation of δ-opioid receptors increased protein kinase Cζ/λ (PKCζ/λ) phosphorylation and a selective PKCζ/λ inhibitor slightly reduced opioid stimulation of glucose uptake.

CONCLUSIONS AND IMPLICATIONS
δ-Opioid receptors stimulated glucose transport probably by enhancing GLUT1 intrinsic activity through a signalling cascade involving G_i/G_o, Src, IGF-1R, PI3Kα, Akt and, to a minor extent, PKCζ/λ. This effect may contribute to the opioid regulation of glucose homeostasis in physio-pathological conditions.

Abbreviations
3-OMG, 3-O-methyl-D-glucose; CHO, Chinese hamster ovary; CHO/DOR, CHO cells stably expressing the human δ-opioid receptor; CHO/DOR Akt DN, CHO/DOR cells stably expressing dominant-negative kinase-deficient Akt1 mutant; dB-cAMP, dibutyryl- cAMP; DPDPE, [D-Pen(2,5)]-enkephalin; EGFR, epidermal growth factor receptor; ERK1/2, extracellular signal-regulated protein kinases 1 and 2; GPCR, G protein-coupled receptors; IGF-1, insulin-like growth factor-1; IGF-1R, IGF-1 receptor; MEK, mitogen-activated protein kinase kinases; NTI, naltrindole; PI3K,

phosphatidylinositol 3-kinase; PKC, protein kinase C; PKCζ-PSI, myristoylated PKCζ pseudosubstrate inhibitor; PMA, phorbol 12-myristate 13-acetate; PMSF, phenylmethylsulphonyl fluoride; PTX, pertussis toxin; SDS, sodium dodecyl sulphate; SDS-PAGE, SDS-polyacrylamide gel electrophoresis

Introduction

Opioid agonists and, in particular β-endorphin, which preferentially acts on μ-opioid receptors, have long been known to regulate glucose homeostasis by exerting central and peripheral effects on glucoregulatory hormones such as insulin, glucagon and catecholamines (Radosevich *et al.*, 1984; Ramabadran and Bansinath, 1990; Appleyard *et al.*, 2003; Wen *et al.*, 2009). In addition, it has been observed that the activation of μ-opioid receptors located on the skeletal muscle of diabetic rats, or expressed in cultured C_2C_{12} myoblast cells, stimulate glucose uptake, thus indicating the possibility of a direct control of glucose homeostasis by μ-opioid receptors independent of action on insulin (Cheng *et al.*, 2001; Yang *et al.*, 2009). These studies also showed that the molecular mechanisms mediating μ-opioid receptor stimulation of glucose uptake appeared to involve the activation of phospholipase C and multiple protein kinase C (PKC) isoforms, including the atypical isoform PKCζ (Liu *et al.*, 2004; Yang *et al.*, 2009).

Like the μ subtype, the δ-opioid receptor has been found to be expressed in rodent skeletal muscles (Evans *et al.*, 1995), and similar to insulin, β-endorphin and the δ-opioid receptor agonist [D-Pen(2,5)]-enkephalin (DPDPE) have been reported to stimulate 2-deoxy-D-glucose uptake in the skeletal muscles of lean and obese-diabetic mice (Evans *et al.*, 2001). Although these observations suggest a role for δ-opioid receptors in peripheral glucose transport, no information has so far been provided on the mechanism(s) mediating this functional response.

Previous studies have shown that Chinese hamster ovary (CHO) cells express glucose transporters of the GLUT family (Harrison *et al.*, 1991; John *et al.*, 2008), which mediates facilitative glucose transport in a wide variety of tissues and cell types (Uldry and Thorens, 2004).

In the present study, we investigated the regulation of glucose uptake by δ-opioid agonists in CHO-K1 cells stably transfected with the human δ-opioid receptor (CHO/DOR cells) as a model system in which to study the coupling of δ-opioid receptor to regulation of GLUT activity.

Methods

Cell culture and transfections

CHO-K1 cells (American Type Culture Collection, Manassas, VA, USA) were grown at 37°C in a humidified atmosphere (5% CO_2) in Ham's F12, containing l-glutamine and sodium bicarbonate and supplemented with 10% foetal calf serum (FCS), 0.5% penicillin/streptomycin. CHO/DOR cells were developed by transfecting CHO-K1 cells with pcDNA3.1-Hygro(+)vector encoding the human δ-opioid receptor (Onali and Olianas, 2007) using PolyFect (Qiagen GmbH, Hilden, Germany) as transfection reagent following the manufacturer's instructions. Cells were selected by their resistance to 1 $mg \cdot mL^{-1}$ of hygromycin for 4 weeks and cell clones were isolated by using cloning cylinders. The cell clone used in the present study had a δ-opioid receptor density of ~1500 $fmol \cdot mg^{-1}$ protein determined by saturation radioligand binding with the δ-opioid receptor antagonist [^{3}H]-naltrindole (PerkinElmer Life and Analytical Sciences, Waltham, MA, USA). Cells were maintained in Ham's F12 medium containing l-glutamine and sodium bicarbonate and supplemented with 10% FCS, 0.5% penicillin/streptomycin and 350 $\mu g \cdot mL^{-1}$ hygromycin (Invitrogen, Carlsbad, CA, USA).

CHO/DOR cells stably expressing dominant-negative kinase-deficient Akt (CHO/DOR Akt DN) were obtained by transfecting the cells with pUSEamp(+) vector encoding Myc-His tagged mouse Akt1 (K179M) mutant (Franke *et al.*, 1995) (Millipore, Temecula, CA, USA) using Lipofectamine 2000 (Invitrogen) as transfectant. The cells were selected by their resistance to 1 $mg \cdot mL^{-1}$ G 418 sulphate (Invitrogen) for 4 weeks, and was maintained in a complete growing medium supplemented with 500 $\mu g \cdot mL^{-1}$ G 418 sulphate and 350 $\mu g \cdot mL^{-1}$ hygromycin.

Assay of glucose uptake

The measurement of 2-deoxy-D-glucose uptake by CHO/DOR cells was performed according to the method described by Asano *et al.* (1989), with some modifications. Briefly, confluent cell monolayers were incubated in serum-free Ham's F12 for 12 h, and, when indicated, treated with either inhibitors or the corresponding vehicles as specified in the text. The concentration of the inhibitor was kept constant throughout the subsequent incubation step. The cells were then washed twice and incubated with Krebs-HEPES buffer containing 25 mM HEPES/NaOH (pH 7.4), 125 mM NaCl, 1.2 mM Mg_2SO_4, 1.2 mM KH_2PO_4, 3.8 mM KCl and 1.2 mM $CaCl_2$ for 20 min at 37°C. Receptor agonists were then added and the incubation was continued for 15 min. Receptor antagonists were added 5 min before the addition of agonists. Control samples received an equal volume of vehicle. The reaction was started by the addition of [^{3}H]-2-deoxy-D-glucose (1 $\mu Ci \cdot mL^{-1}$) together with unlabeled 2-deoxy-D-glucose. Unless otherwise indicated, the final concentration of 2-deoxy-D-glucose was 1 mM and the uptake was measured for a period of 8 min. For the assay of 3-*O*-[methyl-[^{3}H]]-D-glucose ([^{3}H]-3-OMG) uptake, the cells were incubated for 20 min in Krebs-HEPES buffer at 37°C, and exposed to either vehicle or receptor agonist for 10 min at 37°C. Following an additional 10 min incubation at room temperature, [^{3}H]-3-OMG (2 $\mu Ci \cdot mL^{-1}$) was added together with unlabelled 3-OMG to give a final concentration of 1 mM and the incubation was continued for 2 min at room temperature. Preliminary experiments indicated that 3-OMG uptake was linear up to at least 4 min.

The incubation was stopped by aspirating the medium and washing the cells three times with ice-cold Krebs-HEPES

buffer containing 10 mM D-glucose and 0.2 mM phloretin. Cells were solubilized by adding 0.1% sodium dodecyl sulphate (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.

Biotinylation of surface proteins

Surface biotinylation of CHO/DOR cell proteins was performed as described by Samih *et al*. (2000) with some modifications. Cells were grown in 100 mm plates, deprived of serum for 12 h and then treated with either vehicle or δ-opioid receptor agonists for 15 min at 37°C. Thereafter, the cells were washed three times with ice-cold phosphate-buffered saline (PBS, pH 8.0) and incubated for 30 min at 4°C with or without the cell impermeable biotinylating agent sulfosuccinimidyl-6-(biotin-amido)hexanoate (sulpho-NHS-LC-biotin; 0.25 mg·mL^{-1}; Pierce, Rockford, IL, USA). Thereafter, the medium was aspirated and the cells were washed three times with ice-cold PBS containing 20 mM glycine. Cells were then solubilized by incubation for 60 min at ice-bath temperature in a 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 and 1% protease inhibitor cocktail radioimmunoprecipitation assay (RIPA) buffer supplemented with 1% Triton X 100. Cell extracts were centrifuged at 14 000× *g* and the supernatants incubated overnight with streptavidin-conjugated agarose beads with continuous rotation. The samples were then centrifuged to obtain a supernatant and a pellet fraction containing the plasma membrane-associated proteins. The agarose beads were washed three times with ice-cold Tris buffer containing 50 mM Tris-HCl (pH 7.5), 2.5 mM EDTA, 150 mM NaCl and 1% Triton X 100, followed by two washes with 50 mM Tris-HCl (pH 7.5), 2.5 mM EDTA, 500 mM NaCl and 0.1% Triton X 100, and one final wash with 50 mM Tris-HCl (pH 7.5). The pellet was then mixed with sample buffer and incubated 10 min at room temperature and 30 min at 37°C. The proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and analysed by Western blot.

Preparation of cell extracts and Western blot analysis

After treatments, the cells were washed briefly with ice-cold PBS (pH 7.40) and cell extracts were prepared by scraping the cells in RIPA buffer. The samples were sonicated for 5 s in ice-bath and stored at –80°C.

Frontal cortex and soleus muscle tissues were obtained from male Sprague–Dawley rats (200–300 g) maintained in a 12 h light/dark cycle with food and water *ad libitum*. Experiments were performed according to the principles of laboratory animal care (Law on animal experiments in Italy, D.L. 116/92). Freshly dissected tissues were minced in small fragments and homogenized in ice-cold RIPA buffer supplemented with 0.1 mM phenylmethylsulphonyl fluoride (PMSF).

Cell and tissue extracts were analysed for protein content by the method of Bradford (1976), using bovine serum albumin (BSA) as a standard. Aliquots containing equal amounts of protein were subjected to SDS-PAGE, and proteins were electrophoretically transferred to polyvinylidene difluoride membranes (Hybond-P, Amersham Biosciences, Piscataway, NJ, USA). The efficiency of the transfer was controlled by gel staining and by following the transfer of pre-stained protein standards (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). 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 (TBS-T) buffer containing 5% BSA for 1 h. After being washed with TBS-T buffer, the membranes were incubated overnight at 4°C with one of the primary antibodies. 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 (Amersham). 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 NIH ImageJ software (US National Institutes of Health, Bethesda, MA, USA). The optical density of phosphoprotein bands was normalized to the density of the corresponding total protein band or actin band to yield the relative optical density value.

Subcellular membrane preparations

CHO/DOR cells grown in 100 mm dishes were processed as described for the glucose uptake assay and treated for 15 min with either vehicle or 100 nM SNC 80 at 37°C. Thereafter, the medium was removed and the cells were washed once with ice-cold PBS and scraped into an ice-cold homogenization medium containing 0.25 M sucrose in 10 mM Tris-HCl, 1 mM EDTA and 0.1 mM PMSF (pH 7.4). The cells were lysed by using a Dounce glass homogenizer (pestle A, 15 strokes), followed by aspiration through a 26-gauge needle. The cell lysate was centrifuged at 16 600× *g* for 20 min at 4°C. The supernatant was stored at ice-bath temperature, whereas the pellet was resuspended in 10 mM Tris-HCl buffer (pH 7.4) containing 1 mM EDTA and 0.1 mM PMSF (Tris-EDTA buffer) with 10 strokes of Dounce homogenizer and applied over a sucrose cushion (1.12 M sucrose in Tris-EDTA buffer). The samples were centrifuged at 100 000× *g* for 60 min at 4°C in a SW 60 rotor. The plasma membranes were removed from the top of the sucrose cushion, diluted with Tris-EDTA buffer, centrifuged at 30 000× *g* for 30 min and resuspended in the same buffer. The 16 600× *g* supernatant was centrifuged at 150 000× *g* for 2.5 h at 4°C, and the pellet containing the low-density microsomal fraction was resuspended in Tris-EDTA buffer. Aliquots of subcellular fractions containing equal amounts of protein were mixed with sample buffer and incubated for 10 min at room temperature and for 30 min at 37°C. The proteins were separated by SDS-PAGE and analysed by Western blot.

Akt activity assay

Akt activity was assayed by using a non-radioactive assay kit obtained from Cell Signaling Technology. CHO/DOR and CHO/DOR Akt DN were grown in 100 mm Petri dishes to

confluency. Cells were treated with either vehicle or SNC 80 (100 nM) for 10 min, washed with PBS and lysed in ice-cold cell lysis buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 μg·mL^{-1} leupeptin and 1 mM PMSF. Samples were centrifuged and supernatants were assayed for protein content. Aliquots containing equal amount of protein (0.7–0.8 mg) were added to agarose cross-linked to mouse monoclonal anti-Akt antibody and incubated overnight at 4°C with continuous rocking. The beads were then washed with cell lysis buffer and with kinase assay buffer containing 25 mM Tris (pH 7.5), 5 mM β-glycerophosphate, 2 mM dithiothreitol, 0.1 mM sodium orthovanadate and 10 mM $MgCl_2$. Thereafter, the beads were resuspended in kinase assay buffer supplemented with 0.2 mM ATP and 20 μg·mL^{-1} of glycogen kinase synthase (GSK)-3α/β crosstide (corresponding to residues surrounding Ser21/9 and expressed as glutathione S-transferase-fusion protein) and the samples were incubated for 30 min at 30°C. The reaction was stopped by the addition of sample buffer; the samples were heated at 100°C and analysed by Western blot using a rabbit polyclonal antibody against phospho-Ser21/9-GSK-3α/β. Three separate cell preparations were examined.

Statistical analysis

Results are reported as mean ± SEM. Kinetic data and concentration–response curves were analysed by nonlinear regression curve fitting using the program Graph Pad Prism (San Diego, CA, USA.). Antagonist inhibitory constant was calculated according to Cheng and Prusoff (1973). Statistical analysis was performed by either Student's unpaired *t*-test or one-way ANOVA followed by Newman–Keuls *post hoc* test as appropriate.

Materials

[^{3}H]-2-deoxy-D-glucose (8.0 Ci·mmol^{-1}) and [^{3}H]-3-OMG (80.2 Ci·mmol^{-1}) were obtained from PerkinElmer Life and Analytical Sciences. Cell culture supplies including Ham's F12 medium, FCS, penicillin-streptomycin and hygromycin were obtained from Invitrogen. DPDPE, naltrindole (NTI), naloxone, 3-OMG, dibutyryl-cyclic AMP (dB-cAMP), phorbol 12-myristate 13-acetate (PMA), mouse recombinant insulin-like growth factor (IGF-1), pertussis toxin (PTX), wortmannin, tyrphostin I-OMe-AG 538 (I-OMe-AG 538), phloretin, cytochalasin B, phosphatase inhibitor cocktail 1, okadaic acid, protease inhibitor cocktail and streptavidin-conjugated agarose were from Sigma-Life Science (St. Louis, MO, USA). 2-Deoxy-D-glucose, Go 6850 (also termed bisindolylmaleimide I or GF 109203X), Go 6983, PP2, PP3, Akt inhibitor VIII (also known as Akti), phosphatidylinositol-3 kinase (PI3-K)α inhibitor VIII (also known as PIK-75) PI3-Kγ inhibitor II (also known as AS 604850) and myristoylated PKCζ pseudosubstrate inhibitor (PKCζ-PSI) (myr-SIYRRGARRWRKL), tyrphostin AG 1024 and tyrphostin AG 1478 were from Calbiochem (La Jolla, CA, USA). SNC 80, LY294002, LY303511, PD 98059 and U0126 were from Tocris Cookson Ltd (Bristol, UK). Sp-cAMPS was from Biomol GmbH (Hamburg, Germany). The primary antibodies used were from the following sources: (i) rabbit polyclonal anti-GLUT1 (cat. no. 07–1401) from Millipore (Temecula, CA, USA); (ii) mouse monoclonal anti-GLUT3 (sc-74497), mouse monoclonal anti-Na+/K±ATPase α1 subunit (sc-21712), rabbit polyclonal anti-Akt1/2/3 (sc-8312) and anti-PKCζ (which recognizes also PKCλ) (sc-216) from Santa Cruz Biotechnology; (iii) rabbit polyclonal anti-phospho-Thr308-Akt (cat. no. 18–785-210011), rabbit polyclonal anti-PI3K p110α (cat no. 4249), PI3K p110β (cat. no. 3011), PI3K p110γ (cat. no. 4252), p44/42 MAP [kinase extracellular signal-regulated kinases 1 and 2 (ERK1/2); cat. no. 9102], phospho-Tyr416-Src (cat. no.2101), phospho-Thr410/403-PKCζ/λ (cat. no. 9378), rabbit monoclonal anti-Src (cat no. 2109) and anti-phospho-Thr308-Akt (cat. no. 2965) from Cell Signaling Technology (Beverly, MA, USA); (iv) rabbit polyclonal to dually phosphorylated ERK1/2 (cat. no. RA 15002) from Neuromics (Northfield, MN, USA); and (v) rabbit polyclonal anti-GLUT4 (G 4048) and actin (A 2066) from Sigma. The primary antibodies used detected either a single or, in the case of anti-GLUT4, a major immunoreactive band of the expected molecular weight.

Results

Activation of δ-opioid receptors stimulates glucose uptake

As shown in Figure 1A, basal 2-deoxy-D-glucose uptake in CHO/DOR cells increased linearly for at least 12 min of incubation, at a rate of 2.4 ± 0.2 nmol·min^{-1}·mg^{-1} protein. When cells were incubated in the presence of the δ-opioid receptor agonist SNC 80 (100 nM), there was a marked stimulation of 2-deoxy-D-glucose uptake and the rate increased to 5.3 ± 0.3 nmol·min^{-1}·mg^{-1} protein ($P < 0.001$). Cell treatment with either cytochalasin B (20 μM) or phloretin (200 μM), two GLUT inhibitors, reduced basal 2-deoxy-D-glucose uptake by approximately 88% (Figure 1B) and completely blocked the stimulating effect of SNC 80 (100 nM), as there was no significant difference between the amount of radioactivity remaining in the cells following treatment with the δ-opioid receptor agonist as compared with that measured with each inhibitor alone.

As glucose transport across the membranes may depend on hexokinase activity (Naftalin and Rist, 1989), it was important to investigate whether an enhanced uptake by δ-opioid receptor agonist could be observed with the non-metabolized sugar 3-OMG. As shown in Figure 1B, SNC 80 (100 nM) increased [^{3}H]-3-OMG by 130 ± 10% ($P < 0.001$), a magnitude similar to that obtained with [^{3}H]-2-deoxy-D-glucose. [^{3}H]-3-OMG uptake rates were: vehicle 0.49 ± 0.03, SNC 80 1.13 ± 0.05 nmol·min^{-1}·mg^{-1} protein ($P < 0.001$; $n = 5$). As observed with 2-deoxy-D-glucose, 3-OMG uptake was markedly inhibited by cytochalasin B and phloretin, either in both the absence and presence of SNC 80 (Figure 1B).

SNC 80 and DPDPE, another selective δ-opioid receptor agonist, stimulated 2-deoxy-D-glucose uptake in a concentration-dependent and saturable manner with EC_{50} values of 0.68 ± 0.04 nM and 0.23 ± 0.02 nM respectively (Figure 1C). Both agonists showed similar E_{max} values, which corresponded to 135 ± 8% and 140 ± 10% increase of control value ($P < 0.001$). The stimulating effects of SNC 80 (100 nM) and DPDPE (100 nM) were completely blocked by the non-

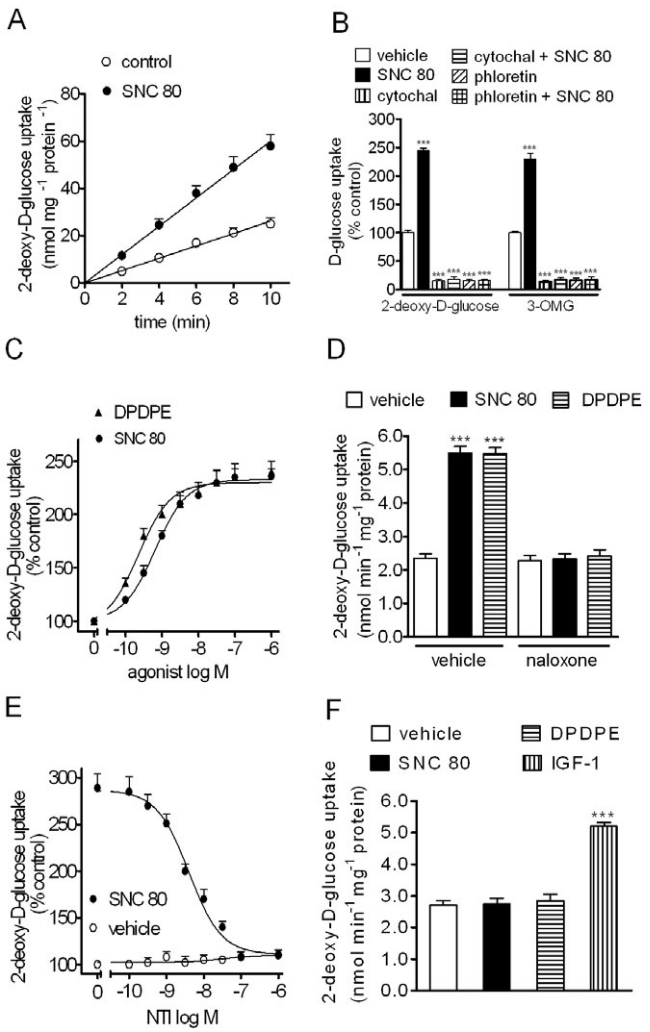

Figure 1

Activation of δ-opioid receptors stimulates glucose uptake in CHO/DOR cells. (A) Time course of basal and SNC 80-stimulated 2-deoxy-D-glucose uptake. Cells were deprived of serum for 12 h and then incubated with Krebs-HEPES buffer (pH 7.4) lacking D-glucose for 20 min at 37°C. Thereafter either vehicle or SNC 80 (100 nM) was added for 15 min followed by the addition of 1 mM [^{3}H]-2-deoxy-D-glucose. The incubation was stopped at the indicated time points. Lines were drawn by linear regression analysis. Values are mean ± SEM of three experiments. (B) SNC 80 stimulates the uptake of either 2-deoxy-D-glucose or 3-OMG and the responses are inhibited by either cytochalasin B (cytochal B) (20 μM) or phloretin (200 μM). GLUT inhibitors were added 5 min before SNC 80. [^{3}H]-2-deoxy-D-glucose and [^{3}H]-3-OMG were present at the concentration of 1 mM and the uptake was measured for a period of 8 and 2 min respectively. Values are mean ± SEM of three (2-deoxy-D-glucose) and five experiments (3-OMG). ***P < 0.001 versus control (vehicle + vehicle). (C) Concentration-dependent stimulation of glucose transport by δ-opioid receptor agonists. Cells were treated with the indicated concentrations of DPDPE and SNC 80 for 15 min before the addition of [^{3}H]-2-deoxy-D-glucose. Values are mean ± SEM of three experiments. (D) Naloxone completely blocked the stimulation of glucose uptake by SNC 80 and DPDPE. Cells were pre-incubated for 5 min with either vehicle or 100 μM naloxone and then treated for 15 min with either vehicle, 100 nM SNC 80 or 100 nM DPDPE. Values are mean ± SEM of three experiments. ***P < 0.001. E: Antagonism of SNC 80-stimulated glucose uptake by the δ-opioid receptor antagonist NTI. Cells were pre-incubated with the indicated concentrations of NTI for 5 min and then treated with either vehicle or SNC 80 (100 nM) for 15 min. Values are mean ± SEM of three experiments. (F) Lack of effects of SNC 80 and DPDPE in untransfected CHO/K1 cells. CHO/K1 cells deprived of serum were treated with either vehicle, SNC 80 (100 nM), DPDPE (100 nM) or IGF-1 (50 ng·mL^{-1}) for 15 min at 37°C and then [^{3}H]-2-deoxy-D-glucose uptake was determined following 8 min incubation. Values are mean ± SEM of three experiments. ***P < 0.001 versus vehicle. 3-OMG, 3-O-methyl-D-glucose; DPDPE, [D-Pen(2,5)]-enkephalin; IGF-1, insulin-like growth factor-1; NTI, naltrindole.

selective opioid receptor antagonist naloxone (100 μM), which *per se* failed to affect 2-deoxy-D-glucose uptake (Figure 1D). The selective δ-opioid receptor antagonist NTI inhibited the SNC 80 (100 nM) stimulating effect in a concentration-dependent manner with an estimated K_i of 16 ± 2 pM (Figure 1E). SNC 80 (100 nM) and DPDPE (100 nM) failed to affect 2-deoxy-D-glucose uptake in untransfected CHO-K1 cells, whereas treatment of the cells with the growth factor IGF-1 (50 ng·mL^{-1}), which acted on endogenously expressed IGF-1 receptors, caused a significant stimulation of hexose transport (93 ± 4% increase of control value, P < 0.001) (Figure 1F).

Effects of δ-opioid receptor activation on 2-deoxy-D-glucose transport kinetic parameters and GLUT1 expression in plasma membranes

Analysis of the kinetics of 2-deoxy-D-glucose uptake indicated that δ-opioid receptor activation increased the V_{max} for transport (vehicle, 10.2 ± 0.8; SNC 80, 24.0 ± 1.1 nmol·min^{-1}·mg^{-1} protein, P < 0.001) without significantly changing the K_m (vehicle, 2.8 ± 0.5 mM, SNC 80 2.6 ± 0.8 mM) (Figure 2A, B).

Western blot analysis of GLUT1, GLUT3 and GLUT4 expression in CHO/DOR cells indicated the presence of GLUT1 immunoreactivity and the absence of GLUT3 and GLUT4 proteins (Figure 2C). As expected, an immunoreactive band of ~55 kDa was detected by anti-GLUT3 and anti-GLUT4 antibodies in rat frontal cortex and rat soleus extracts respectively (Figure 2C).

To assess whether the enhanced hexose transport was associated with a change in the cellular distribution of the GLUT1 transporter, plasma membrane proteins were biotinylated and isolated from cytosolic proteins by streptavidin-agarose precipitation. As shown in Figure 2D, cell treatment with SNC 80 (100 nM) under conditions similar to those employed for hexose uptake failed to change the content of GLUT1 either in plasma membrane or in the cytosol fraction. No GLUT1 immunoreactivity was detected in samples incubated in the absence of biotinylating reagent (Figure 2D). Analysis of GLUT1 distribution in CHO/DOR subcellular fractions isolated by ultracentrifugation indicated that under basal conditions, the transporter expression was higher in plasma membrane than microsomal fraction and this cellular distribution was not significantly affected by SNC 80 treatment (Figure 2E).

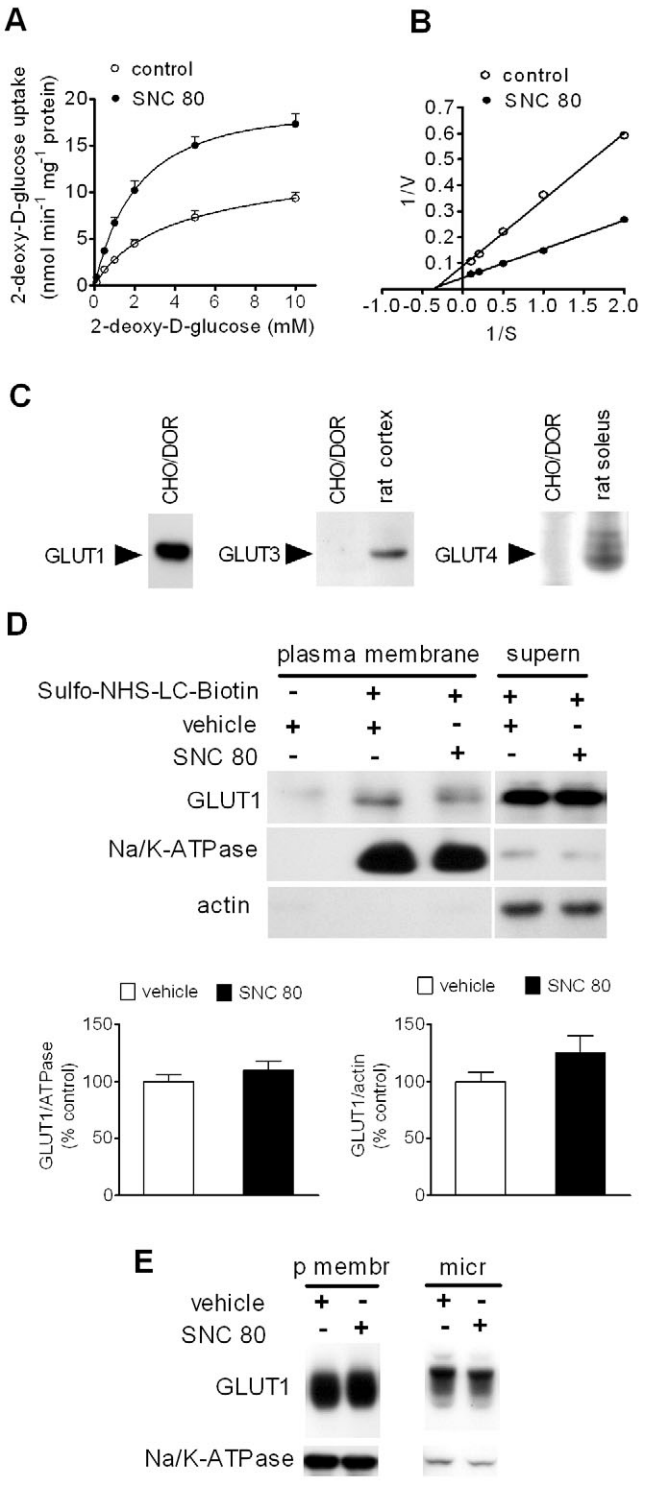

Figure 2

SNC 80 increases maximal glucose transport rate without changing membrane GLUT1 expression. (A) Cells were incubated with either vehicle (control) or 100 nM SNC 80 for 15 min. Thereafter, [^{3}H]-2-deoxy-D-glucose uptake was determined in the presence of the indicated concentrations of unlabelled hexose. Values are mean ± SEM of three experiments. (B) Lineweaver-Burk plot of the data reported in (A). (C) Western blot analysis of GLUT1, GLUT3 and GLUT4 expression in CHO/DOR whole-cell extracts. GLUT3 and GLUT4 immunoreactivities in rat frontal cortex and soleus, respectively, are also shown. Each lane was loaded with 5 μg of cell or tissue protein. Data are representative of three similar experiments. (D) Cells (deprived of serum) were treated as in glucose uptake experiments and incubated for 15 min with either vehicle or 100 nM SNC 80. Cells were washed and incubated for 30 min at 4°C without or with 0.25 mg·mL^{-1} sulpho-NHS-LC-biotin. Cell extracts were incubated overnight with streptavidin-conjugated agarose beads and the samples were then centrifuged to obtain a supernatant (supern) and a pellet fraction. Following repeated washing, the biotinylated proteins were separated by SDS-PAGE and immunoblotted with antibodies against GLUT1, α1 subunit of Na^+/K^+ ATPase (Na/K-ATPase) as plasma membrane marker protein, and actin as cytosolic marker protein. The density of GLUT1 immunoreactive bands was measured and normalized to the density of either the Na/K-ATPase or actin for plasma membrane and cytosolic samples respectively. Values are mean ± SEM of four experiments. (E) Western blot analysis of GLUT1 content in plasma membrane (p membr) and microsomal (micr) fractions of cells treated with either vehicle or SNC 80 (100 nM) for 15 min. Data are representative of three experiments. CHO, Chinese hamster ovary; CHO/DOR, CHO cells stably expressing the human δ-opioid receptor; SDS, sodium dodecyl sulphate; SDS-PAGE, SDS-polyacrylamide gel electrophoresis.

Effects of PTX, cAMP analogues, Src and ERK1/2 protein kinase inhibitors on δ-opioid receptor stimulation of glucose uptake

To investigate the molecular mechanisms mediating the δ-opioid receptor stimulation of 2-deoxy-D-glucose uptake, we first examined the involvement of the G proteins G_i/G_o, which have been shown to couple the receptors with multiple signal transduction pathways (Quock *et al.*, 1999). Cell treatment with PTX, which uncouples G_i/G_o from receptors, completely prevented the stimulation of glucose transport (Figure 3A).

As the coupling to adenylyl cyclase activity is a major signalling mechanism of δ-opioid receptors (Quock *et al.*, 1999) and cAMP has been shown to control glucose transport (Clancy and Czech, 1990; Kelada *et al.*, 1992), it was important to explore whether this pathway was involved in δ-opioid receptor regulation of GLUT1. Incubation of CHO/DOR cells with either dB-cAMP (2 mM) or Sp-cAMPS (0.5 mM), two cell permeant and stable cAMP analogues, caused a significant increase in 2-deoxy-D-glucose uptake (52 ± 2 and 35 ± 3%, respectively, $P < 0.05$), but failed to affect the stimulating effect of SNC 80 (Figure 3B). Moreover, δ-opioid receptor regulation of GLUT1 was not affected by blockade of protein kinase A with the selective inhibitor KT 5720 (5 μM) (Figure 3B).

Previous studies have demonstrated that Src tyrosine kinases play a critical role in conveying stimulating inputs from G protein-coupled receptors (GPCR) to ERK1/2 and PI3K (Wan *et al.*, 1996; Ma *et al.*, 2000; Luttrell and Luttrell, 2004). Both ERK1/2 and PI3K signalling pathways are known to be involved in the hormonal control of glucose transport (Shepherd *et al.*, 1998; Harmon *et al.*, 2004) and have been shown to be regulated by opioid receptors (Tegeder and Geisslinger, 2004). We found that treatment of CHO/DOR cells with the selective Src family tyrosine kinase inhibitor PP2 (10 μM) (Hanke *et al.*, 1996) reduced basal and δ-opioid receptor

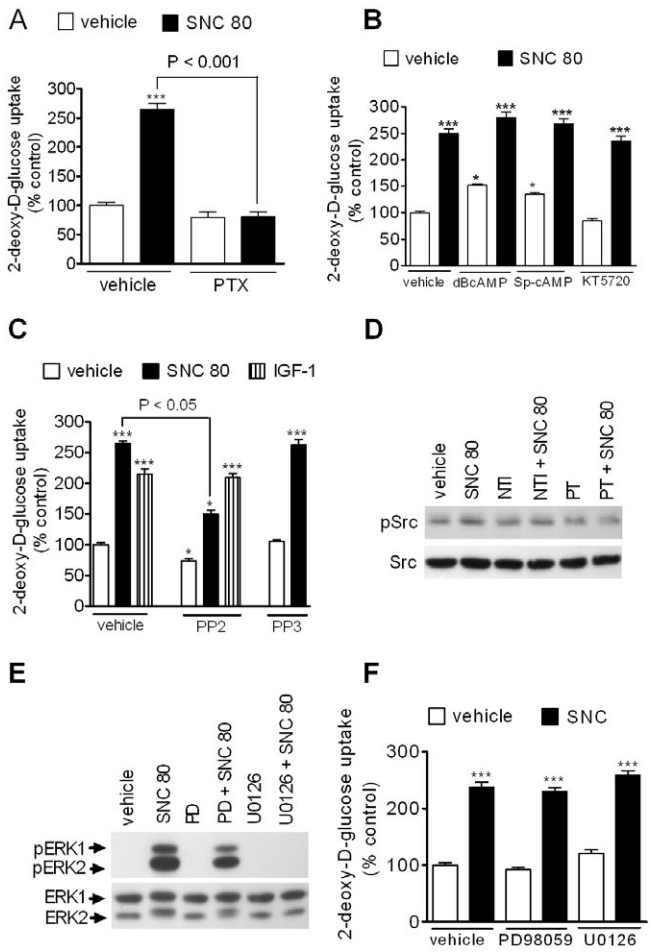

Figure 3

Sensitivity of δ-opioid receptor stimulation of glucose uptake to pertussis toxin (PTX) and agents affecting cAMP, Src and ERK1/2 signalling. (A) PTX prevents δ-opioid receptor stimulation of glucose uptake. Cells were incubated for 24 h with either vehicle or 250 ng·mL^{-1} PTX, washed and treated with either vehicle or 100 nM SNC 80 for 15 min. Values are mean ± SEM of three experiments. ***P < 0.001 versus control (vehicle + vehicle). (B) Effects of dB-cAMP, Sp-cAMP and KT 5720. Cells were pre-incubated with either vehicle, dB-cAMP (2 mM), Sp-cAMP (0.5 mM) or KT 5720 (5 μM) for 20 min before the addition of either vehicle or SNC 80 (100 nM). Values are mean ± SEM of three experiments. *P < 0.05, ***P < 0.001 versus control. (C) The Src family tyrosine kinase inhibitor PP2 reduces δ-opioid receptor stimulation of glucose uptake. Cells were pre-incubated with either vehicle, 10 μM PP2 or 10 μM PP3 for 1 h before the addition of either vehicle, SNC 80 (100 nM) or IGF-1 (50 ng·mL^{-1}). Values are mean ± SEM of five experiments. *P < 0.05, ***P < 0.001 versus control. (D) Stimulation of δ-opioid receptors induces Src phosphorylation on Tyr416. Cells were pre-incubated with vehicle or PTX (250 ng·mL^{-1}) for 24 h and vehicle or NTI (100 nM) for 5 min and then treated with vehicle or SNC 80 (100 nM) for 10 min. Cell extracts were then analysed for phospho-Src (pSrc) and total Src (Src) by Western blot. Data are representative of three similar experiments. (E) Inhibiton of δ-opioid receptor stimulation of ERK1/2 phosphorylation by MEK inhibitors. Cells were pre-incubated with either vehicle, PD 98059 (10 μM) for 1 h or U0126 (10 μM) for 30 min and then treated with either vehicle or SNC 80 (100 nM) for 10 min. Cell extracts were analysed for phospho-ERK1/2 (pERK) and total ERK1/2 by Western blot. Data are representative of three similar experiments. (F) MEK inhibitors fail to affect δ-opioid receptor stimulation of glucose transport. Cells were pre-incubated as described in (E) and then treated with either vehicle or SNC 80 (100 nM). Values are mean ± SEM of three experiments. ***P < 0.001 versus control. dB-cAMP, dibutyryl-cAMP; ERK1/2, extracellular signal-regulated protein kinases 1 and 2; IGF-1, insulin-like growth factor-1; MEK, mitogen-activated protein kinase kinases; NTI, naltrindole.

stimulation of 2-deoxy-D-glucose uptake by 26 ± 3 (P < 0.05) and 53 ± 5% respectively (P < 0.05) (Figure 3C). Conversely, PP2 (10 μM) did not affect the IGF-1 (50 ng·mL^{-1}) stimulant effect. Moreover, PP3 (10 μM), an analogue of PP2 that does not inhibit Src kinase, failed to affect either basal or δ-opioid receptor stimulation of 2-deoxy-D-glucose uptake. To assess whether activation of human δ-opioid receptors regulated Src, the effect of SNC 80 on Src autophosphorylation at Tyr416, an event associated with the kinase activation (Bjorge *et al.*, 2000), was examined. As shown in Figure 3D, SNC 80 (100 nM) enhanced the level of phospho-Tyr416-Src (89 ± 8% increase of basal value, P < 0.001), and this effect was completely blocked by either NTI or cell pretreatment with PTX, indicating that Src may act as downstream effector of human δ-opioid receptors.

We next examined the involvement of the ERK1/2 pathway in the δ-opioid receptor regulation of glucose transport. As shown in Figure 3E, SNC 80 (100 nM) induced ERK 1/2 phosphorylation and this effect was either inhibited by 50 ± 6% (P < 0.05) or was completely blocked by pretreatment with PD 98059 (10 μM) or U0126 (10 μM), respectively, two agents that interrupt the ERK1/2 pathway by inhibiting the upstream mitogen-activated protein kinase kinases (MEK). However, the MEK inhibitors failed to significantly affect SNC 80-induced increase of hexose transport (Figure 3F).

Involvement of PI3K/Akt pathway in δ-opioid receptor stimulation of glucose uptake

Among the different isoforms of PI3K, class I PI3Ks are known to be acutely regulated by extracellular stimuli and comprise class IA PI3Kα, PI3Kβ and PI3Kδ, which are characterized by having a Src-homology 2 domain-containing regulatory subunit p85 that binds phosphorylated tyrosine residues of intracellular proteins, and class IB PI3Kγ, which is instead regulated by G protein βγ subunits (Hennessy *et al.*, 2005). PI3K-catalysed formation of 3'-phosphoinositides recruit the protein kinase Akt to the membranes and allows its activation through dual phosphorylation on Thr308 and Ser473 by phosphoinositide-dependent protein kinase (PDK) 1 and 2 respectively.

In CHO/DOR cells, SNC 80 and DPDPE stimulated Akt phosphorylation on Thr308 and this effect was inhibited by pretreatment with PP2 (Figure 4A). To explore the involvement of PI3K in δ-opioid receptor stimulation of glucose uptake, we examined the effect of two well-characterized inhibitors of PI3K, wortmannin and LY 294002 (Hennessy *et al.*, 2005). Both compounds caused a concentration-dependent inhibition of SNC 80-stimulated hexose transport,

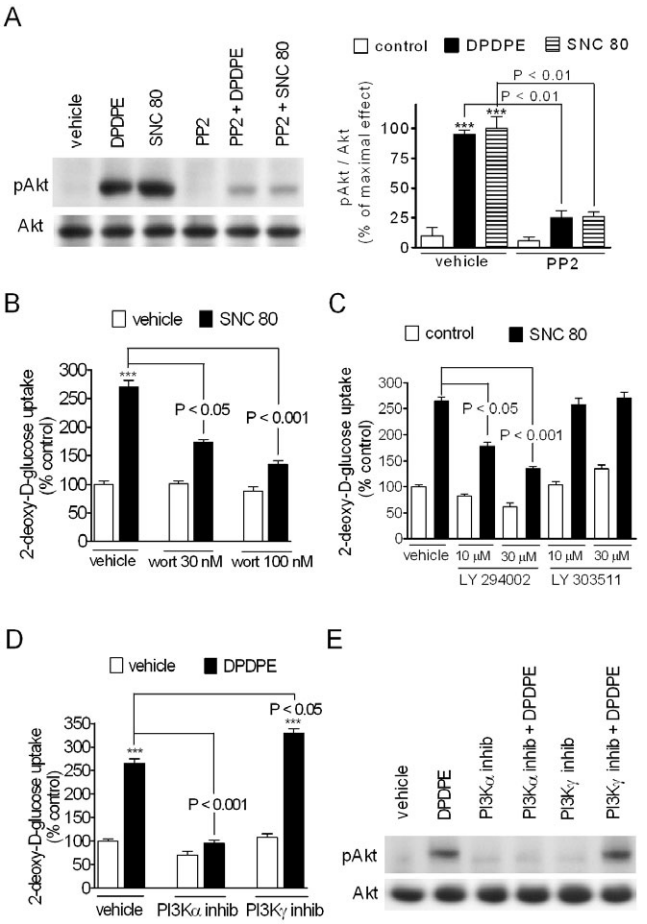

Figure 4

The coupling of δ-opioid receptor to PI3K is required for stimulation of glucose uptake. (A) Stimulation of Akt phosphorylation on Thr308 and inhibition by PP2. Cells were pre-incubated with either vehicle or PP2 (10 μM) for 1 h and then treated with either vehicle, SNC 80 (100 nM) or DPDPE (100 nM) for 10 min. Cell extracts were analysed for phospho-Akt (pAkt) and total Akt by Western blot. Densitometric values of pAkt/Akt ratios are reported as % of the maximal effect and are mean ± SEM of three experiments. ***$P < 0.001$ versus control. (B) Inhibition of δ-opioid receptor stimulation of glucose transport by wortmannin. Cells were pre-incubated with either vehicle or the indicated concentrations of wortmannin (wort) for 2 h and then treated with either vehicle or SNC 80 (100 nM). Values are mean ± SEM of four experiments. ***$P < 0.001$ versus control. (C) LY294002, but not LY303511, inhibits δ-opioid receptor stimulation of glucose transport. Cells were pre-incubated with either vehicle or the indicated concentrations of the two compounds for 1 h and then treated with either vehicle or SNC 80 (100 nM). Values are mean ± SEM of four experiments. (D) Differential effect of PI3Kα and PI3Kγ inhibitors on δ-opioid receptor stimulation of glucose transport. Cells were pre-incubated with either vehicle, PI3Kα inhibitor (300 nM) or PI3Kγ inhibitor (300 nM) for 1 h and then treated with either vehicle or DPDPE (100 nM). Values are mean ± SEM of four experiments. ***$P < 0.001$ versus control. (E) PI3Kα inhibitor but not PI3Kγ inhibitor blocks DPDPE stimulation of AKt phosphorylation on Thr308. Cells were pre-incubated with PI3Kα inhibitor (500 nM) or PI3Kγ inhibitor (500 nM) for 1 h and then treated with either vehicle or DPDPE (100 nM) for 10 min. Data are representative of three experiments. DPDPE, [D-Pen(2,5)]-enkephalin; PI3K, phosphatidylinositol 3-kinase.

whereas LY 303511, an inactive analogue of LY 294002, was without effect (Figure 4 B and C).

Because cells contain different PI3Ks, it was important to know which isoform was regulated by δ-opioid receptor and involved in the stimulation of glucose transport. Western blot analysis indicated that CHO-K1 cells expressed PI3Kα and, at a lower level, PI3Kγ, but no PI3Kβ immunoreactivity (results not shown). To investigate the role of PI3Kα and PI3Kγ, isoform-selective inhibitors were employed. Cell treatment with the PI3Kα inhibitor VIII (300 nM) (Knight *et al.*, 2006) markedly reduced DPDPE-stimulated 2-deoxy-D-glucose uptake, whereas the PI3Kγ inhibitor II (300 nM) (Camps *et al.*, 2005) caused a small but significant enhancement of the agonist effect (Figure 4D). In line with this finding, the PI3Kα inhibitor VIII (500 nM) completely prevented DPDPE-stimulated Akt phosphorylation, whereas PI3Kγ inhibitor II (500 nM) was without effect (Figure 4E).

We next examined the role of Akt in δ-opioid receptor stimulation of 2-deoxy-D-glucose uptake by using CHO/DOR Akt-DN cells. Functional assays showed that in CHO/DOR Akt-DN cells, SNC 80 (100 nM) stimulated Akt activity less efficiently than in untransfected (wt) cells (Figure 5A), indicating that overexpression of the Akt mutant indeed exerted a dominant negative effect. In CHO/DOR Akt-DN cells, the maximal stimulation of 2-deoxy-D-glucose uptake by SNC 80 was reduced by 45 ± 5% ($P < 0.01$) as compared with the response observed in untransfected cells, with no significant changes in the agonist EC_{50} values (CHO/DOR wt 0.60 ± 0.07 nM, CHO/DOR Akt-DN 0.75 ± 0.09 nM) (Figure 5B). The reduction of SNC 80-stimulated hexose transport observed in CHO/DOR Akt-DN cells was not associated with a reduction in the level of whole-cell expression of GLUT1 protein (Figure 5C).

To further examine the involvement of Akt, CHO/DOR cells were treated with the Akt inhibitor VIII, which suppresses the activity of Akt1, Akt2 and Akt3 (Green *et al.*, 2008). As shown in Figure 5D, cell treatment with this Akt inhibitor (500 nM) reduced the SNC 80 (100 nM) stimulation of 2-deoxy-D-glucose uptake by 51 ± 3% ($P < 0.01$).

Effects of receptor tyrosine kinase inhibitors on δ-opioid receptor stimulation of glucose uptake

As PI3Kα, but not G protein-regulated PI3Kγ, appeared to be regulated by δ-opioid receptors in CHO-K1 cells, it was important to understand how the receptor could trigger the activation of this PI3K isoform. Previous studies have shown that in different cell types various GPCR can induce Src-dependent transactivation of receptor tyrosine kinases (Luttrell and Luttrell, 2004; Gavi *et al.*, 2006), which then may provide the phospho-tyrosine docking sites for the recruitment and activation of class IA PI3Ks. We investigated the involvement of this mechanism by examining the effect of tyrphostin AG 1024 and tyrphostin I-OMe-AG 538, two structurally different inhibitors of IGF-1R tyrosine kinase activity (Parrizas *et al.*, 1997; Blum *et al.*, 2000). As shown in Figure 6A and B, cell treatment with either tyrphostin AG 1024 (0.3 μM) or tyrphostin I-OMe-AG 538 (30 μM) completely blocked the stimulation of glucose uptake induced by IGF-1 and SNC-80. Moreover, tyrphostin AG 1024 (0.3 μM) and tyrphostin

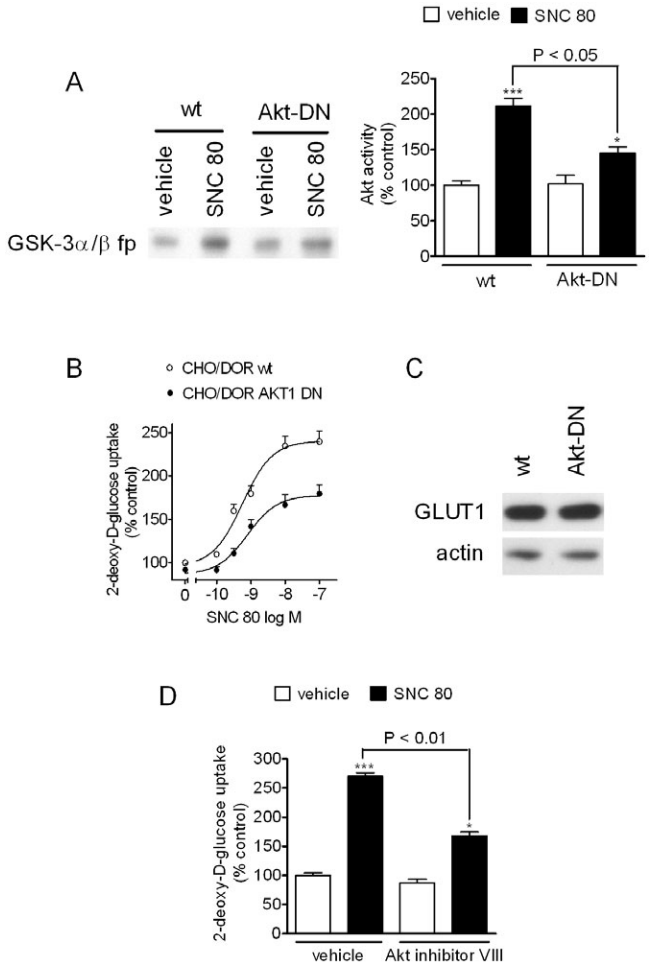

Figure 5

Expression of DN Akt1 and chemical inhibition of Akt curtail δ-opioid receptor stimulation of glucose uptake. (A) Inhibition of δ-opioid receptor stimulation of Akt activity in cells transfected with DN Akt. Untransfected (wt) and CHO/DOR Akt DN cells (Akt-DN) were incubated with either vehicle or SNC 80 (100 nM) and Akt activity was determined in immunoprecipitates of cell extracts as described in Methods. Values are mean ± SEM of three experiments. ***$P < 0.001$ versus control. (B) Reduced stimulation of glucose transport in CHO/DOR Akt DN cells. [^{3}H]-2-deoxy-D-glucose uptake was determined in untransfected and CHO/DOR Akt DN cells in the presence of the indicated concentrations of SNC 80. Values are mean ± SEM of four experiments. (C) Expression of DN Akt1 fails to alter whole-cell GLUT1 levels. Cell extracts of untransfected (wt) and CHO/DOR Akt DN cells were analysed for GLUT1 content by Western blot. Data are representative of three experiments. (D) Chemical inhibition of Akt attenuates δ-opioid receptor stimulation of glucose uptake. Cells were pre-incubated with either vehicle or Akt inhibitor VIII (500 nM) for 90 min and then treated with either vehicle or SNC 80 (100 nM). Values are mean ± SEM of five experiments. CHO, Chinese hamster ovary; CHO/DOR, CHO cells stably expressing the human δ-opioid receptor; CHO/DOR Akt DN, CHO/DOR cells stably expressing dominant-negative kinase-deficient Akt1 mutant.

I-OMe-AG 538 (30 μM) completely suppressed the induction of Akt phosphorylation elicited by SNC 80 (Figure 6C and D). Conversely, tyrphostin AG 1478 (1 μM), which selectively inhibits epidermal growth factor receptor (EGFR) tyrosine kinase (Levitzki and Gazit, 1995), failed to affect the δ-opioid stimulation of glucose uptake (Figure 6E).

Effects of PKC inhibitors on δ-opioid receptor stimulation of glucose uptake

In different cell types, it has been shown that activation of PKC promotes glucose transport, and selective inhibitors have been employed to assess the relative contribution of the various PKC family members, and in particular PKCζ, to this cellular process (Bandyopadhyay *et al.*, 1997; Fryer *et al.*, 2000; Yang *et al.*, 2009). Acute treatment of CHO/DOR cells with PMA (100 nM), a potent stimulator of conventional (α, β1, β 2, and γ) and novel (δ, ε, η, θ and μ) PKC isoforms, induced a marked increase in glucose uptake (265 ± 5% increase of basal rate, $P < 0.001$) (Figure 7A). Pretreatment with either Go 6850 (100 nM), which preferentially inhibits α and β1 PKC isozymes, or Go 6983 (1 μM), which inhibits several conventional and novel PKC isoforms, inhibited PMA-induced glucose uptake by 25 ± 5% ($P < 0.05$) and 55 ± 3% ($P < 0.001$) respectively. Under similar experimental conditions, both PKC inhibitors failed to affect the stimulation response to SNC 80 (Figure 7A).

The atypical PKCζ isoform is activated downstream of PI3K through PDK1-dependent phosphorylation on Thr410 located in the activation loop (Chou *et al.*, 1998). Several studies indicate that PKCζ plays a critical role in regulating glucose transport and participates in insulin signalling in different cell types (Liu *et al.*, 2006). Recently, PKCζ has also been shown to be involved in the μ-opioid receptor-induced stimulation of glucose uptake in myoblast C_2C_{12} cells (Yang *et al.*, 2009). To investigate whether δ-opioid receptors acutely regulate PKCζ/λ, we examined whether SNC 80 and DPDPE could induce PKCζ/λ phosphorylation on Thr410/403. As shown in Figure 7B, the two δ-opioid receptor agonists increased the phosphorylation state of PKCζ/λ by 50 ± 6 and 48 ± 4% ($P < 0.05$, $n = 3$) respectively. The SNC 80 stimulating effect was prevented by cell treatment with either AG 1024 (300 nM) (Figure 7C), wortmannin (100 nM), or PP2 (10 μM) (Figure 7D). To assess whether PKCζ/λ contributed to δ-opioid stimulation of glucose uptake, we used the selective inhibitor PKCζ-PSI (Bandyopadhyay *et al.*, 1997). The addition of PKCζ-PSI (3 μM) reduced the δ-opioid stimulation by 22 ± 3% ($P < 0.05$) (Figure 7E). When PKCζ-PSI (3 μM) was combined with the Akt inhibitor VIII (500 nM), an additive effect was observed, reaching an overall 70 ± 5% inhibition of the δ-opioid response (Figure 7E).

Discussion

In the present study, we show that activation of human δ-opioid receptor stably expressed in CHO cells acutely stimulated glucose uptake. This effect was elicited by both SNC 80 – a non-peptide agonist – and DPDPE with potencies consistent with their receptor affinities, and was completely blocked by either naloxone or NTI and was absent in untransfected

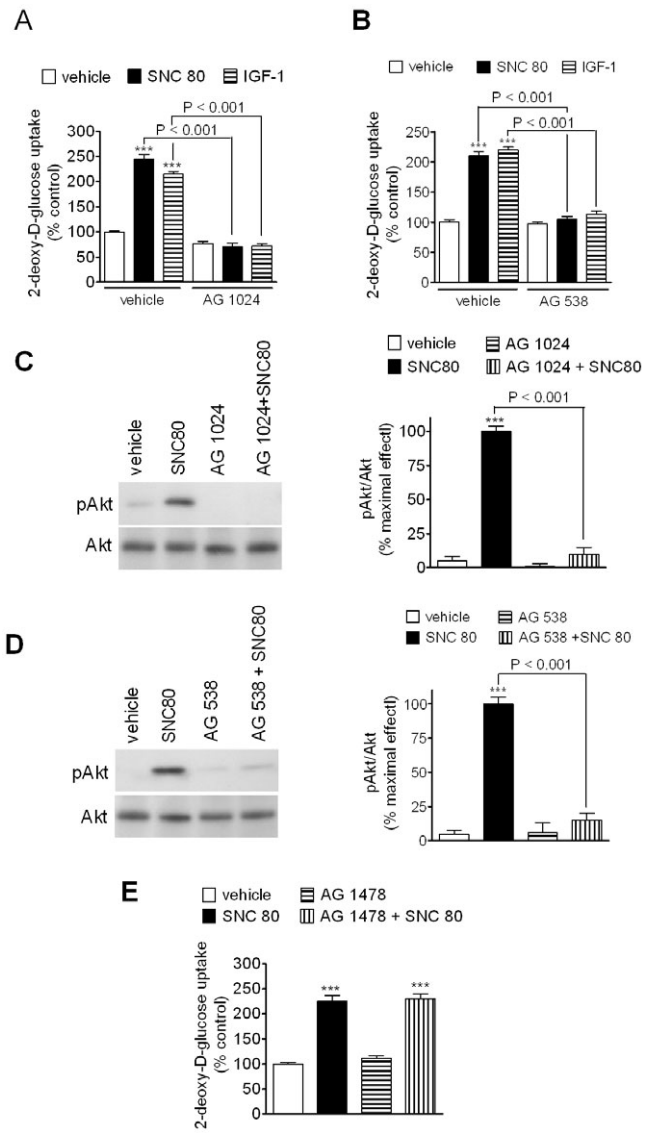

Figure 6

Effects of IGF-1R and EGFR tyrosine kinase inhibitors on δ-opioid receptor stimulation of glucose uptake and Akt phosphorylation on Thr308. (A) Inhibitory effects of tyrphostin AG 1024 (AG 1024) on glucose uptake. Cells were pre-incubated with either vehicle or 300 nM AG 1024 for 30 min and then treated with either vehicle, SNC 80 (100 nM) or IGF-1 (50 ng·mL^{-1}) for 15 min. Values are mean ± SEM of four experiments. ***P < 0.001 versus control. (B) Inhibitory effects of tyrphostin I-OMe-AG 538 (AG 538) on glucose uptake. Cells were pre-incubated with either vehicle or 30 μM AG 538 for 16 h and then treated with either vehicle, SNC 80 (100 nM) or IGF-1 (50 ng·mL^{-1}) for 15 min. Values are mean ± SEM of four experiments. ***P < 0.001 versus control. (C) AG 1024 blocks δ-opioid receptor stimulation of Akt phosphorylation. Cells were pre-incubated with either vehicle or AG 1024 (300 nM) for 30 min and then treated with either vehicle or SNC 80 (100 nM) for 10 min. Densitometric values of pAkt/Akt ratios are reported as % of maximal effect and are mean ± SEM of four experiments. ***P < 0.001 versus control. (D) Blockade of δ-opioid receptor stimulation of Akt phosphorylation by AG 538. Cells were pre-incubated with either vehicle or 30 μM AG 538 for 16 h and then treated with either vehicle, SNC 80 (100 nM) or IGF-1 (50 ng·mL^{-1}) for 10 min. Values are mean ± SEM of four experiments. ***P < 0.001 versus control. (E) Lack of effect on δ-opioid receptor stimulation of glucose uptake by tyrphostin AG 1478. Cells were pre-incubated with either vehicle or 1 μM AG 1478 for 1 h and then treated with either vehicle or SNC 80 (100 nM). Values are mean ± SEM of three experiments. ***P < 0.001 versus control. ; EGFR, epidermal growth factor receptor; IGF-1, insulin-like growth factor-1; IGF-1R, IGF-1 receptor.

CHO-K1 cells, demonstrating its dependence on δ-opioid receptor activity. The complete blockade of the response by cytochalasin B and phloretin, two inhibitors of glucose transport by GLUT family members (Uldry and Thorens, 2004), indicates that δ-opioid receptors increased glucose uptake through GLUT proteins rather than sodium/glucose cotransporters or non-specific alteration of membrane permeability.

GLUT-mediated glucose transport across plasma membrane is gradient-dependent and hexokinase activity can increase the rate of glucose uptake by transforming the permeant sugar into an impermeant hexose phosphate (Naftalin and Rist, 1989). As hexokinase can be affected by different signalling molecules regulated by δ-opioid receptors (Robey *et al.*, 1999; Rathmell *et al.*, 2003), it was critical to assess whether the δ-opioid stimulation was dependent on sugar metabolism. We found that SNC 80 increased the uptake of 3-OMG, which is not metabolized by hexokinase, to the same extent as that of 2-deoxy-D-glucose, indicating that the effect was not dependent on enhanced hexokinase activity.

Kinetic analysis indicated that δ-opioid receptor activation induced an increase in the maximal rate of glucose transport without affecting the apparent affinity for the substrate. These changes may suggest that δ-opioid receptor stimulated the uptake by enhancing the number of transport molecules in the plasma membrane. It is well known that in skeletal muscle and adipose tissue, insulin stimulates glucose transport primarily by promoting GLUT4 redistribution from cytoplasmic stores to plasma membrane (Saltiel and Khan, 2001). In CHO cells overexpressing the human insulin receptor, insulin stimulation of glucose uptake was found to be accompanied by an increase in cell surface GLUT1 levels (Hara *et al.*, 1994). To study the effects of δ-opioid receptor stimulation on cellular GLUT dynamics, we initially investigated the nature of GLUT molecular forms present in CHO/DOR cells. Early functional studies reported the presence of only GLUT1 in CHO-K1 cells (Harrison *et al.*, 1991), whereas a recent study using reverse transcription-polymerase chain reaction and primers for the human cDNA sequence also reported the presence of GLUT3 messenger RNA, although at a level lower than GLUT1 messenger RNA (John *et al.*, 2008). In CHO/DOR cells, we detected strong GLUT1, but no GLUT3 and GLUT4, immunoreactivity. These data are consistent with previous studies reporting the absence of endogenous GLUT3 and GLUT4 proteins in CHO/K1 cells (Asano *et al.*, 1992; Piper *et al.*, 1992; Shibasaki *et al.*, 1992).

By using either surface protein biotinylation or subcellular membrane fractionation, we found that δ-opioid receptor stimulation of glucose uptake occurred in the absence of significant changes in GLUT1 plasma membrane expression. A possible explanation of this finding is that the methods employed failed to detect subtle but functionally significant

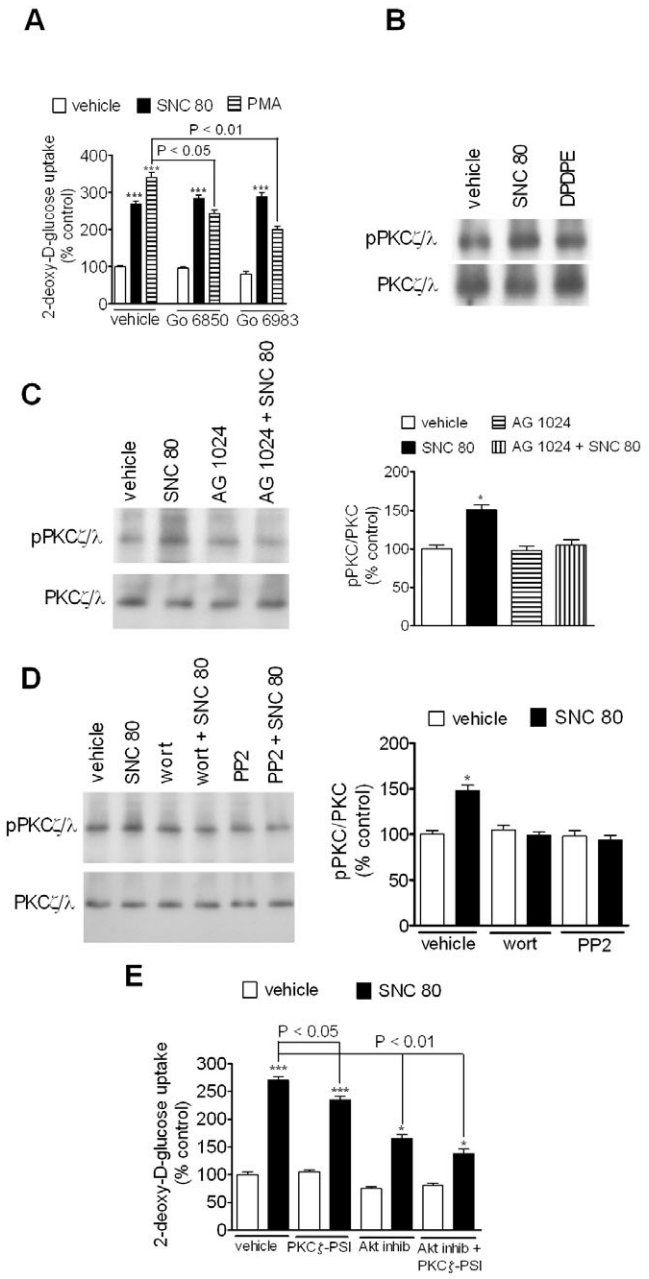

Figure 7

Involvement of PKC isoforms in δ-opioid receptor stimulation of glucose uptake. (A) Effects of Go 6850 and Go 6983 on SNC 80 and PMA stimulations of glucose transport. Cells were pre-incubated with either vehicle, Go 6850 (100 nM) or Go 6983 (1 μM) for 1 h and then treated with either vehicle, SNC 80 (100 nM) or PMA (100 nM) for 15 min. Values are mean ± SEM of four experiments. ***$P < 0.001$ versus control. (B) Stimulation of PKCζ/λ phosphorylation by δ-opioid receptor agonists. Cells were treated with either vehicle, SNC 80 (100 nM) or DPDPE (100 nM) for 15 min and cell extracts were analysed for phospho-Thr410/403-PKCζ/λ and total PKCζ/λ levels by Western blot. Data are representative of three experiments. (C) AG 1024 prevents SNC 80 stimulation of PKCζ/λ phosphorylation. Cells were treated with either vehicle or AG 1024 (300 nM) for 30 min and then exposed to either vehicle or 100 nM SNC 80 for 10 min. Densitometric values are expressed as percent of control and are mean ± SEM of four experiments. *$P < 0.05$ versus control. (D) SNC 80 stimulation of PKCζ/λ phosphorylation is blocked by wortmannin and PP2. Cells were treated with either vehicle, 100 nM wortmannin (wort) or 10 μM PP2 for 1 h and then exposed to either vehicle or 100 nM SNC 80 for 10 min. Densitometric values are mean ± SEM of five experiments. *$P < 0.05$ versus control. (E) Effects of the selective PKCζ/λ inhibitor PKCζ-PSI on δ-opioid receptor stimulation of glucose uptake. Cells were pre-incubated with either vehicle, PKCζ-PSI (3 μM), Akt inhibitor VIII (Akt inhib) (500 nM) or the combination of the two inhibitors for 1 h and then treated with either vehicle or SNC 80 (100 nM). Values are mean ± SEM of four experiments. ***$P < 0.001$ versus control. DPDPE, [D-Pen(2,5)]-enkephalin; PKC, protein kinase C; PKCζ-PSI, myristoylated PKCζ pseudosubstrate inhibitor; PMA, phorbol 12-myristate 13-acetate.

changes in glucose transporter trafficking to the cell surface. By using the same methods, however, other studies found alterations in cellular GLUT1 distribution following hormonal stimulation (Shetty *et al.*, 1993; Samih *et al.*, 2000). Alternatively, δ-opioid receptors might have stimulated glucose transport by increasing the catalytic activity of GLUT1 already present in the plasma membranes. This type of regulation has been proposed for other stimuli, such as inhibition of oxidative phosphorylation and osmotic stress, which have also been found to increase glucose transport without affecting membrane GLUT1 levels (Shetty *et al.*, 1993; Koseoglu and Ismail Beigi, 1999; Barros *et al.*, 2001). However, the precise mechanisms affecting GLUT1 intrinsic catalytic activity have not yet been elucidated and remain to be defined also for the regulation by δ-opioid receptors.

Investigation of the molecular pathways mediating the stimulation of glucose transport by δ-opioid receptors suggests the occurrence of a signalling cascade transduced by PTX-sensitive G proteins G_i/G_o, Src, IGF-1R, PI3Kα, Akt and PKCζ/λ (Figure 8). cAMP- and ERK1/2-dependent pathways, although known to be regulated by δ-opioid receptor (Quock *et al.*, 1999; Tegeder and Geisslinger, 2004) and to participate in the control of GLUT1 activity (Clancy and Czech, 1990; Kelada *et al.*, 1992; Harmon *et al.*, 2004), did not appear to contribute to the development of the stimulation response. Thus, the regulation of GLUT1 involved the engagement of particular signalling components among the multiple transduction molecules that can be regulated by δ-opioid receptors in CHO cells.

The activity of the Src family of tyrosine kinases appeared to play a major role in δ-opioid receptor regulation of glucose transport. Stimulation of δ-opioid receptors induced Src activation, as indicated by increased Src autophosphorylation, and the selective Src inhibitor PP2, but not the inactive analogue PP3, attenuated the enhancement of glucose uptake. Moreover, PP2 suppressed δ-opioid receptor-induced Akt phosphorylation, indicating that Src mediated the coupling of δ-opioid receptor to the PI3K/Akt signalling system. PP2 failed to affect IGF-1 stimulation of glucose uptake, suggesting that this inhibitor had no effect on PI3K/Akt and other pathways downstream of IGF-1R activation. Previous studies have shown that GPCR can directly activate Src through different mechanisms, including Src recruitment by β-arrestin bound to receptors, stimu-

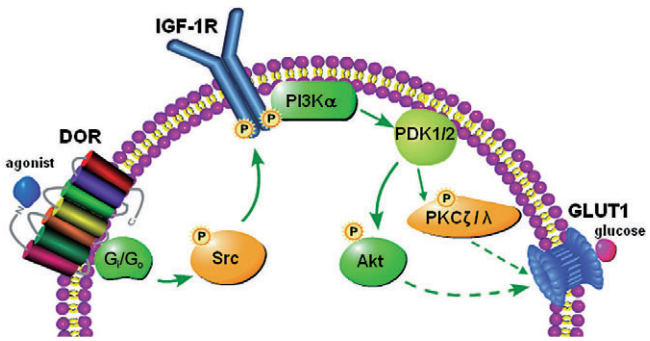

Figure 8

Proposed intracellular mechanisms mediating δ-opioid receptor stimulation of glucose uptake in CHO/DOR cells. Through activation of PTX-sensitive G_i/G_o proteins δ-opioid receptors stimulate Src tyrosine kinases, which in turn activate IGF-1R tyrosine kinase activity. This event recruits PI3Kα to plasma membrane with consequent PDK1/2-mediated phosphorylation and activation of Akt and PKCζ/λ. These protein kinases may regulate GLUT1 intrinsic activity through yet unidentified mechanisms, as indicated by the broken lines. The minor contribution of PKCζ/λ as compared with Akt is indicated by the thinner line. CHO, Chinese hamster ovary; CHO/DOR, CHO cells stably expressing the human δ-opioid receptor; IGF-1, insulin-like growth factor-1; IGF-1R, IGF-1 receptor; PDK, phosphoinositide-dependent protein kinase ; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; PTX, pertussis toxin.

lation by the α subunits of Gi and Gs proteins, and interaction with intracellular GPCR domains (Luttrell and Luttrell, 2004; Gavi *et al.*, 2006). These data support the idea that Src activation was a proximal event in the signalling cascade linking δ-opioid receptors to glucose uptake regulation.

The results obtained with tyrphostin AG 1024 and tyrphostin I-OMe-AG 538 indicated that IGF-1R tyrosine kinase activity was absolutely required for δ-opioid receptors stimulation of glucose transport. Moreover, both inhibitors completely blocked SNC 80-induced Akt phosphorylation, indicating that IGF-1R activity was required for opioid stimulation of PI3K/Akt. Previous studies have shown that Src can induce tyrosine phosphorylation and activation of IGF-1R, and that the receptor sites of Src-induced phosphorylation are the same as the ligand-induced autophosphorylation sites (Peterson *et al.*, 1996). Thus, it is possible that δ-opioid receptor regulation of glucose transport involved the Src-dependent transactivation of IGF-1R. This possibility may also explain the unexpected finding that both stimulations of Akt phosphorylation and glucose transport required the activity of PI3Kα, which is activated through the binding of the regulatory subunit to phospho-tyrosine sites, rather than that of PI3Kγ, which is stimulated by G protein βγ subunits and more likely to be subjected to regulation by δ-opioid receptors. An upstream role of Src in transactivation of receptor tyrosine kinase has been reported for several GPCR (Luttrell and Luttrell, 2004; Gavi *et al.*, 2006). Many GPCR, including δ-opioid receptors, have been shown to signal through EGFR transactivation (Gschwind *et al.*, 2001; Forster *et al.*, 2007). However, in CHO/DOR cells, δ-opioid receptor agonists stimulated glucose transport through a molecular pathway independent of EGFR tyrosine kinase activity, as tyrphostin AG 1478 was completely inactive.

Downstream of PI3K, both Akt and PKCζ/λ contributed to δ-opioid receptor stimulation of glucose transport, although to a different extent. In fact, inhibition of Akt activity by either overexpression of a dominant-negative form of Akt1 or the exposure to Akt inhibitor VIII was associated with a robust decrease in the stimulation response to δ-opioid agonists. This indicates that activation of Akt constituted a major mechanism for glucose transport regulation. Stimulation of δ-opioid receptors elicited a significant increase in the levels of phospho-Thr410/403-PKCζ/λ, which was prevented by inhibition of Src, IGF-1R or PI3K, indicating that this response was triggered by the same signalling pathway regulating Akt. However, δ-opioid stimulation of PKCζ/λ phosphorylation was consistently weak, indicating that this PDK-1-dependent reaction was not efficiently transduced. Accordingly, the PKCζ/λ inhibitor PKCζ-PSI, used at a concentration effective in completely inhibiting insulin-stimulated glucose transport in L5 myotubes (Bandyopadhyay *et al.*, 1997), caused only a modest decrease of the opioid stimulating effect, suggesting a minor contribution by the atypical PKC isoforms. Nonetheless, the present data are consistent with the study by Yang *et al.* (2009), who found that PKCζ-PSI partially reduced μ-opioid receptor stimulation of glucose uptake in C_2C_{12} myoblast cells. However, in the study by Yang *et al.* (2009) and Liu *et al.* (2004), μ-opioid receptor stimulation of glucose uptake was also found to be inhibited by GF 109203X (Go 6850 in the present study), whereas in CHO/DOR cells we found that the PKC inhibitors Go 6850 and Go 6983 failed to affect the δ-opioid response. Taken together, these data suggest that the various PKC isoforms may differentially contribute to opioid regulation of glucose transport as a function of the opioid receptor subtype, rather than the cell type involved. Further studies are required to more specifically address this issue, and to understand how Akt and PKC signals are translated into an increased GLUT1 activity. Moreover, the combination of Akt and PKCζ/λ inhibitors, both used at concentrations fully suppressing receptor-regulated glucose transport in other cell systems (Bandyopadhyay *et al.*, 1997; Green *et al.*, 2008; Yang *et al.*, 2009), left about one-third of the maximal δ-opioid response unaffected, suggesting the possibility that yet unidentified mechanisms mediate this residual component of δ-opioid receptor regulation of glucose transport.

δ-Opioid receptor agonists have been shown to exert neuroprotective and cardioprotective effects under hypoxic and ischaemic insults (Bofetiado *et al.*, 1996; Zhang *et al.*, 2002; Gross *et al.*, 2005; Forster *et al.*, 2007). As GLUT1 is widely expressed (Carruthers *et al.*, 2009), it is important to investigate whether an increased GLUT1 activity may contribute to the beneficial effects of δ-opioid receptor agonists in conditions of limited energy supply, and whether this property could be exploited to develop new pharmacological strategies for enhancing glucose utilization in diseases characterized by altered glucose homeostasis.

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Conflicts of interest

The authors declare no conflict of interest.

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